

Peer Review File

**Manuscript Title:** NASH limits anti-tumor surveillance in immunotherapy-treated HCC

**Editorial Notes:** *none*

## Reviewer Comments & Author Rebuttals

### Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

Using two different mouse models of NASH-induced HCC as well as data from patients with NASH-associated HCC, the authors suggest the concept that CD8+PD1+ T cells promote NASH development and that treatment with checkpoint inhibitors may release the brake in these NASH-promoting cells, resulting in disease exacerbation and more HCC, which they proposed is confirmed by their findings of absent response to checkpoint inhibitors Nivolumab and Pembroluzimab in patients with NASH-associated HCC but not in patients with HCC due to other causes. While the analyses are carefully performed and raise the question of harmful effects of checkpoints in NASH-associated HCC, both the mouse and patient studies have major limitations, and it cannot be excluded that this paper sends the wrong message to the community and will negatively impact the field.

1. The NASH-HCC mouse models represent a major weakness of this paper and may lead to premature conclusions on the effect of PD-1 therapy in NASH-associated HCC. While the employed mouse models may be among the best to study various aspects of NASH, there are several limitations that preclude them from serving as useful preclinical models for HCC:

1a. Many mouse models of cancer are simply not responsive to checkpoint inhibition because of low mutational load and lacking tumor antigens/neoantigens. The authors do not provide evidence that the employed models have a mutational load that is at least as high as in that seen in HCC patients.

1b. The mouse model - albeit taking over a year - is not comparable to HCC development in patients, which takes decades and mostly occurs in the setting of advanced fibrosis or cirrhosis (even though a subset of NASH-associated HCC patients do not have cirrhosis, most of them have advanced fibrosis). Importantly, in most of these patients the underlying NASH is much less activate than in earlier disease stages/burnt out - meaning that the risk of increasing NASH activity and thereby worsening not only NASH but also increasing NASH-HCC is much lower and possibly not even relevant. The authors' conclusions would be relevant if one employed checkpoint inhibitors for HCC prevention but are likely not applicable to patients except for those, in whom HCC develops in the absence of cirrhosis and with high NAS.

2. In relation to above-described limitations of the model, the paper does not sufficiently focus on dual functions of CD8+PD1+ T cells, promoting NASH but possibly also restricting HCC. These functions are likely to occur at different stages in patients.

3. The data on the NASH- and NASH-HCC-promoting role of CD8+ T cells is similar to a previous study from the last author (Wolf et al, Cancer Cell). Hence a number of the findings presented in this manuscript are incremental with, adding PD1 into this context, with somewhat expected results, as well as novel techniques such as scRNA-seq.

4. The human data are based on a very small and poorly analyzed cohort of patients with NASH-associated HCC (n=10-11). While the underlying question is important, pairing data from this small cohort with the data from the mouse model with its above-described limitations and confounders may send a wrong and potentially deleterious message to the community, and much more careful analysis as well as larger cohorts are needed to put the provided message on a solid scientific foundation: The authors should analyzed outcomes for NASH-HCC patients with or without cirrhosis to account for the possibility of worsened NASH in patients without cirrhosis (for which the cohort is much too small).

A. A cohort of n=10-11 NASH-associated HCC patients is unacceptable. Many of the parameters

such as PFS are not significant and it cannot be excluded that inclusion of a larger number of NASH-HCC patients may change the data significantly.

B. The authors do not answer the question whether the differences in survival are due to failed checkpoint therapy or due to other differences between the two cohorts. Most likely, the differences in survival would persist if the authors removed all responders from the "other etiologies" group. Control groups that did not receive checkpoint inhibitors are missing to determine if survival is different between NASH and non-NASH HCC in patients who did not receive checkpoint inhibitors.

C. Is there any indication of increase NASH activity in patients receiving Pembro or Nivo?

D. There is no proper analysis of confounding factors.

E. Another problem is mixing Pembro and Nivo groups. Even though the target is the same, the authors need to provide subgroup analysis for this and increase the number far beyond what they have to make any meaningful conclusions in these subgroups.

F. Characterization of patients is insufficient - how were other liver diseases excluded, including ALD, which is not trivial, and especially important in such small cohorts?

5. Do the authors get the same results when blocking CTLA-4 - which was, even though not approved for HCC - the first approach and published study to show efficacy of checkpoint inhibitors in HCC?

Referee #2 (Remarks to the Author):

In their manuscript, Pfister and colleagues aim to show that CD8+PD-1+ T cells expand during progressing, diet-induced NAFLD and, upon treatment with anti-PD-1 antibodies, that these cells can promote carcinogenesis by establishing an inflammatory tumor microenvironment in a diet-induced, murine model of advanced NAFLD. Additionally, the authors observe a similar, intratumoral CD8+CD103+PD-1+ T cell subset in NASH-induced human HCC patients and claim that patients with NASH-induced HCC respond worse to anti-PD-1 therapy compared to HCC of other origin.

While the seminal observation in this paper is intriguing, namely that anti-PD-1 treatment can exacerbate tumorigenesis in a murine model of NASH-induced HCC, the authors fail to demonstrate clear causal relationships between the implicated cell types, liver inflammation and tumor development in the vast amount of the data they present, which therefore remain largely correlative. I will highlight my major concerns below.

1. In the reporting summary, the authors state that "Exclusion criteria was pre-established and the CD-HFD fed mice which did not show the NASH phenotype, high ALT, AST and body weight, were excluded from the analysis". I fail to understand why this decision was taken as these mice offer valuable insight in the author's proposed mechanism. Do CD-HFD mice without overt signs of NASH have reduced CD8+PD-1+ T cells? Do these mice also less frequently grow tumors upon anti-PD-1 blockade? Do the T cells in the livers of these mice fail display an enhanced effector phenotype? Aside from the valuable experimental insights that could be gained from these mice, the decision to exclude these CD-HFD but non-NASH mice from analysis also invalidates any claim that links a given diet to a given phenotype since mice that did not fit the authors' desired phenotype were excluded.

2. The data presented by the authors fail to demonstrate clear causal relationships. As an example, the authors note in lines 341-343 that a pro-inflammatory hepatic environment is created by TNF upon anti-PD-1 treatment, yet fail to show supporting evidence that this indeed drives "necro-inflammation" and accelerated hepatocarcinogenesis. The authors should neutralize TNF in their in vivo models to determine whether this molecule is indeed required for their phenotype, i.e., inflammatory microenvironment, liver damage and increased tumorigenicity.

3. Based on the authors' presented data, this problem can be further expanded. In Figure S9d and S9m, the authors show an increase in the number of antigen-presenting cells and increased MHC-

II expression. Are these recruited upon liver inflammation? Are they required for liver inflammation?

4. In Figure S11 the authors show an increase in many inflammatory mediators upon anti-PD-1 therapy; which of these are required for the accelerated carcinogenesis? While the authors propose a mechanism based on liver inflammation leading to increased hepatocarcinogenesis upon anti-PD-1 blockade, they provide little if any conclusive evidence for this hypothesis.

5. Some of the data the authors present seems internally inconsistent. As an example, the authors postulate that the pro-inflammatory hepatic environment is responsible for the increase in liver cancer incidence in anti-PD-1-treated mice, which they underscore by an increase in inflammatory cytokines in the liver microenvironment (Figure S11). However, they also show that upon CD8 depletion, which reduces cancer incidence, the inflammatory cytokines do not significantly reduce compared to the CD-HFD diet mice alone. This implies that the inflammatory microenvironment is not actually responsible for increased cancer incidence. How do the authors harmonize these findings?

6. Crucially, and related to my previous point, the authors also did not perform CD8 depletion in the context of anti-PD-1 treatment to show that CD8 cells are indeed the cells that are responsible for increased carcinogenesis upon anti-PD-1 therapy.

7. At times, the authors are (highly) selective in the data they choose to discuss and interpret. As an example, regarding Figure 1i, the authors describe the CD8<sup>+</sup> T cells in CD-HFD mice to demonstrate profiles of cytotoxicity and effector function because of increased expression of GzmK/M and Pcd1. However, in the same plot shows that these cells have reduced expression of GzmA/B, Klrp1, Il2ra, TNF and Il2; all markers of effector/cytotoxicity. How do the authors harmonize these observations?

8. Regarding Figure 1e, the authors state that CD-HFD contain a significantly altered immune composition that mainly affects the CD8<sup>+</sup> T cell compartment. However, this finding was not significant ( $p=0.09$  for CD8<sup>+</sup>PD-1<sup>+</sup> T cells and ns for CD8<sup>+</sup> T cells). In this plot, the authors do show significant differences in frequency of CD4<sup>+</sup> T cells ( $p<0.01$ ), classical monocytes ( $p<0.01$ ) and MDMs Ly6CHigh ( $p=0.01$ ). Why are these cell types not regarded as interesting? Are these cells responsible for the authors' proposed phenotype? In line 259 the authors state that there are only minor differences in the CD4 compartment, yet when looking at the data (Figure S9h and Figure S9f) the difference in the CD4 subset of CD62L-CD44<sup>+</sup>CD69<sup>+</sup> upon anti-PD-1 blockade is as strong as, if not stronger than, in the same subset of CD8 T cells, which the authors do deem interesting.

9. Along these lines, in line 387 the authors state that consistent with previous results, effects on the CD4<sup>+</sup>PD-1<sup>+</sup> T cell compartment remained minor, yet the differences observed for matching analyses (i.e. S17a vs S17g, S17b vs S17f, S17i vs S17j) of CD4 and CD8 populations show similar, if not stronger, effects for the CD4 T cell population. Why are these differences disregarded by the authors?

10. Similarly, in Figure 5a, the authors claim that a CD8<sup>+</sup>PD-1<sup>+</sup> T cell population arises upon NASH. However, there is a, perhaps even stronger, depletion of an Eomes<sup>+</sup> gamma-delta T cell subset. Additionally, a very strong induction of a CD4<sup>+</sup>CD27<sup>+</sup> population is observed in NASH samples. Why are these not discussed? Can these populations also be identified in the authors' murine models? Do these contribute to the authors' described phenotype? The authors should deplete CD4 T cells and gamma-delta T cells in their murine models, as these cell types may, at the very least, contribute to what occurs in patients.

11. The patient data is not convincing, but also does not match their murine models. In Figure 5a, the authors show that CD8<sup>+</sup>GzmB<sup>+</sup> cells are specifically lost in NASH samples which seems to counteract the claim made by the authors that inflammatory CD8 T cells cause liver inflammation and associated carcinogenesis. The authors similarly show in S19a that IFN $\gamma$ , Ccl3 and PD-L1 are in fact reduced in advanced NASH samples; does the loss of these inflammatory genes not counteract the claims made in Figure 3g, S4d, S10, S11 and S13a?

12. Lastly, the majority of patient data are not significant and show weak effect sizes; is it fair to draw strong conclusions on the basis of these data as the authors do?

Minor points:

- Figure 1j lacks a color scale bar and proper description. How does one interpret the difference between ND and CD-HFD in this plot?
- Where is the ND + PD-1<sup>-/-</sup> in Figure 3b? Do these mice also get accelerated carcinogenesis?
- There is no color scale bar in Figure 3e.
- In Figure 5k, shouldn't progression-free survival and time to progression plots yield the exact same data, but inversed? Why don't these curves match?
- In Figure S1i, what is the parent population?
- In Figure S4a, how does one distinguish ND from CD-HFD mice? The y-axis lacks a label.
- Figure 5c is plotted in a confusing manner (as the z-score scale is red independent of whether it goes up or down), but it seems that the TNF signaling gene sets are actually decreasing in expression.
- Why do the PD-1<sup>-/-</sup> mice still express PD-1 (Fig. S12e)?
- In Figure S13k, the authors should present cleaved Caspase 3 and cleaved Caspase 8 if they want to conclude something about cell death, as total, uncleaved levels of these proteins do not indicate cell death.
- In Figure S16f, the FACS plot does not match the quantification on the left.
- Regarding Figure S17b, the authors claim an increase in calcium levels in line 383 of their manuscript, but this difference is not significant.
- In Figure S18b, how does one interpret the difference between healthy, borderline NASH or NASH patients? There is no explanation of the color scale bar. Also, what are "randomly chosen CD45+ cells" as mentioned in the corresponding Figure Legend?
- Figure S19b is not legible.
- In lines 237-246 the authors describe that NK1.1-based depletion of immune populations did not result in changed liver pathology, body weight, fibrosis ALT, hepatic cytokines and hepatic chemokines. However, the animals who underwent this depletion also completely lacked liver cancer development. How does this happen if the authors did not detect any changes? The authors should perform NK1.1 depletion by itself to see if NK1.1+ cells, potentially depending on CD8 cells, are in fact responsible for the authors' phenotype.
- Sentence 289-292 is unclear.
- When discussing GSEA, the authors frequently use the wording 'reduced enrichment (e.g. line 241)' when talking about enrichment in the opposite phenotype. This is incorrect, as the absolute amount of enrichment is often similar just, as mentioned, in the opposite direction.

#### Referee #3 (Remarks to the Author):

This full article manuscript is novel, and the experimentation to support the conclusions is exhaustive and solid for the most part. In essence, the findings indicate that, in NASH livers, there is an accumulation/expansion of a pathogenic CD8 T cell population that expresses PD-1 and exacerbates NASH pathology and fosters hepatocellular carcinogenesis and progression. The inflammatory and tissue-damaging functions of this pathogenic CD8 T cells are repressed by PD-1 blockade that is common clinical practice for second-line treatment of advanced HCC and is under clinical trials for earlier stages of the disease. In fact, PD-L1 blockade plus anti-VEGF will soon become the standard of treatment for advanced HCC in first line. According to the findings in this paper upon PD-1 blockade, authors document an exacerbation of carcinogenesis and liver damage that questions the indication of PD-1 blockade in NASH-associated liver cancer. A balanced presentation of preclinical and supportive clinical results in patient specimens very much enhances the significance of this study.

#### Questions and comments:

1. TNF seems to be an actionable therapeutic target for the observed harmful effects of this CD8 T cell population. It would be interesting to know if TNF could be blocked preserving anti-cancer immunity (especially under checkpoint inhibition therapy) but preventing tissue damage and

carcinogenesis promotion.

2. Would PD-L1 blockade enhance liver cancer and tissue damage as well? Which cells are expressing PD-L1 in the system. This becomes important given the recent approval of atezolizumab + bevacizumab.
3. Results on NASH in human samples are compelling and supportive of the relevance of the findings. It would be interesting to know in such livers which cells express PD-L1.
4. What do you think is the fibrogenic factor/s promoted by pathogenic CD8 cells? Any candidates from the extensive transcriptomic analyses?
5. Are Kupffer cells involved in the CD8-dependent pathogenesis mechanisms?
6. Obesity and response to PD-1 associations have been reported (PMID: 30420753 and PMID: 30813970). According to these studies, obesity relates to T cell dysfunction that PD-1 blockade derepresses and results in better responsiveness. The models of NASH should suffer overweight as well as perhaps the patients in the reported series. This point should be addressed if possible and at least discussed. Authors may gain insight with their comparisons of the models with and without choline in the diet. As a potential consequence, would it be the case that in HCC patients, obese patients respond worse to treatment contrary to other indications? Of clinical note, advanced HCC patients frequently experience cachexia but perhaps less frequently so those with presumed or documented NASH etiology.
7. The retrospective series of patients with advanced HCC treated cannot be considered conclusive at this point and only hypothesis-generating. The wording there needs to be carefully down-toned.
8. An important message of this paper is that progression following PD-(L) treatment in NASH patients could be the development of a second primary malignancy rather than from the same one. Can this point be addressed in the models? Is multifocal cancer more common in those cases? The more CD8 pathogenic T cells in the infiltrate, the more multifocal the tumors?
9. The companion back to back paper shows more data on the physiology of the pathogenic CD8 T cells that I would otherwise ask to this article. Therefore, proper cross-reference of those findings is needed at least in discussion.

Referee #4 (Remarks to the Author):

This is an interesting and quite original study of the role of immunity in promoting liver cancer. There are data from the mouse models presented which show that CD8+ T cells can contribute to the pathology of NASH and the risk of cancers. The implication is that checkpoint blockade which can accentuate the function of CD8 populations can worsen disease. There are also some human data which are fairly consistent with this idea. It is perhaps not surprising that checkpoint inhibition might worsen an inflammatory condition, although inducing a cancer risk is very interesting.

Overall the authors do a very good job in describing the cellular responses and the impact of depletion/blockade. There seemed to be a bit of a gap around defining the mechanisms in terms of how the CD8+ T cell population induced cancer. Also it was somewhat unclear what the specificity of these T cells was and what was triggering their initial responsiveness in NASH. So although a strong case is made for the pro-tumor role the actual pathways to disease were less concrete.

Figure 1: There do not appear to be any iNKT cells in the UMAP or tSNE plots – these are discussed latter in the text. That seems a little surprising as they are quite dominant in the mouse liver and have a clear transcriptional profile. Could the authors clarify where these cells lie. It would be also useful to know whether other unconventional cell subsets including GD T cells and MAIT cells are incorporated in this, although they are likely much rarer. The latter may be relevant even if rare as they have been linked to liver fibrosis. The same questions would also apply to the scRNAseq of the human samples

Figure 1e: What are the p values on the right referencing? The difference in the PD1+ population does not appear to be significant. How valid is the PD1+ subset as a subcluster and also what are

the critical significant differences apart from elevated PD1 expression – some justification for this early on would be helpful. Often PD1 expression is more of a gradient (even within PD1+ cells) so a binary distinction needs a bit more justification. Does this group of cells have distinct TCRs from the non-PD1 (or lower PD1) subset or are they the same population with distinct expression? Some data on this would address the question about specificity – although this would be better addressed by defining actual TCR-specific (or independent) functionality.

Figure 1f: The stains are both single stains. It should be possible to show a double staining CD8+PD1+ population and enumerate them as this seems like the critical part of the study.

Figure 1j: One of the most upregulated genes in the PD1+ subset is IL-10. Do the authors have any data on whether this is secreted by this subset. Although the subset is labelled as “PD1+” it is not the top upregulated gene here (as above). A side-by-side broader functional study would add a bit of resolution here and if they do secrete IL-10 this may impact on the overall interpretation. The interpretations about function are all via the screening approaches so some further specific back up by FACS/ELISA would be helpful in confirming functionality, especially in the context of an “exhausted” phenotype – this would clarify the statement on line 199 about “potential effector function”. Such an experiment would also be valuable in the anti-PD1 treated mice in later parts of the manuscript.

Figure 2: It was not that clear why depleting CD8s had no impact on ALT, suggesting they are not playing a role in vivo, while blocking PD1 had some impact (AST is not shown for the anti-CD8 treatment).

Line 202 – lack of impact of anti-PD1. Is there a control for this experiment? The implication is that this lack of impact is aetiology-specific but it may also be that the intervention does not work well in other HCC models.

Figure 5b and the text are presented in a slightly confusing way. It would be easier to understand the disease associations of %CD8 (of CD3), and % PD1+ (or MFI) of CD3+CD8+ first. The association of CD103 with tissue residency in the liver is not as good as other tissues, so a broader look at the CD8+PD1+ population by flow would be better as well as some caution in interpretation.

Figure 5e could include some study of CD4s as well for reference. That subset has been linked to NASH pathogenesis as well. As above, it should be possible to perform some dual CD8 and PD1 staining to map the subset of interest.

Figure 5f is not really that convincing of a relationship with TNF – the r-squared value would be better to illustrate and would be very low. If the authors think TNF secretion is critical it would be possible to explore this further in the mouse model.

For Figure 5G some disease controls would be valuable.

Line 493+: This sentence is perhaps overstating the data, which were not significant in all those parameters. It is likely quite hard to make the firmest comparisons, especially in such a retrospective analysis, where the heterogeneous group of patients with eg viral aetiologies will be on effective therapies - the actual aetiologies were not obvious in the supplementary data. This interpretation could be a bit more cautious throughout (eg it is in the abstract).

**Author Rebuttals to Initial Comments**

**FULL AUTHOR REBUTTAL**

**(please note that the authors have quoted the reviewers in black and responded in blue)**





1 **Referee #1 (Remarks to the Author):**

2  
3 Using two different mouse models of NASH-induced HCC as well as data from patients with  
4 NASH-associated HCC, the authors suggest the concept that CD8+PD1+ T-cells promote  
5 NASH development and that treatment with checkpoint inhibitors may release the brake in  
6 these NASH-promoting cells, resulting in disease exacerbation and more HCC, which they  
7 proposed is confirmed by their findings of absent response to checkpoint inhibitors Nivolumab  
8 and Pembrolizumab in patients with NASH-associated HCC but not in patients with HCC due  
9 to other causes. While the analyses are carefully performed and raise the question of harmful  
10 effects of checkpoints in NASH-associated HCC, both the mouse and patient studies have  
11 major limitations, and it cannot be excluded that this paper sends the wrong message to the  
12 community and will negatively impact the field.

13

14 We thank Referee #1 for appreciating that our experiments have been “carefully performed”  
15 experiments as well as for outlining the potential clinical impact of our study on PD-1 targeted  
16 immunotherapy in HCC. Also, we thank Referee #1 for pointing out the current limitations of  
17 the applied mouse models and clinical cohorts of our study, which we have taken utmost  
18 seriously and improved both. Statements on the role of checkpoint inhibitors in non-viral  
19 etiologies in HCC have been tempered, but nonetheless reflect the results of the meta-  
20 analysis, which is aligned with the pre-clinical findings.

21

22 In short:

23 (i) We have added a third preclinical mouse model of NASH with NASH to HCC transition  
24 (Gomes et al., 2016; Tummala et al., 2014). Analysis of this model corroborated the link  
25 between CD8+PD1+ T-cells and NASH development

26 (ii) We have extended our preclinical experiments with six novel treatment groups and  
27 performed in detail analyses on the mechanism and functional link of liver damage,  
28 inflammation, and responsiveness to anti-PD1-targeted immunotherapy in liver cancer.

29 (iii) We have added human clinical data sets (with 1656 HCC patients on immunotherapy  
30 involving the important clinical trials - IMbrave 150; Checkmate 459; Keynote  
31 240), enlarged our initial retrospective clinical cohort, and validated results  
32 obtained from this cohort in a second cohort of HCC patients under  
33 immunotherapy. Moreover, we corroborated our findings of CD8+PD1+  
34 increasing by NASH in now in total 3 independent patient cohorts across  
35 Europe by flow cytometry or single-cell RNA-seq.

36 Furthermore, we have performed CYTOF and scRNA Seq analysis of lymphocytes from livers  
37 derived from human NAFLD/NASH and steatosis and compared these data with our preclinical  
38 models – corroborating our data.

39

40 We hereby address the Referee’s concerns in the following section point-by-point.

41 We agree with the Referee that additional analyses of patient cohorts and mouse experiments  
42 have been necessary to strengthen and corroborate our data.

43 We believe that we have achieved this in the new version of our manuscript by examining a  
44 very large number of HCC patients on immunotherapy with viral and non-viral/NASH/NAFLD  
45 origin – adding both individual cohorts from independent centers as well as a meta-analysis  
46 from the most important published trials on immunotherapy on HCC. Furthermore, we have  
47 strongly increased our *in vivo* analyses applying several different treatments in combination  
48 with anti-PD1 treatment, and a third NASH mouse model, validating further the reliability of our  
49 pre-clinical mouse models.

50 In particular, we have now added a meta-analysis including 1656 HCC patients with different  
51 underlying etiologies (viral and non-viral) treated with immunotherapy derived from three large  
52 clinical trials (included in **Figure 6, Extended Data 30-32** and **Rebuttal Figure 1, 2**).  
53 (Comment from our side: The total number of patients in the combined cohort is 1656.  
54 However, one patient in the CheckMate-459 had unknown etiology, and could therefore not be  
55 included in the quantitative meta-analysis). We conducted this meta-analysis to support the  
56 experimental data suggesting that anti-PD1/anti-PDL1 checkpoint inhibitors would have a  
57 distinct effect in non-viral (NASH-related) HCC as opposed to viral-related HCC (included in  
58 **Figure 6, Extended Data 30-32** and **Supplementary Table 7** and **Rebuttal Figure 1, 2**). Out  
59 of eight studies identified in the search, only three fulfill the pre-established criteria (included  
60 in **Extended data 30** and **Rebuttal Figure 1a, b**), including a total of 1656 HCC patients.

61 These randomized controlled trials (RCT) included **A**) CheckMate-459 (Yau et al., 2019), a  
62 first-line, randomized, sorafenib-controlled trial testing nivolumab (an anti-PD1 monoclonal  
63 antibody) in monotherapy (n=742), **B**) IMbrave150 (Finn et al., 2020), a first-line, randomized,  
64 sorafenib-controlled trial testing the combination of atezolizumab (an anti-PD-L1 monoclonal  
65 antibody) and bevacizumab (an anti-VEGF-A monoclonal antibody) (n=501), **C**) KEYNOTE-  
66 240 (Finn et al., 2019), a second-line, randomized, placebo-controlled trial testing  
67 pembrolizumab (an anti-PD1 monoclonal antibody) monotherapy.

68

69 All three trials reported a subgroup analysis of survival data stratified according to disease  
70 etiology: hepatitis B virus (HBV), hepatitis C virus (HCV), and non-viral, which mostly includes  
71 both NASH and alcohol intake.

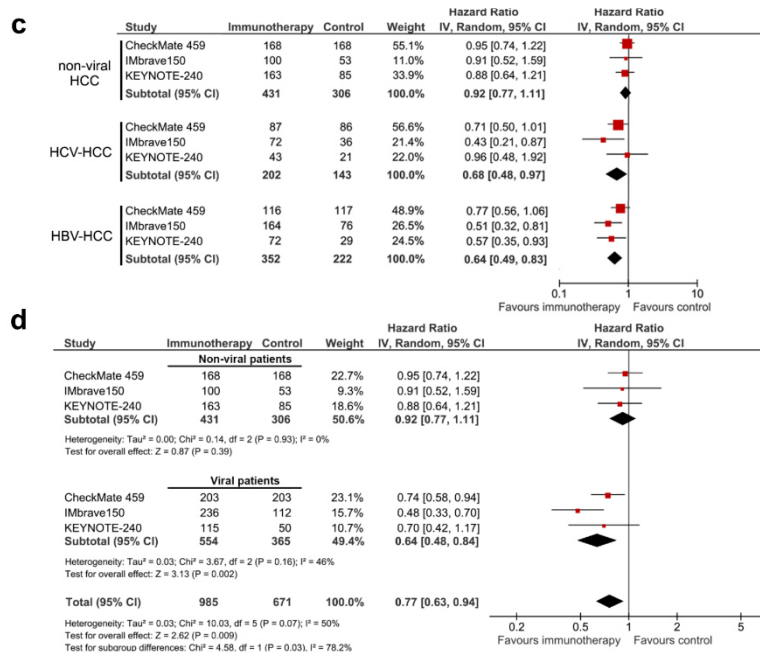
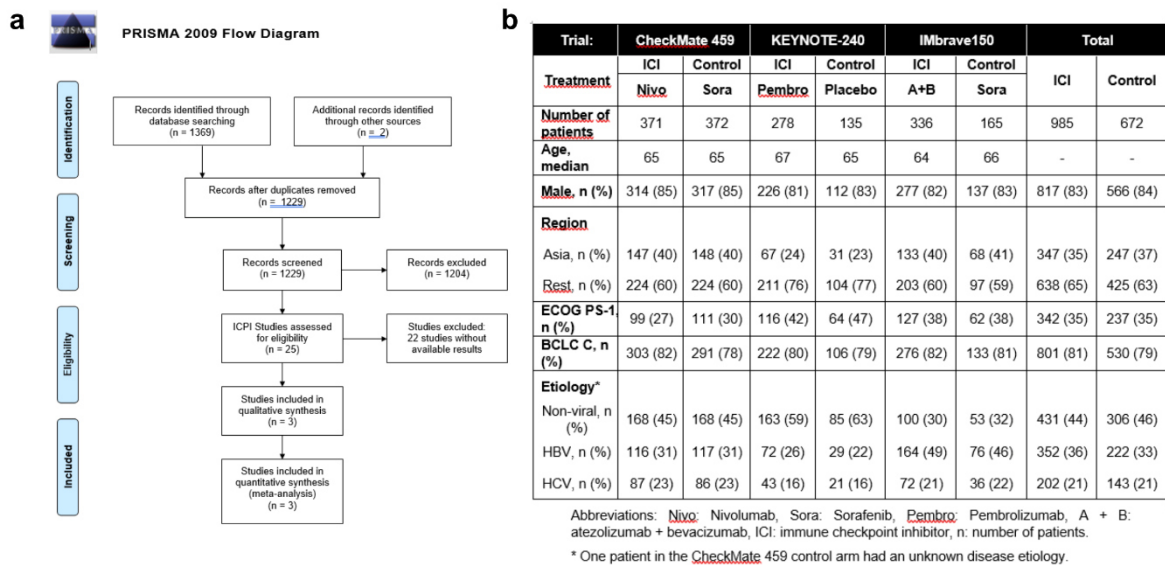
72 First, we analyzed whether checkpoint inhibitors were effective in each of three etiologies  
73 (HBV, HCV, and non-viral) and then compared the efficacy by categorizing patients with viral  
74 vs non-viral etiology HCC in all three phase III studies including a total of 1656 patients.  
75 Immunotherapy was superior to the control arm in both HBV (n= 574; p=0.0008) and HCV-  
76 related HCC patients (n= 350; p=0.04), **but not in non-viral** HCCs (n=737; p=0.39). The  
77 magnitude of the benefit with checkpoint treatment according to etiology was significantly  
78 better in viral etiology (pooled HBV and HCV cases) [HR: 0.64; 95%CI 0.48-0.94] than non-  
79 viral etiology [HR: 0.92; 95%CI 0.77-1.11]; p of interaction= 0.03 (Rebuttal Figure 1d). Then,  
80 we dissected the specific effect by each viral type in a subgroup analysis. Comparison of  
81 magnitude of effect was significant comparing HBV vs. non- viral etiology (n=1311; p  
82 interaction= 0.03), and there was a non-significant trend for HCV vs. non-viral etiology  
83 (n=1082; p of interaction=0.14) (Rebuttal Figure 2a,b).

84 Second, considering that two out of three RCT were conducted in first-line treatment of  
85 advanced HCC with a homogeneous control arm (sorafenib), we conducted a subgroup  
86 analysis specifically with these two studies (n= 1234). This approach allowed us to control for  
87 biases related to the study population and distinct control arms. Immunotherapy was superior  
88 to sorafenib in both HBV (n= 473; p=0.03) and HCV-related HCC patients (n= 281; p=0.03),  
89 but not in non-viral HCC (n=489; p=0.62). (Rebuttal Figure 2d,e). The magnitude of the  
90 checkpoint treatment effect vs sorafenib according to etiology showed a non-significant trend  
91 favoring viral etiology (n=754; HR: 0.61 (95%CI 0.40-0.93)] when compared to non-viral  
92 etiology [n=489; HR: 0.94 (95%CI 0.75-1.18) (p of interaction= 0.08) (Rebuttal Figure 2c). As  
93 a result, we have included these data in the main text and main figure (**Figure 6**) of the  
94 resubmitted manuscript.

95 Based on these data we want to point out that it is - as indicated by Referee#1 - of the highest  
96 importance to us to specifically define/tone down appropriately the message of our manuscript:  
97 Our manuscript does not indicate that immunotherapy is not beneficial for HCC patients at all.  
98 Our manuscript rather demonstrates that HCC patients with viral etiologies do respond well  
99 and achieve survival benefits - however, that patients with non-viral etiologies (e.g. NASH) do  
100 not achieve a significant outcome benefit.

101 We thus propose to stratify HCC patients who are very likely to profit from immunotherapy and  
102 strengthen the argumentation to use immunotherapy in specific cohorts of HCC patients. We

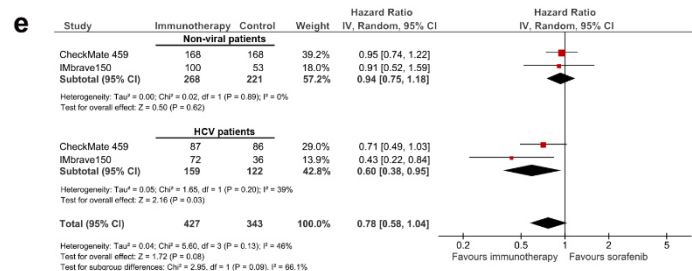
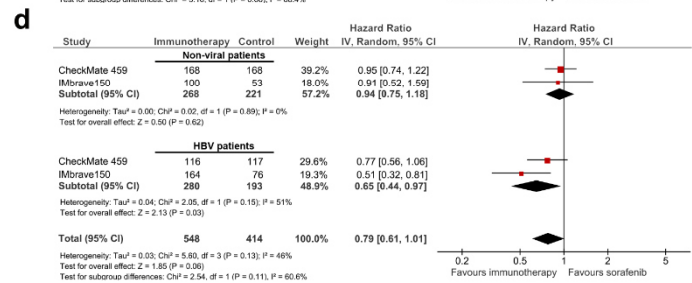
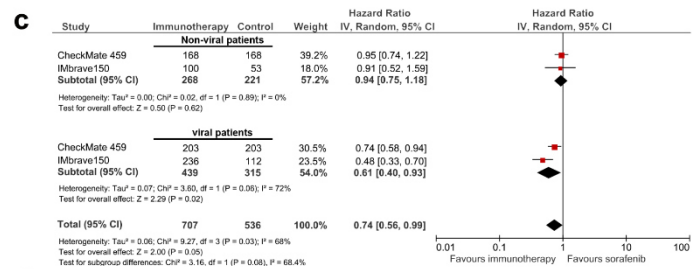
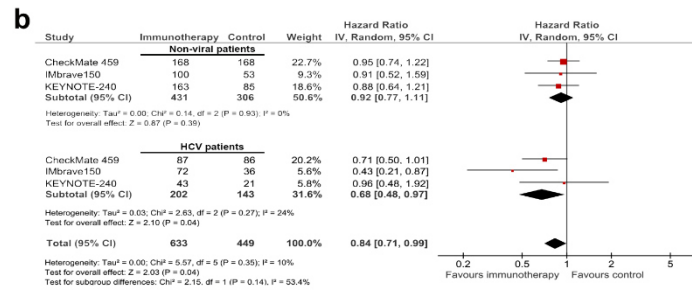
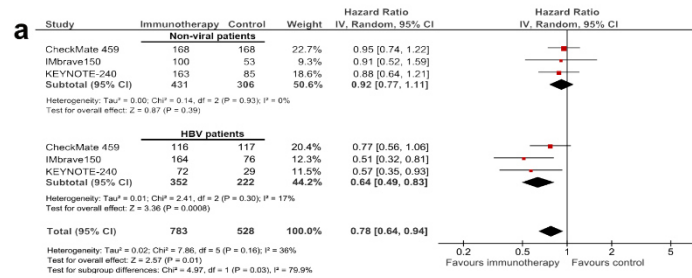
103 agree with Referee#1 that this information needs to be articulated in the paper appropriately  
 104 not to deliver wrong messages but to be very specific.  
 105 We truly believe that these are important clinical data, also providing the basis to test our  
 106 hypotheses in prospective studies on non-significantly beneficial effects in terms of OS for  
 107 immunotherapy in HCC patients with non-viral and NAFLD/NASH etiology, in particular.



108  
 109 **Rebuttal Figure 1**

110 (a) Selection of articles assessing the clinical outcome of immune checkpoint inhibitors in  
 111 advanced HCC for inclusion in the systematic review and meta-analysis. ICPI: Immune  
 112 checkpoint inhibitor. (b) Pooled baseline characteristics of the patients included in the meta-  
 113 analysis (total n= 1656). (c) A total of 1656 patients were included in all three randomized trials,

114 and 985 patients received a checkpoint inhibitor (Supplementary Table 7). (c) Separate meta-  
 115 analyses were performed for each of the three etiologies: non-viral (including mostly NASH  
 116 and alcohol intake), HCV and HBV. (d) HCV and HBV were pooled into a separate category,  
 117 termed “viral”, and a subsequent meta-analysis comparing viral (n=919) and non-viral,  
 118 including mostly NASH and alcohol intake (n=737) was performed. Hazard ratios for each trial  
 119 are represented by squares, the size of the square represents the weight of the trial in the  
 120 meta-analysis. The horizontal line crossing the square represents the 95% confidence interval  
 121 (CI). The diamonds represent the estimated overall effect based on the meta-analysis random  
 122 effect of all trials.



**124 Rebuttal Figure 2**

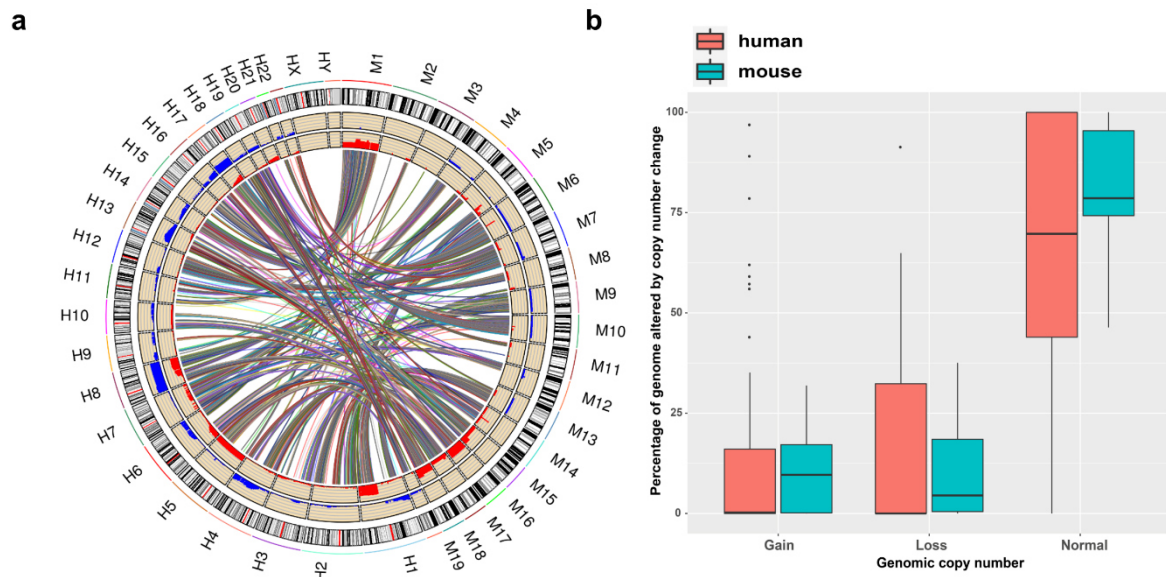
125 A total of 1656 patients were included in all three randomized trials, and 985 patients received  
126 a checkpoint inhibitor. Subgroup analysis was performed to study the specific effects of  
127 immunotherapy comparing non-viral etiologies (n=737) with (a) HBV (n=574) or (b) HCV  
128 (n=345). A total of 1243 patients were included in two first-line trials comparing PD-1 or PD-L1  
129 targeted immunotherapy to sorafenib. 707 patients received an immune checkpoint inhibitor  
130 (either PD-1 or anti-PD-1). (c) HCV and HBV were pooled into a separate category, termed  
131 “viral”, and a subsequent meta-analysis comparing viral (n=754) and non-viral (n=489), mostly  
132 NASH and alcohol intake, was performed. A subgroup analysis studying the specific effects of  
133 non-viral etiologies (n=489) on the magnitude of effect of immunotherapy are presented, when  
134 compared to (d) HBV (n=473) or (e) HCV (n=281). Hazard ratios for each trial are represented  
135 by squares, the size of the square represents the weight of the trial in the meta-analysis. The  
136 horizontal line crossing the square represents the 95% confidence interval (CI). The diamonds  
137 represent the estimated overall effect based on the meta-analysis random effect of all trials.  
138

**139 Specific points:**

140 1. The NASH-HCC mouse models represent a major weakness of this paper and may lead to  
141 premature conclusions on the effect of PD-1 therapy in NASH-associated HCC. While the  
142 employed mouse models may be among the best to study various aspects of NASH, several  
143 limitations preclude them from serving as useful preclinical models for HCC:  
144

145 We thank Referee #1 for appreciating the used NASH-HCC models as “among the best to  
146 study various aspect of NASH”, and we agree in general that studies in preclinical models have  
147 their limitations, especially in the context of chronic inflammation-induced cancer. These  
148 limitations of preclinical models are pronounced if mouse models are not used chronically (e.g.  
149  $\geq 1$  year).

150 However, we would like to point out that the model(s) used in our paper reflect sporadic liver  
151 cancer development with similar immune cell signature, pathophysiology, and the  
152 heterogeneous genetic landscape found in humans (Ma et al., 2016; Malehmir et al., 2019;  
153 Wolf et al., 2014 - and the data reported in this manuscript). In response to Referee #1, we  
154 have performed synteny analyses comparing HCC nodules from individual mice with human  
155 HCC (included in **Extended Data 6** and **Rebuttal Figure 3**). These data indicated no  
156 significant changes in genomic aberrations and thus a comparable character between human  
157 HCC and mouse liver tumors.



158

159 **Rebuttal Figure 3**

160 (a) Synteny analysis of mouse-HCC and (b) quantification of genomic aberrations by array  
 161 comparative genomic hybridization (aCGH) after 12 months on CD-HFD (n= 19) and human  
 162 NALFD/NASH-HCC (n= 78).  
 163

164 1a. Many mouse models of cancer are simply not altered responsive to checkpoint inhibition because  
 165 of low mutational load and lacking tumor antigens/neo-antigens. The authors do not provide  
 166 evidence that the employed models have a mutational load that is at least as high as in that  
 167 seen in HCC patients.  
 168

169 We thank and agree with Referee #1 for pointing out the possible unresponsiveness of clinical  
 170 models to checkpoint inhibition due to low mutational load. The mutational load HCC of most  
 171 conventional preclinical models is indeed very low, or lower compared to human HCC. This is  
 172 the case, in particular when taking into account liver cancer models triggered through  
 173 transgenesis, e.g. c-myc transgenic mice or preclinical mouse models with hydrodynamic tail  
 174 vein injection (HTDVi) of oncogenic drivers and tumor suppressors. In those models, pre-  
 175 existing genetic drivers and tumor suppressor deficiencies can be a major drawback  
 176 concerning additional mutations and increased mutational load.

177 In a chronic model of liver inflammation, we could show that mutational load increases over  
 178 time - comparing 9, 12, and 15 months (Finkin et al., 2015).

179 Our chronic, spontaneous NASH-HCC models develop liver cancer in the absence of specific  
 180 genetic drivers – but rather through chronic liver damage triggering DNA instability, ER and  
 181 mitochondrial stress, accumulating genetic hits over time stochastically triggering liver cancer  
 182 formation, like has been shown in human NASH (Boege et al., 2017).

183 In light of the important question of Referee #1, we have now included a further genetic  
184 screening of 19 mouse HCC nodules in our revised manuscript and compared them to human  
185 HCC nodules and their mutational landscape (included in **Extended Data 6** and **Rebuttal**  
186 **Figure 3**). Data from this study confirm that quality, degree of heterogeneity, and load of  
187 chromosomal aberrations (gains and deletions) of the used NASH to HCC mouse model is  
188 similar to human HCC (Wolf et al., 2014 and this manuscript). Strikingly, also the immune cell  
189 populations revealed by scRNA Seq are comparable in mouse and human NASH underscoring  
190 that the used NASH-HCC mouse model reflects the basic immune landscape of NASH and  
191 subsequently NASH-HCC transition.

192 Furthermore, we would like to point out, that overall in human HCC so far a responder rate of  
193 17-20% for PD-1-targeted monotherapy was observed, potentially due to a generally low  
194 amount or lack of broad-scale tumor antigens in HCC (El-Khoueiry et al., 2017; Zhu et al.,  
195 2018).

196  
197 1b. The mouse model - albeit taking over a year - is not comparable to HCC development in  
198 patients, which takes decades and mostly occurs in the setting of advanced fibrosis or cirrhosis  
199 (even though a subset of NASH-associated HCC patients do not have cirrhosis, most of them  
200 have advanced fibrosis). Importantly, in most of these patients, the underlying NASH is much  
201 less activate than in earlier disease stages/burnt out - meaning that the risk of increasing NASH  
202 activity and thereby worsening not only NASH but also increasing NASH-HCC is much lower  
203 and possibly not even relevant. The authors' conclusions would be relevant if one employed  
204 checkpoint inhibitors for HCC prevention but are likely not applicable to patients except for  
205 those, in whom HCC develops in the absence of cirrhosis and with high NAS.

206  
207 We thank Referee #1 to point out the limitations of preclinical models in comparison to patient-  
208 derived data. We agree that preclinical models do not take decades to develop HCC (averages  
209 mouse life-time ~ 2 years), however, mouse models have helped in the identification of  
210 molecular and cellular mechanisms leading to liver cancer (Ringelhan et al., 2018) - and if used  
211 in a long term fashion - up to 2 years - they do recapitulate in part the chronicity of inflammatory  
212 etiologies driving liver cancer. Moreover, mouse liver cancer occurs in age comparable to the  
213 life-span of patients (we applied 12 - 15 months of NASH-diet feeding months from 2 months  
214 of age onwards), which is comparable with the 4<sup>th</sup> to 5<sup>th</sup> life decade in humans regarding the  
215 age of HCC onset/HCC disease (Llovet et al., 2016).





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216 We would like to highlight, that preclinical models implemented in our study develop fibrosis to  
217 different degrees (mostly mild peri-cellular fibrosis to periportal streets and cirrhosis (Malehmir  
218 et al., 2019; Wolf et al., 2014)).

219 Thus, we agree with Referee #1, that the preclinical model might represent a subgroup of  
220 patients developing HCC in the background of fibrosis.

221 Moreover, we agree with Referee#1, that underlying NASH in HCC patients might be less  
222 activated compared to earlier stages and burnt-out.

223 Of note, clinical state-of-the-art care includes the use of corticosteroids for the treatment of  
224 adverse effects (Weiler-Normann and Lohse, 2016), which can also induce NASH-like  
225 pathologies. Thus, understanding mechanisms of underlying NASH in NASH-HCC in  
226 preclinical models is of vital interest. Furthermore, current studies explore checkpoint inhibitors  
227 for HCC as prevention of recurrence (Kudo, 2018).

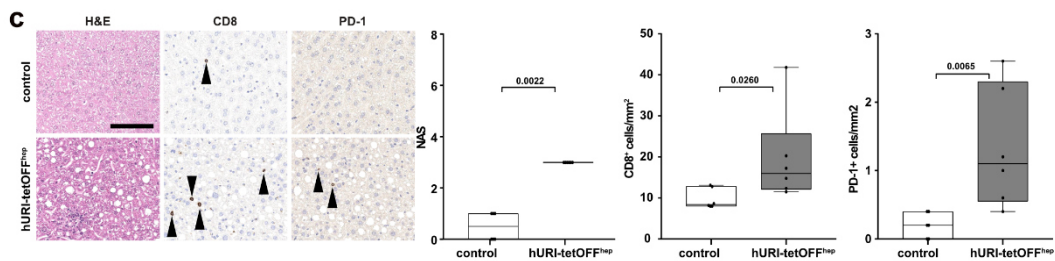
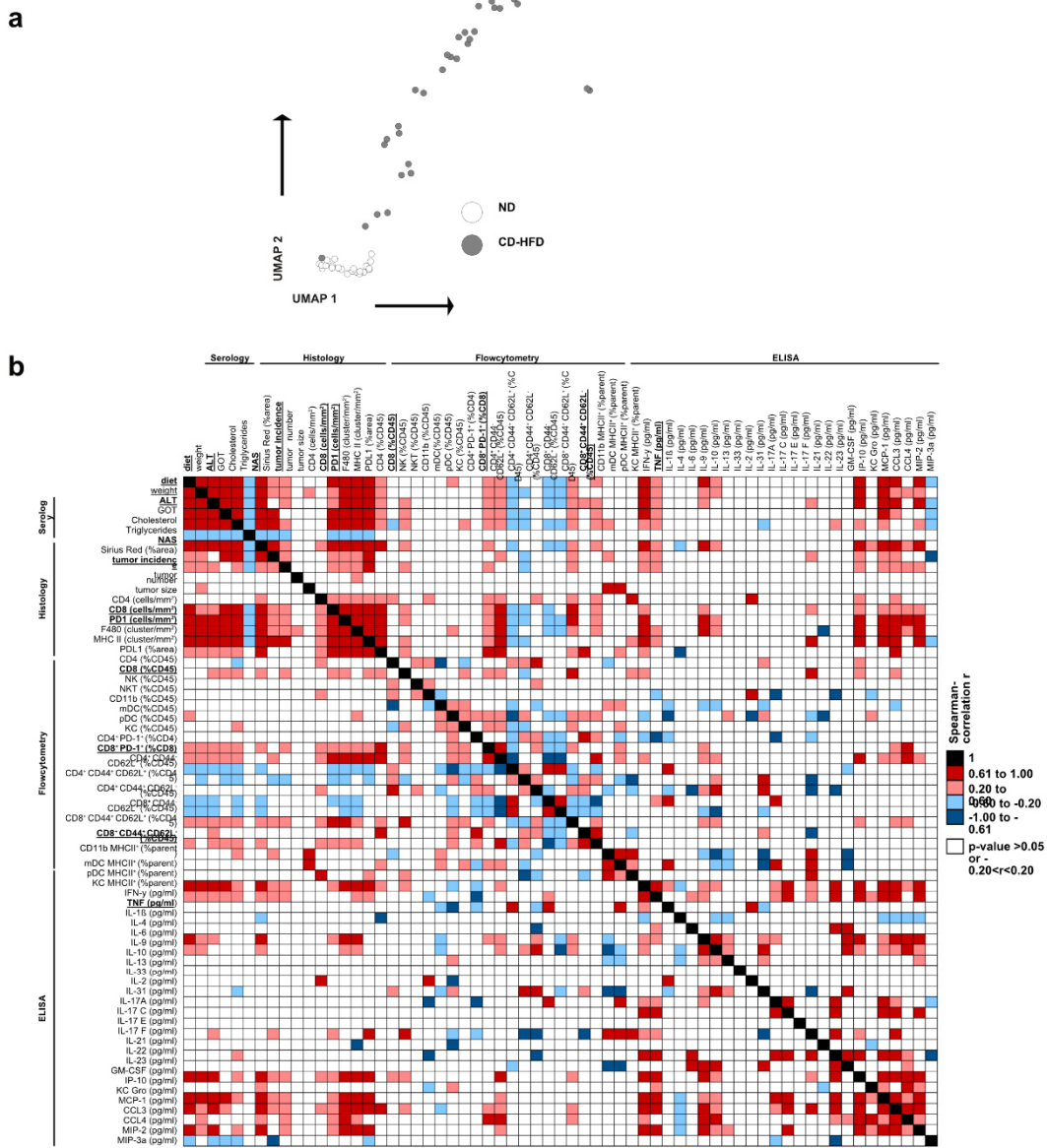
228 We take this point of Referee #1 utmost seriously and devised importance for this critique in  
229 the discussion section of our manuscript. We toned down our interpretations from human  
230 cohorts analyzed in a retrospective design, although we believe the points raised in our  
231 manuscript address important points like a potential stratification for etiology, the need for  
232 biomarkers, and clinical awareness of potential unfavorable side-effects of checkpoint inhibitor  
233 usage (e.g. similar to hyper progressive disease during PD-1 blockade in advanced HCC (Kim  
234 et al., 2020)).

235 In line with the suggestion of Referee #1 to explore the limitations of our mouse models and to  
236 understand the link between liver inflammation and tumor development better, we have re-  
237 analyzed our mouse data sets to dissect potential correlations of fibrosis, tumor size, tumor  
238 nodule number, flow cytometry data of livers, ALT, NAS, CD8, and PD-1 expression using  
239 artificial intelligence, machine learning and neuronal networking (included in **Figures 1** and  
240 **Extended Data 4 and 24** and **Rebuttal Figure 4a,b, 5**).

241 Moreover, we have added a third NASH-HCC mouse model, which corroborates the link  
242 between the amount of CD8+, PD1+ T-cells, and NASH (included in **Extended Data 3e** and  
243 **Rebuttal Figure 4c**).

244 Of note, we now underlined that our preclinical NASH models recapitulate in part the alterations  
245 of hepatic immune cells in NASH by performing correlative analyses and machine learning of  
246 liver-derived lymphocytes of NASH patients by CYTOF, classical flow cytometry, and scRNA-  
247 seq (included in **Figure 5**, **Extended Data 25-27** and **Rebuttal Figure 6-9**). Data from these  
248 analyses demonstrate that the pro-tumorigenic T cell population found in our preclinical NASH  
249 mouse models livers (CD8+PD1+CXCR6+) are also found in / and correlate with NASH in  
250 human livers (CD8+PD1+CD103+).

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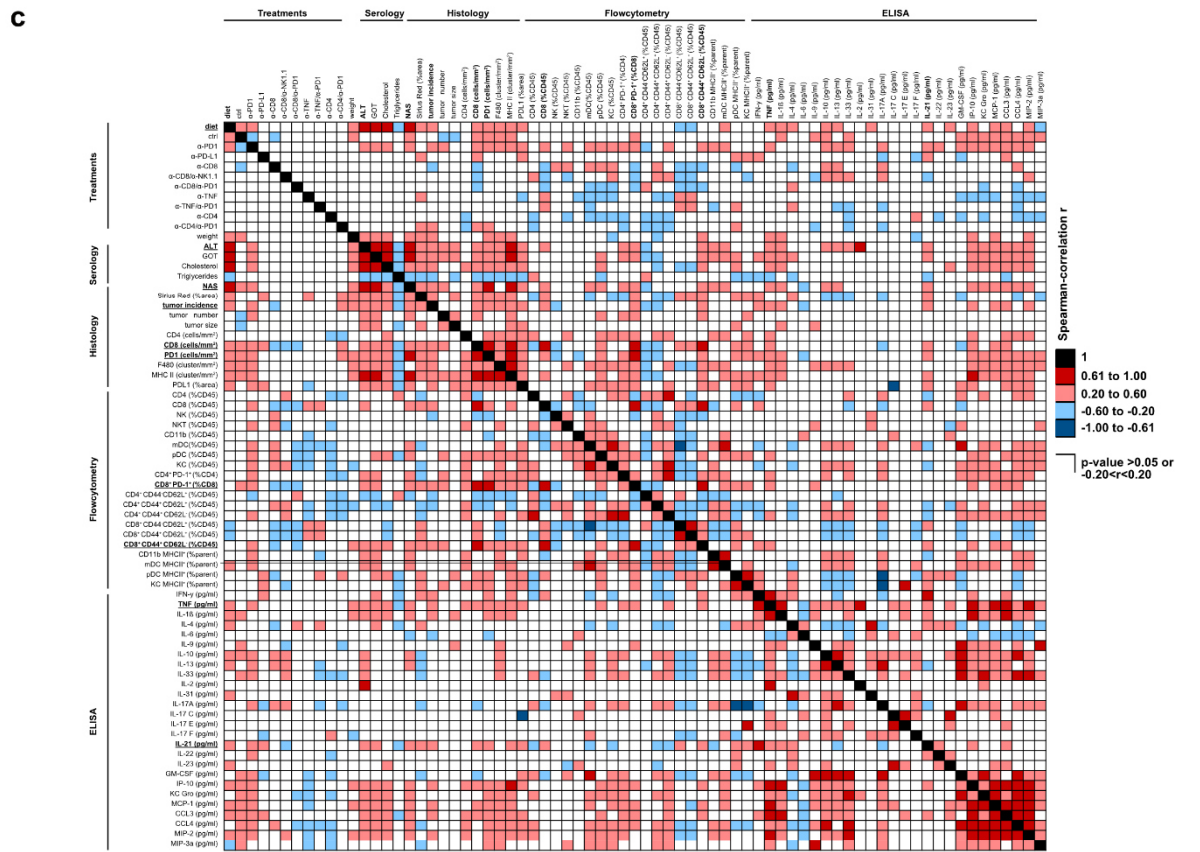
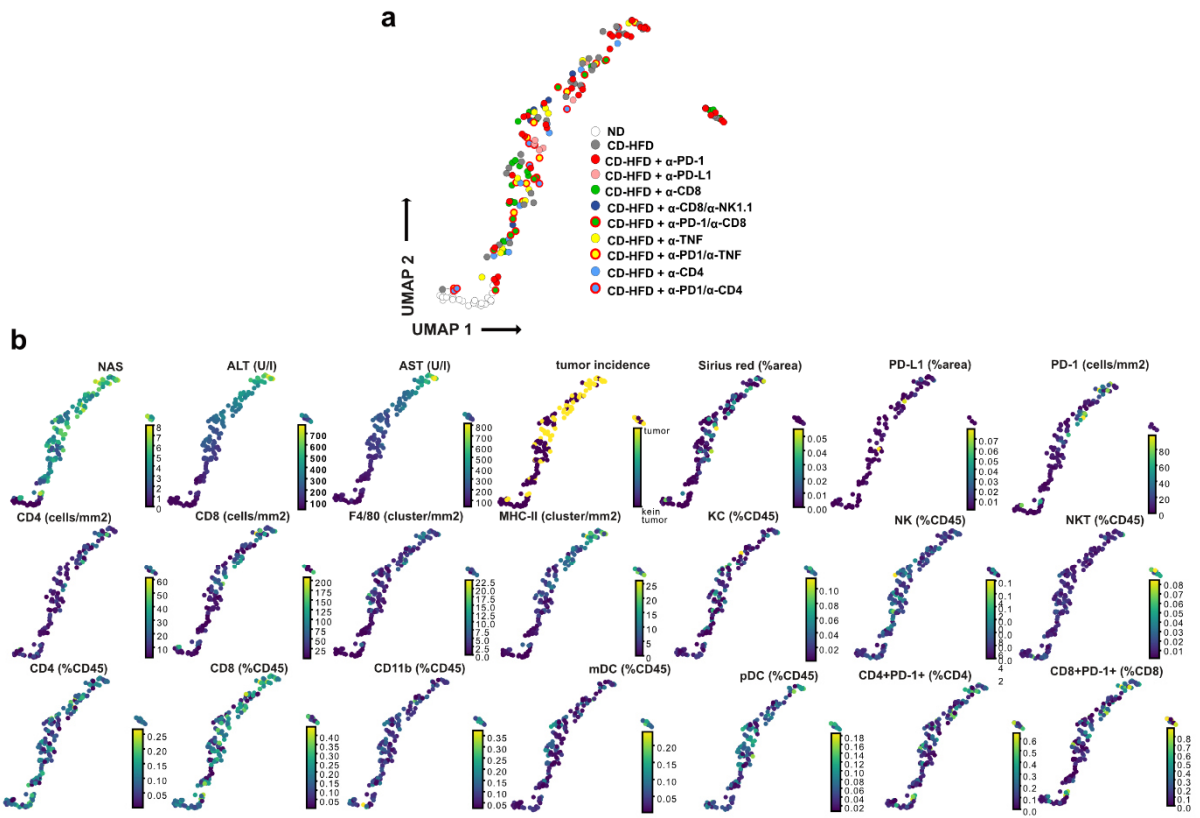
252 **Rebuttal Figure 4**

253 (a) UMAP representation of 63 parameters (serology, flow cytometry, histology) indicating  
 254 NASH pathology severity measured of 12 months ND or CD-HFD fed mice (ND n= 22 mice;  
 255 CD-HFD n= 31 mice). (b) Data gathered from hepatic tissue analyses was binary correlated  
 256 with each other of 6- or 12-months ND or CD-HFD fed mice (ND n= 47 mice; CD-HFD n= 72  
 257 mice). (c) H&E, CD8 and PD-1 staining, evaluation by NAS and quantification of CD8+ cells



258 and PD-1+ expressing cells by immunohistochemistry of 32-weeks old hURI-tetOFFhep and  
259 non-transgenic litter control mice (n=6 mice/group). Arrowheads indicate specific staining  
260 positive cells. Scale bar: 100  $\mu$ m.

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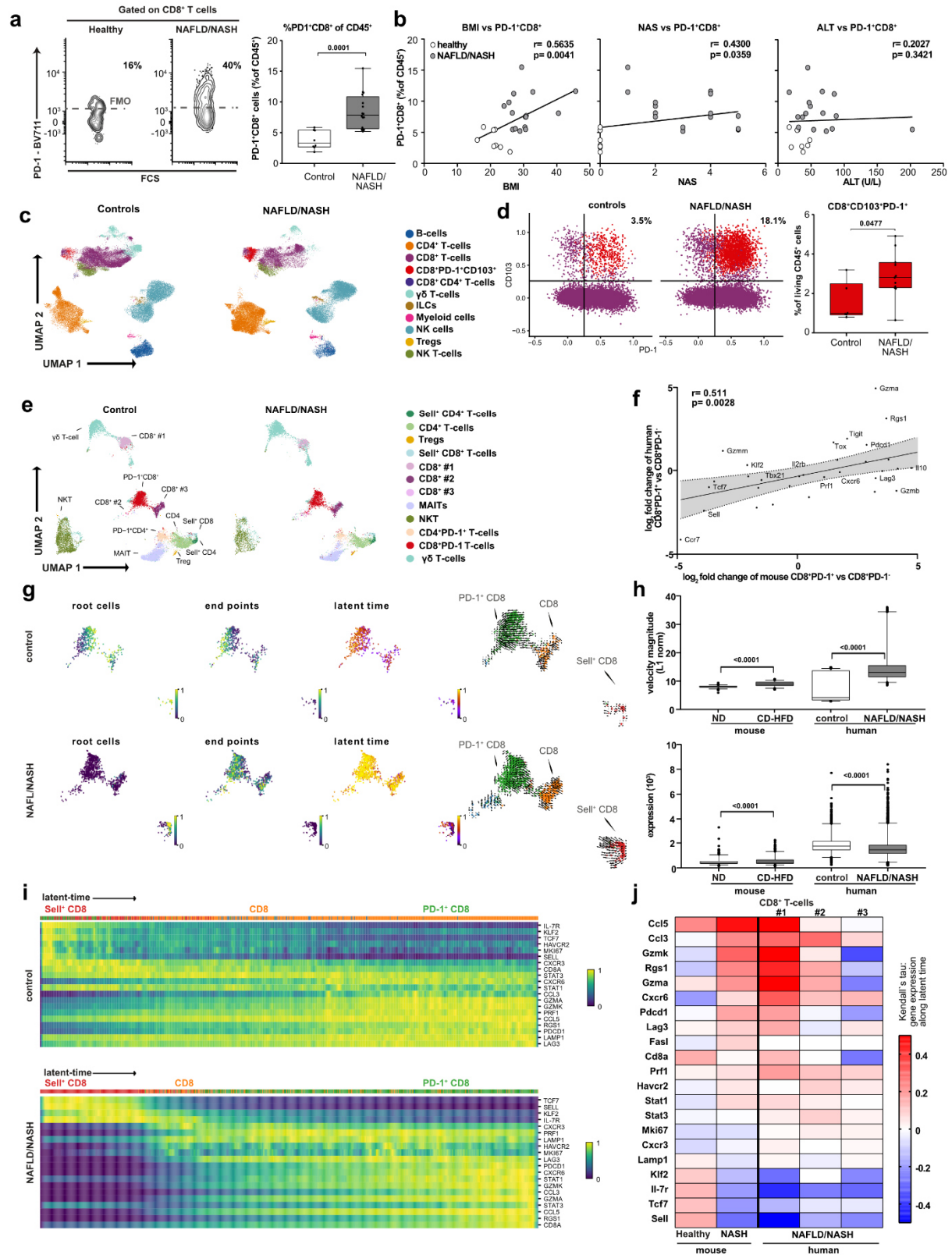




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**263 Rebuttal Figure 5**

264 (a) UMAP representation of 63 parameters (serology, flow cytometry, histology) and (b)  
265 selected display of analyzed parameters indicating NASH pathology severity measured of 12  
266 months ND or CD-HFD fed mice (ND n= 22 mice; CD-HFD n= 31 mice; CD-HFD +  $\alpha$ -PD-1 n=  
267 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; CD-HFD +  $\alpha$ -CD8 n= 24 mice; CD-HFD +  $\alpha$ -  
268 CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice;  
269 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4  
270 n= 9 mice). (c) Data gathered from hepatic tissue analyses was binary correlated with each  
271 other of 6- or 12-months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8,  $\alpha$ -CD8/ $\alpha$ -  
272 NK1.1;  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or  $\alpha$ -PD-1/ $\alpha$ -CD4 fed mice (ND  
273 n= 47 mice; CD-HFD n= 72 mice; CD-HFD +  $\alpha$ -PD-1 n= 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6  
274 mice; CD-HFD +  $\alpha$ -CD8 n= 29 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -  
275 CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD  
276 +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).

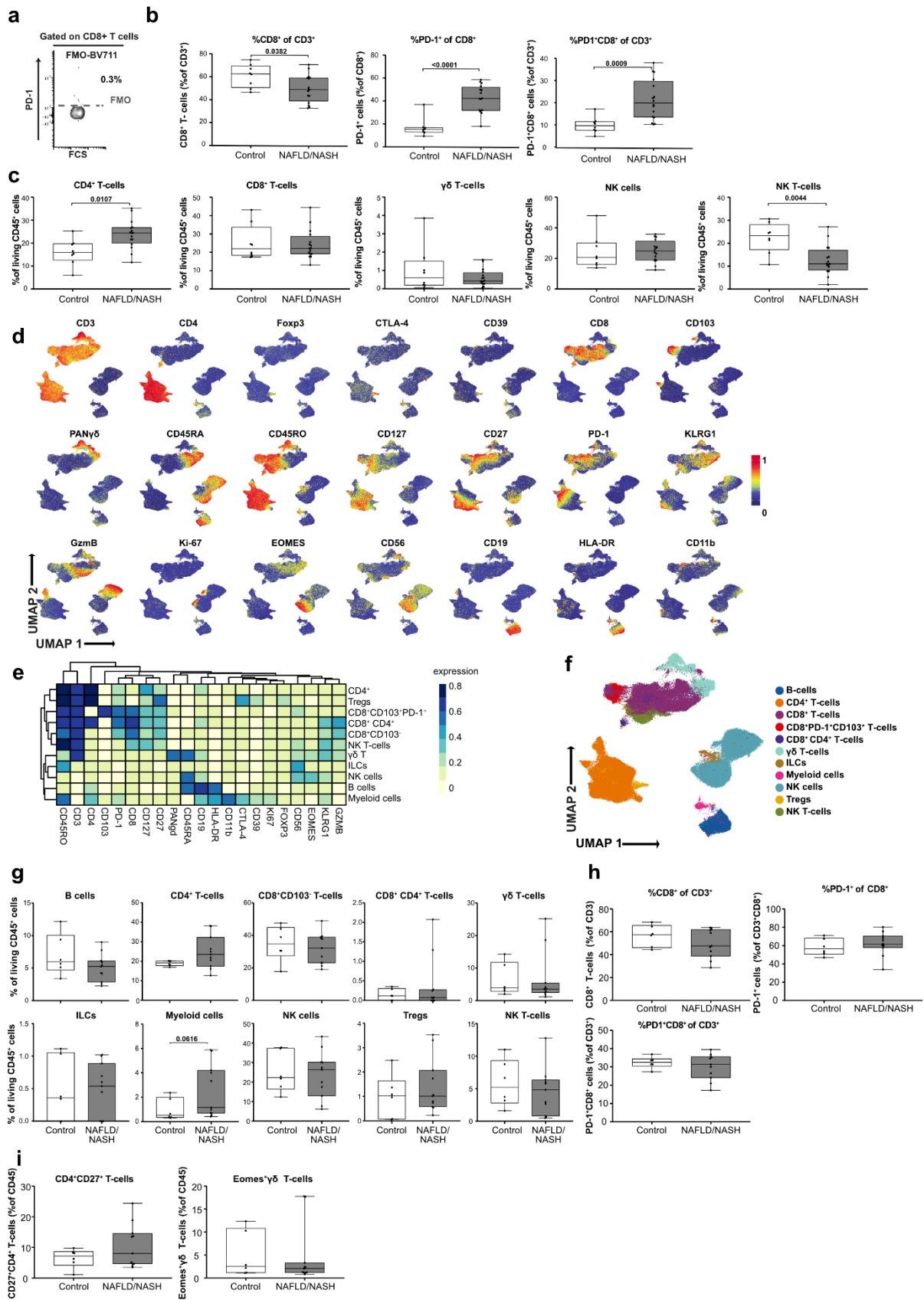
277  
278

## Rebuttal Figure 6

279 (a) Flow cytometry plots, quantification of patient-liver-derived PD-1+CD8+ T-cells, and (b)  
 280 correlation of PD-1+CD8+ T-cells with BMI, NAS and ALT of healthy or NAFLD/NASH patients  
 281 (Supplementary Table 1: healthy n= 8 patients; NAFLD/NASH n= 16 patients). (c) UMAP



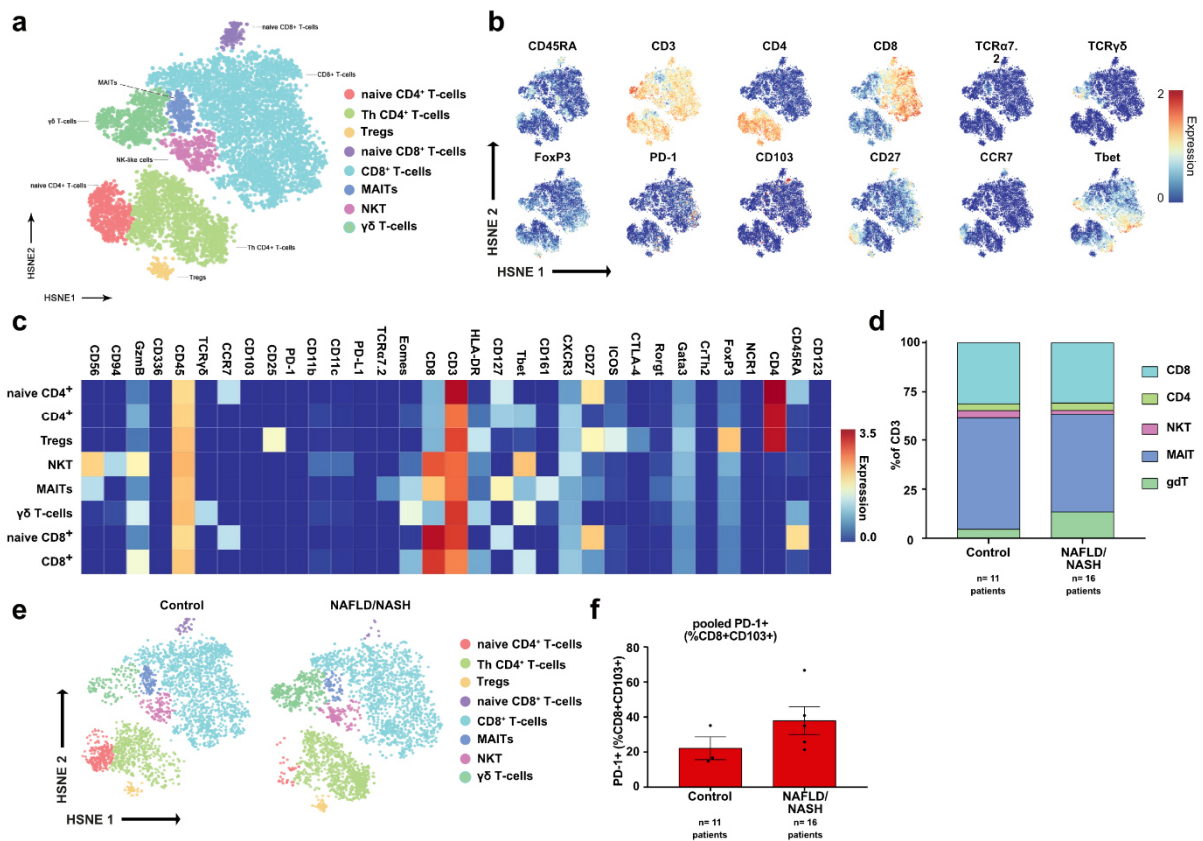
282 representation of randomly chosen CD45+ cells and (b) flow cytometry plots and quantification  
283 of CD8+PD-1+CD103+ derived from hepatic biopsies of control, or NAFLD/NASH patients  
284 (Supplementary Table 2: control n= 6 patients; NAFLD/NASH n= 11 patients) Populations:  
285 CD8+ (violet), CD8+PD-1+CD103+ (red). (e) UMAP representation of CD3+ cells and (f)  
286 analyses of differential gene expression by scRNA-seq of control, or NAFLD/NASH patients  
287 (control n= 4 patients; NAFLD/NASH n= 7 patients). (f) Correlation of significant differentially  
288 expressed genes in liver-derived CD8+PD-1+ compared to CD8+PD-1- T-cells subsets of 12  
289 months CD-HFD fed mice and NAFLD/NASH patients (mouse: n= 3 mice; human: n= 3  
290 patients). (g) RNA Velocity analyses of scRNA-seq data showing (h) expression,  
291 transcriptional activity, (i) gene expression and (j) correlation of expression along the latent-  
292 time of selected genes along the latent-time of patient-liver-derived CD8+ T-cells of control, or  
293 NAFLD/NASH patients in comparison to mouse-liver-derived CD8+ T-cells (patients:  
294 NAFLD/NASH n= 3 patients; mouse: n= 3 mice/group).  
295





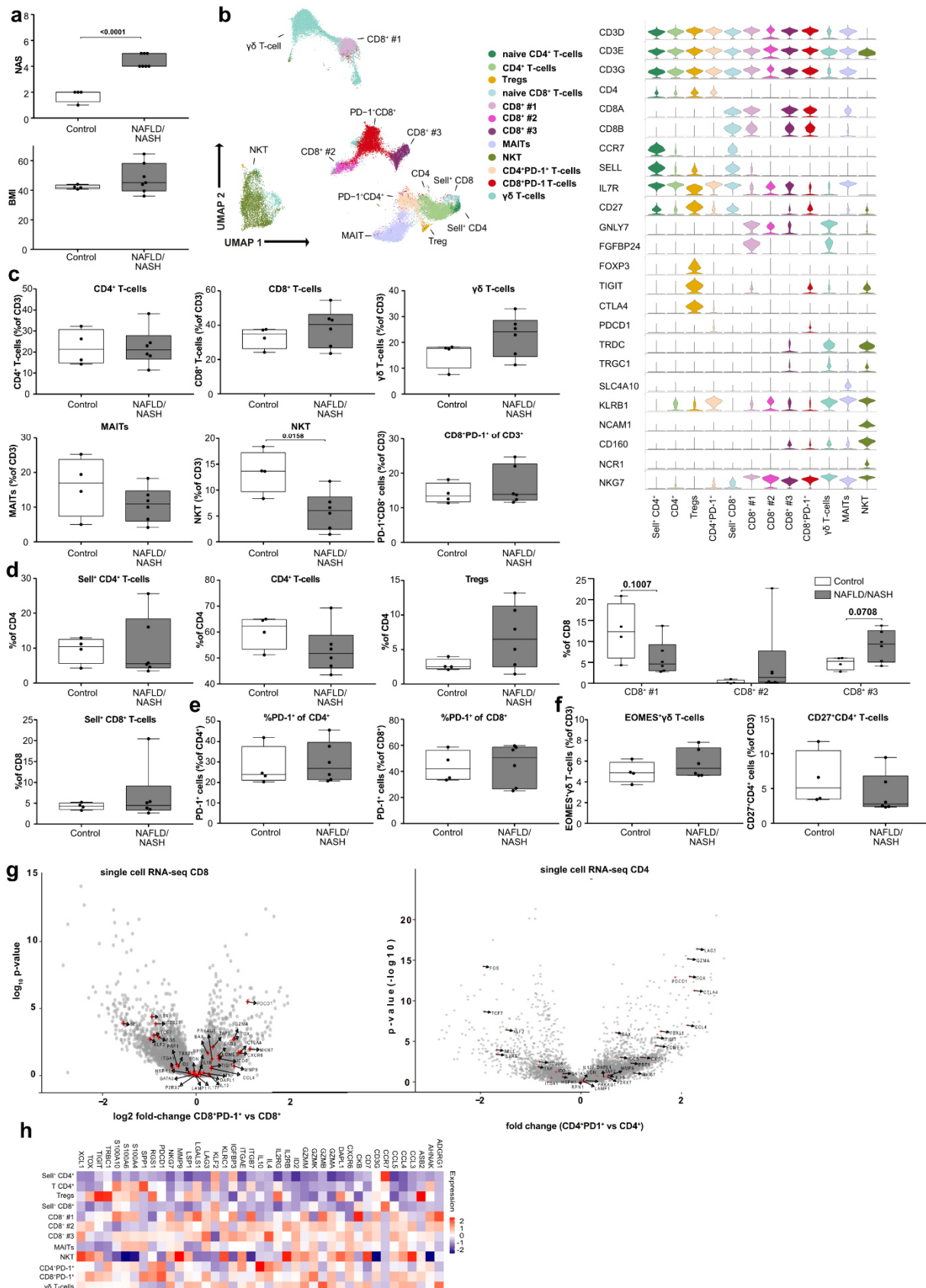
298 **Rebuttal Figure 7**

299 (a) Flow cytometry plot of FMO control, (b) quantification of patient-liver-derived PD-1+CD8+  
300 T-cells, and (c) quantification of CD4, CD8,  $\gamma\delta$ , NK and NKT cells healthy or NAFLD/NASH  
301 patients (Supplementary Table 1: healthy n= 8 patients; NAFLD/NASH n= 16 patients). (d)  
302 Analysis of randomly chosen CD45+ cells and (e) average marker expression of defined  
303 CD45+ subsets by flow cytometry derived from hepatic biopsies of control and NAFLD/NASH  
304 patients to define distinct marker expression (Supplementary Table 2: control n= 6 patients;  
305 NAFLD/NASH n= 11 patients). (f) Definition of cellular subsets, (g) relative quantification of  
306 defined cellular subsets of randomly chosen CD45+ cells, (h) polarization of CD8+ T-cells and  
307 (i) quantification of CD4+CD27+, or  $\gamma\delta$  TCR+Eomes+, T-cells by flow cytometry derived from  
308 hepatic biopsies of healthy and NAFLD/NASH patients (Supplementary Table 2: control n= 6  
309 patients; NAFLD/NASH n= 11 patients).



310 **Rebuttal Figure 8**

312 (a) tSNE representation, (b) marker expression, (c) average marker expression of defined T-  
313 cell subsets of patient-liver-derived T-cells analyzed by CYTOF of control and NAFLD/NASH  
314 patients (control n= 11 patients pooled in 3 analyses; NAFLD/NASH n= 16 patients pooled in  
315 5 analyses). (d) Composition, (e) HSNE representation of defined T-cell subsets and (f)  
316 quantification of CD8+CD103+PD-1+ cells of patient-liver-derived T-cells analyzed by CyTOF  
317 of control and NAFLD/NASH patients (control n= 11 patients pooled in 3 analyses;  
318 NAFLD/NASH n= 16 patients pooled in 5 analyses).



**322 Rebuttal Figure 9**

323 (a) NAS and BMI of patients used for scRNA-seq analyses of patient-liver-derived T-cells of  
324 control and NAFLD/NASH patients (control n= 4 patients; NAFLD/NASH n= 7 patients). (b)  
325 UMAP representation, marker expression, (c) relative quantification and (d), (e), (f) polarization  
326 of defined T-cell subsets of defined T-cell subsets of patient-liver-derived T-cells by scRNA-  
327 seq of control and NAFLD/NASH patients (control n= 4 patients; NAFLD/NASH n= 7 patients).  
328 (g) Differential gene expression of CD4+PD-1+ vs CD4+ T-cells and (h) selected average  
329 marker expression in CD4+ and CD8+ T-cell subsets of by scRNA-seq of control and  
330 NAFL/NA2SH patients (control n= 4 patients; NAFLD/NASH n= 7 patients).  
331

332 2. In relation to above-described limitations of the model, the paper does not sufficiently focus  
333 on dual functions of CD8+PD1+ T-cells, promoting NASH but possibly also restricting HCC.  
334 These functions are likely to occur at different stages in patients.

335

336 We thank Referee #1 for this important concern. We agree that the effects of CD8+PD1+ cells  
337 are executed at different time points. However, we would like to draw attention to the point that  
338 immunotherapy is considered to boost pre-existing inflammation (determined e.g. by  
339 evaluation of liver infiltration by immune cells using immunohistochemistry or flow cytometry  
340 for CD3, CD8, and PD-L1). Our data rather indicate that this certain population has no impact  
341 in restricting HCC development - in the context of NASH - and even immunotherapy. In fact,  
342 we show that depletion of CD8+ T-cells in NASH prevents NASH to HCC transition.

343 Thus, CD8+PD1+ T cells drive NASH which is exacerbated in the context of anti-PD1-related  
344 immunotherapy. We have now pointed this out more clearly, executed novel experiments to  
345 underline this point of early (NASH) and late time points (NASH to HCC transition) and have  
346 further discussed this in the discussion section (see also below) as well analyzed these cells  
347 in the context of human NASH.

348

349 To mirror the clinical status of the majority of patients at the time of diagnosis, we performed  
350 PD-1-targeted checkpoint inhibition in mice with pre-existing liver tumors (**Extended Data 6**  
351 **and 7** and **Rebuttal Figure 10, 11**) and performed now MRI-guided follow up.

352 Our data clearly show, that anti-PD1 or anti-PDL1-related immunotherapy does not stop or  
353 revert tumor burden but rather supports further tumor abundance. In contrast, when anti-CD8  
354 antibody therapy was applied, it decreased tumor incidence and thus development (**Figure 2,**  
355 **Extended Data 8** and **Rebuttal Figure 12q**). Furthermore, we underlined the importance of  
356 hepatic CD8+ T-cells abundance driving NASH-induced hepatocarcinogenesis by antibody-  
357 based treatments in our mouse model (anti-CD8/anti-NK1.1, anti-CD4, anti-TNF; included in  
358 **Figure 2 and 4, Extended Data 8, 9, 20-23** and **Rebuttal Figure 12b-d, 13-18**), as well as  
359 cross-referencing to the co-submitted manuscript Dudek et al., which describes molecular

360 mechanisms of CD8+ T-cell-mediated liver damage. Additionally, we dissected CD8+ T-cell  
361 mediated mechanisms driving NASH-induced hepatocarcinogenesis in PD1-targeted  
362 immunotherapy by antibody-based treatments (anti-CD8/anti-PD1, anti-TNF/anti-PD1, anti-  
363 CD4/anti-PD1; included in **Figure 4, Extended Data 20-23** and **Rebuttal Figure 12b-d, 15-  
364 18**). These data indicated that the abundance of CD8+ T-cells, as well as CD8+ T-cell-derived  
365 TNF plays an important role in boosting liver cancer in the context of NASH/HCC related  
366 immunotherapy. Of note, velocity analyses of scRNA-seq for transcriptional activation, or  
367 proteome analyses of sorted cells could not detect different phenotypes between CD8+PD1+  
368 T-cells derived from mice fed CDHFD with NASH or CDHFD treated with an anti-PD1 related  
369 therapy in the context of HCC development, indicating that the main proportion of CD8+PD1+  
370 T-cells in our preclinical models drive hepatocarcinogenesis and do not restrict HCC (included  
371 in **Figure 4, Extended Data 4 and 24** and **Rebuttal Figure 4b, 5c, 19**).

372 Further, our data show that anti-PDL1 therapy lead (included in **Extended Data 7** and **Rebuttal  
373 Figure 11**) to the same effects as observed in the anti-PD1 therapy (included in **Extended  
374 Data 6** and **Rebuttal Figure 10**) or in the context of our analyses using PD1 knock-out mice  
375 developing NASH/HCC (included in **Figure 3, Extended Data 14** and **Rebuttal Figure 20**).

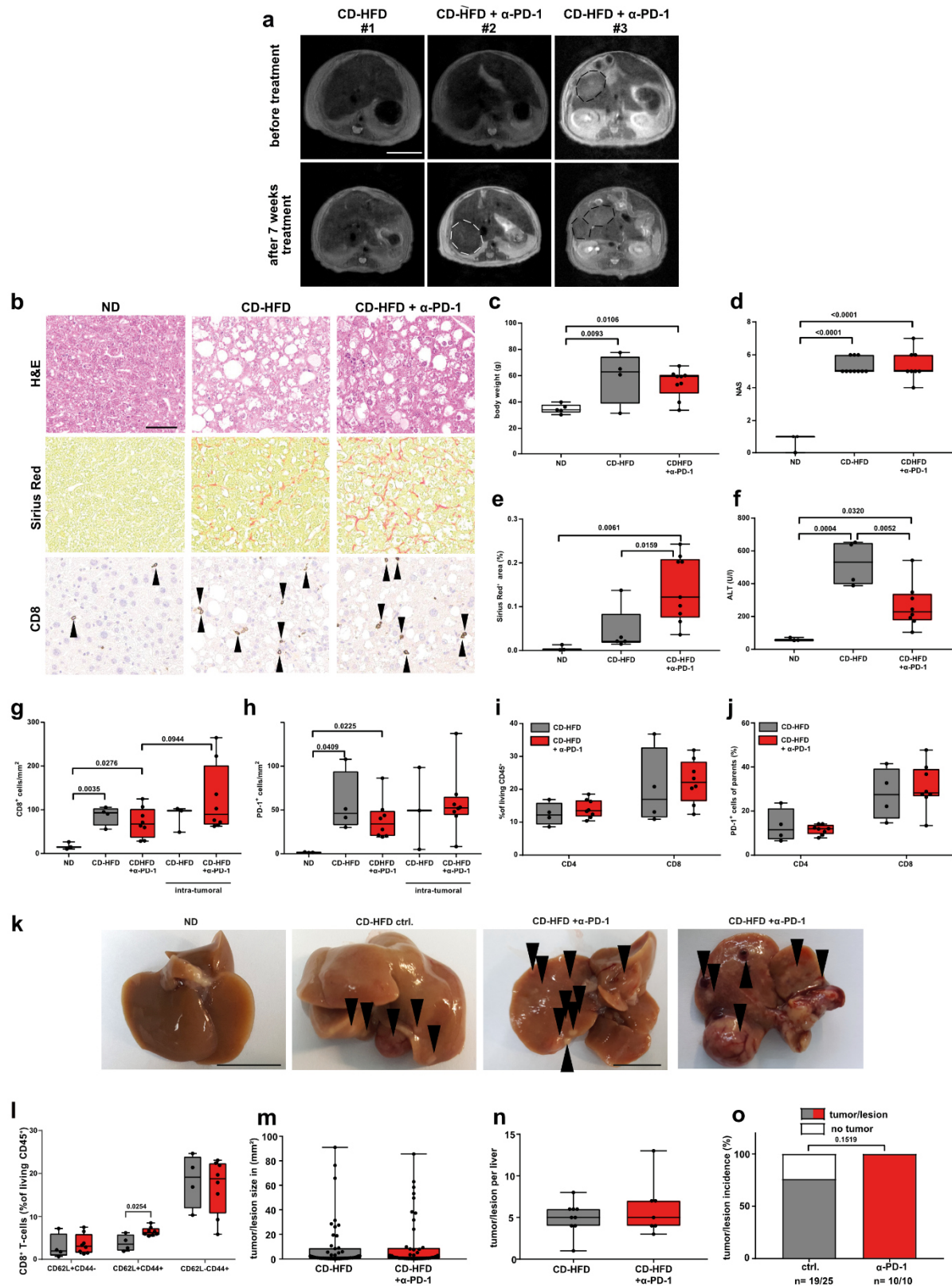
376 Data that have not been included in the initial submission of the manuscript indicate that PD-1  
377 targeted immunotherapy-induced hepatic inflammation triggers the enrichment of central  
378 memory-like cells (CD44+CD62L+CD8+) but not T-cells with a naïve character (CD62L+CD8+)  
379 (included in **Extended Data 6** and **Rebuttal Figure 10I**). This enrichment of memory-like  
380 CD44+CD62L+CD8+ T-cells can be explained by one of two options: these cells might be  
381 expanded and infiltrate the liver upon the anti-PD-1 targeted immunotherapy to either drive  
382 hepatic inflammation or these memory-like T-cells might be indicative of a subset of T-cells  
383 reactive to tumor-associated antigens and thus of CD8+ T-cells of a dual role (included in  
384 **Extended Data 6** and **Rebuttal Figure 10I**). In respect of the co-submitted manuscript Dudek  
385 et al., CD8+ T-cells drive liver damage and subsequently liver cancer in NASH in an antigen-  
386 independent manner, thus the enrichment of memory-like CD44+CD62L+CD8+ T-cells upon  
387 PD-1 targeted immunotherapy might argue in favor of a dual role of CD8 T-cells. However,  
388 tumor size, tumor number per liver, and tumor incidence are not affected by increased  
389 CD44+CD62L+CD8+ T-cells, arguing against a tumor restricting function of CD8 T-cells in this  
390 context.

391 Finally, we would like to draw again the attention to the improved cross-referencing of the  
392 revised manuscript to the co-submitted manuscript Dudek et al..

393 Data described in this manuscript demonstrate that the NASH-induced microenvironment  
394 drives hepatic inflammation in a TCR-independent manner and thus rather describes a



395 mechanism that activates CD8+T-cells downstream of the TCR through environmental  
396 signaling (e.g. acetate, IL21 signaling), arguing against a tumor antigen-specific CD8+ T-cells  
397 mediated HCC restriction in the context of NASH. It is exactly these CD8+ T-cells which –  
398 altered by the NASH liver microenvironment acquired a pro-tumorigenic phenotype – which  
399 we can detect also by analysis of the ICF signature – predictive of inflammation triggered liver  
400 cancer in humans. Notably, CD8 depletion eliminates this signature – strongly underlining that  
401 CD8 T cells are the main source of driving the pro-tumorigenic environment.



402

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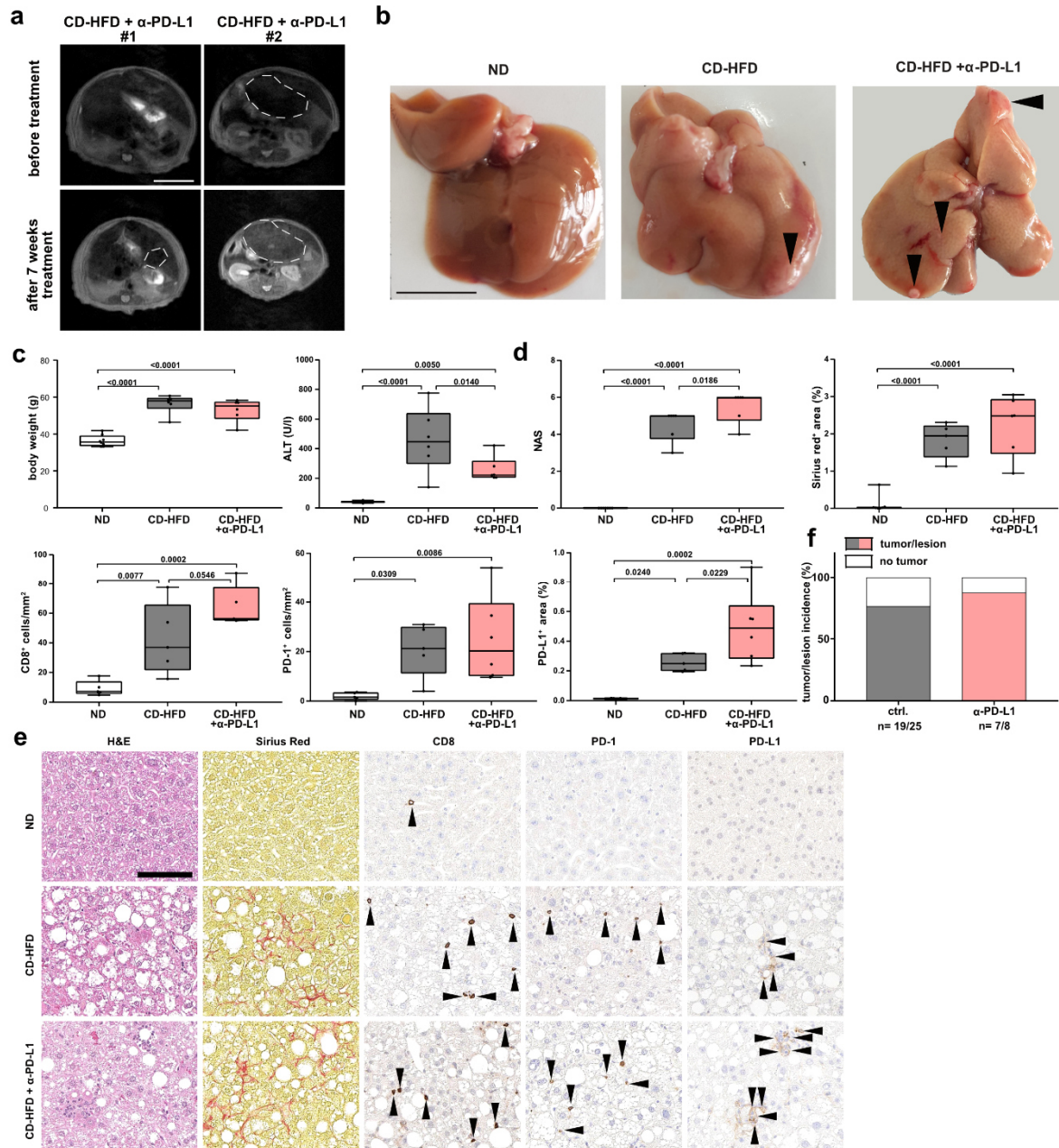
**Rebuttal Figure 10**

404

405

(a) MRI pictures of liver of mice after 13- months CD-HFD-fed mice followed by 7 weeks treatment of CD-HFD or CD-HFD + 7 weeks by  $\alpha$ -PD-1 antibodies (CD-HFD n= 6 mice; CD-

406 HFD +  $\alpha$ -PD-1 n= 4 mice). Lines indicate tumor nodule. Scale bar: 10 mm. (b) Histological  
407 staining of hepatic tissue by H&E, Sirius Red and CD8 of 15 months ND, CD-HFD or CD-HFD  
408 + 8 weeks treatment of  $\alpha$ -PD-1 (H&E: ND n= 3 mice; CD-HFD n= 10 mice; CD-HFD +  $\alpha$ -PD-1  
409 n= 8 mice; Sirius Red: ND n= 3 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 9 mice; CD8:  
410 ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 3 mice). Scale bar: 50  $\mu$ m.  
411 Arrowheads indicate CD8+ cells. (c) Body weight of 15 months ND, CD-HFD or CD-HFD + 8  
412 weeks treatment of  $\alpha$ -PD-1 (ND n= 5 mice; CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 9 mice).  
413 (d) NAS evaluation by H&E of 15 months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -  
414 PD-1 (ND n= 3 mice; CD-HFD n= 10 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). (e) Fibrosis  
415 evaluation of Sirius Red staining of 15 months ND, CD-HFD or CD-HFD + 8 weeks treatment  
416 of  $\alpha$ -PD-1 (ND n= 3 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 9 mice). (f) ALT levels of  
417 15 months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1 (ND n= 3 mice; CD-HFD  
418 n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). (g) Quantification of CD8 and (h) PD-1 staining of  
419 hepatic tissue by immunohistochemistry of 15 months ND, CD-HFD or CD-HFD + 8 weeks  
420 treatment of  $\alpha$ -PD-1 (ND n= 3 mice; CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice; intra-  
421 tumoral staining: CD-HFD n= 3 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). (i) Quantification and (j)  
422 expression of PD-1 of hepatic CD4+ and CD8+ T-cells by flow cytometry of 15 months CD-  
423 HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1 (CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 8  
424 mice). (k) Macroscopic images of liver of 15 months ND, CD-HFD or CD-HFD + 8 weeks  
425 treatment of  $\alpha$ -PD-1. Arrowheads indicate tumor/lesions. Scale bar: 10 mm. (l) Quantification  
426 of CD8+ T-cells by flow cytometry of 15 months CD-HFD or CD-HFD + 8 weeks treatment of  
427  $\alpha$ -PD-1 (ND n= 3 mice; CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). (m) Quantification  
428 of tumor/lesion size, (n) tumor load and (o) tumor incidence of 15 months CD-HFD or CD-HFD  
429 + 8 weeks treatment of  $\alpha$ -PD-1 (tumor/lesion size and tumor load: CD-HFD n= 9 mice; CD-  
430 HFD +  $\alpha$ -PD-1 n= 7 mice; tumor incidence: CD-HFD n= 17 tumors/lesions in 22 mice; CD-HFD  
431 +  $\alpha$ -PD-1 n= 10 tumors/lesions in 10 mice).



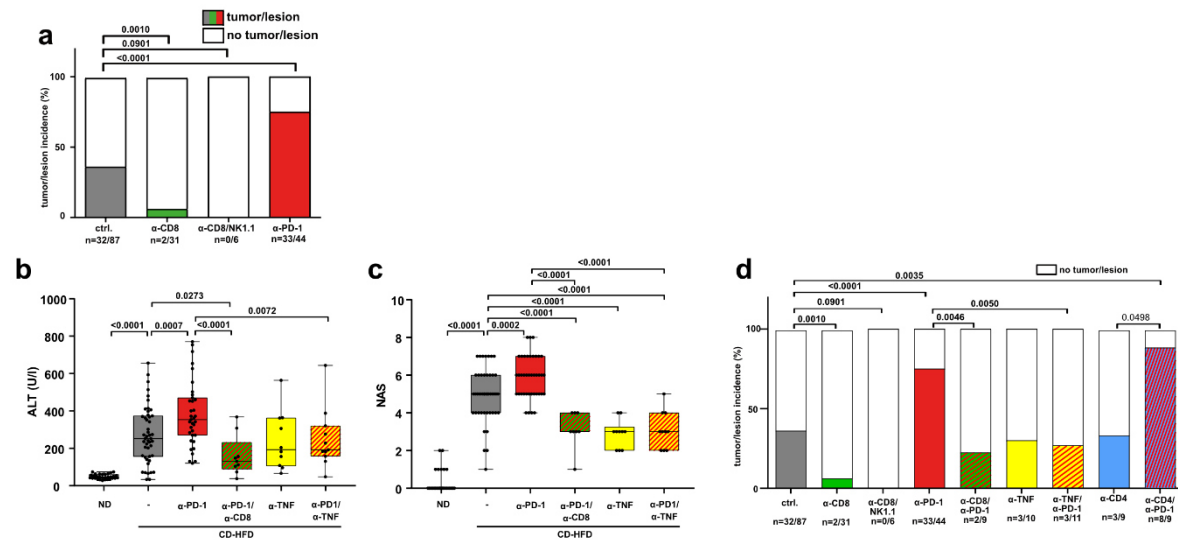
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433 **Rebuttal Figure 11**

434 (a) MRI pictures of liver of mice after 13 months CD-HFD followed by 7 weeks treatment to  
 435 CD-HFD or CD-HFD-fed mice + 7 weeks by  $\alpha$ -PD-L1 antibodies (CD-HFD n= 6 mice; CD-HFD  
 436 +  $\alpha$ -PD-L1 n= 8 mice). Lines indicate tumor nodule. Scale bar: 10 mm. (b) Macroscopic images  
 437 of liver of 12 months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-L1. Arrowheads  
 438 indicate tumor/lesions. Scale bar: 10 mm. (c) Body weight, ALT levels of 12 months ND, CD-  
 439 HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-L1 (Body weight, ALT, : ND n= 8 mice; CD-HFD  
 440 n= 6 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice) (d) and (e) NAS evaluation by H&E, Fibrosis  
 441 evaluation of Sirius Red staining, quantification of CD8, PD-1 and PD-L1 staining of hepatic  
 442 tissue by immunohistochemistry of 12 months ND, CD-HFD or CD-HFD + 8 weeks treatment  
 443 of  $\alpha$ -PD-L1 (NAS: ND n= 7 mice; CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; Sirius  
 444 Red: ND n= 7 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; CD8, : ND n= 5 mice;  
 445 CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 5 mice; PD-1, PD-L1: ND n= 5 mice; CD-HFD n=



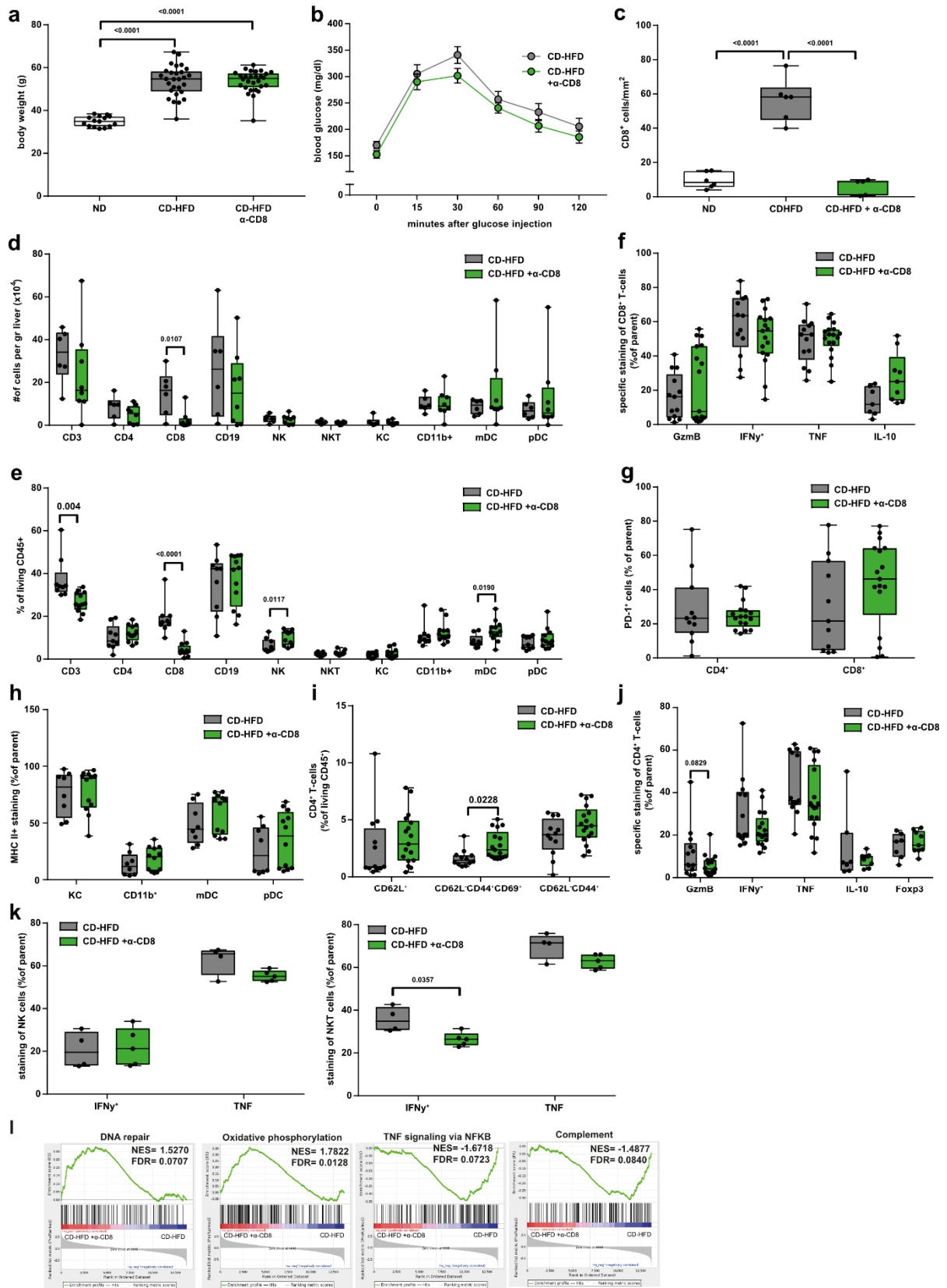
446 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice). Scale bar: 100  $\mu$ m. (f) Tumor/Lesion incidence in CD-  
 447 HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-L1 fed mice (CD-HFD n= 19 tumors/lesions in  
 448 25 mice; CD-HFD +  $\alpha$ -PD-L1 n= 7 tumors/lesions in 8 mice). Arrowheads indicate specific  
 449 staining positive cells.  
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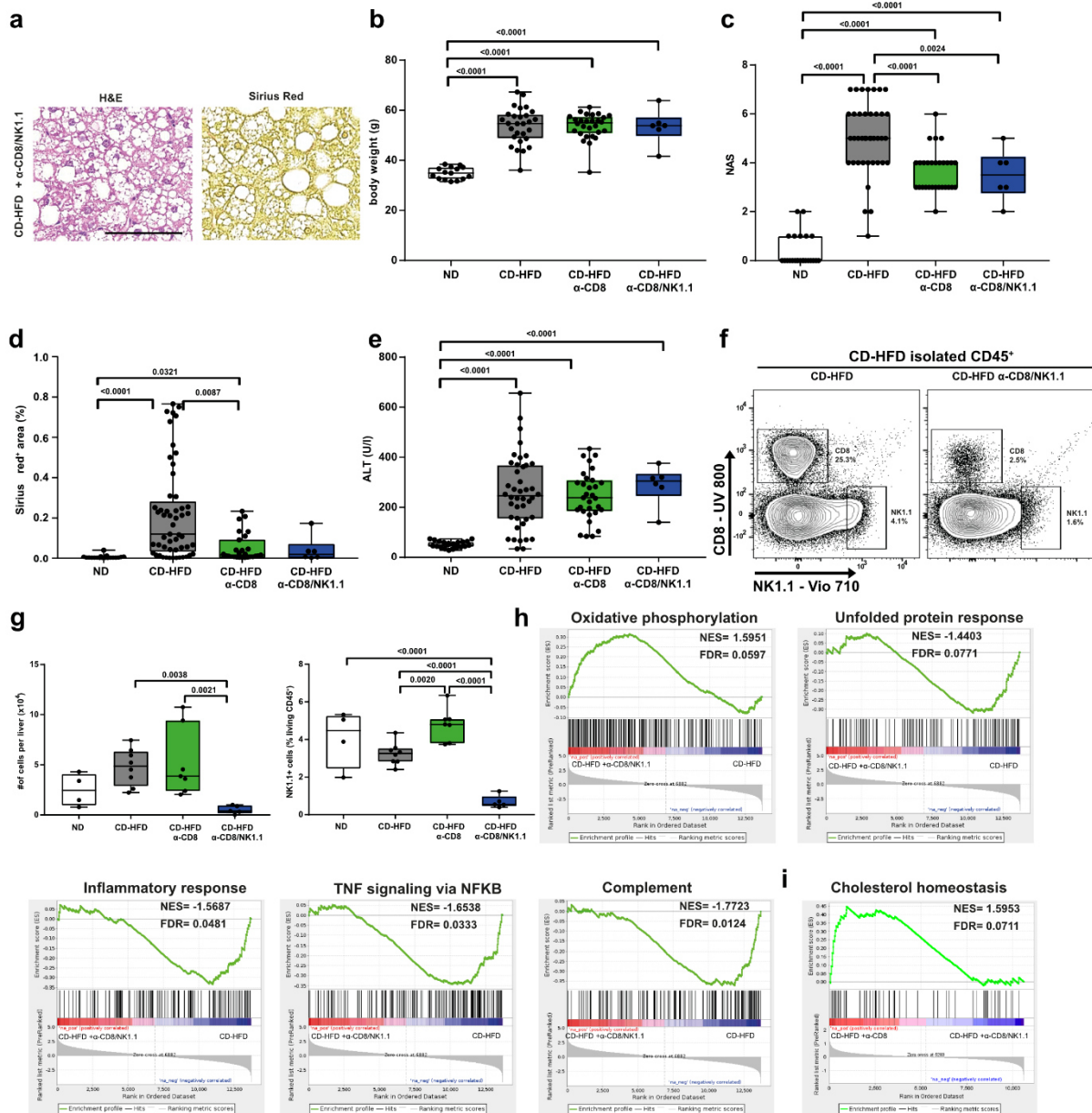
## Rebuttal Figure 12

453 (a) Quantification of tumor incidence of 12 months CD-HFD or CD-HFD + 8 weeks treatment  
 454 of  $\alpha$ -CD8, co-depletion of  $\alpha$ -CD8/NK1, or  $\alpha$ -PD-1 (tumor incidence: CD-HFD n= 32  
 455 tumors/lesions in 87 mice; CD-HFD +  $\alpha$ -CD8 n= 2 tumors/lesions in 31 mice; CD-HFD +  $\alpha$ -  
 456 CD8/NK1.1 n= n= 0 tumors/lesions in 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 33 tumors/lesions in 44  
 457 mice). (b) ALT and (c) NAS evaluation of 12 months ND, CD-HFD, CD-HFD + 8 weeks  
 458 treatment of  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF, or  $\alpha$ -PD-1/ $\alpha$ -TNF fed mice (ND n= 30 mice; CD-  
 459 HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-  
 460 HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). (d) Quantification of tumor  
 461 incidence of 12 months CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8,  $\alpha$ -CD8/NK1.1,  $\alpha$ -  
 462 PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF fed mice,  $\alpha$ -CD4, or  $\alpha$ -PD-1/ $\alpha$ -CD fed mice 1  
 463 (tumor incidence: CD-HFD n= 32 tumors/lesions in 87 mice; CD-HFD +  $\alpha$ -CD8 n= 2  
 464 tumors/lesions in 31 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 0 tumors/lesions in 6 mice; CD-HFD +  
 465  $\alpha$ -PD-1 n= 33 tumors/lesions in 44 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 2 tumors/lesions in 9  
 466 mice; CD-HFD +  $\alpha$ -TNF n= 3 tumors/lesions in 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 3  
 467 tumors/lesions in 11 mice); CD-HFD +  $\alpha$ -CD4 n= 3 tumors/lesions in 9 mice; CD-HFD +  $\alpha$ -PD-  
 468 1/ $\alpha$ -CD4 n= 8 tumors/lesions in 9 mice).



**472 Rebuttal Figure 13**

473 (a) Body weight of 12 months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 (ND n=  
474 15 mice; CD-HFD n= 28 mice; CD-HFD +  $\alpha$ -CD8 n= 28 mice). (b) Assessment of metabolic  
475 tolerance by intra peritoneal glucose tolerance test of 12 months CD-HFD or CD-HFD + 8  
476 weeks treatment of  $\alpha$ -CD8 (CD-HFD n= 8 mice; CD-HFD +  $\alpha$ -CD8 n= 10 mice). (c)  
477 Quantification of CD8 staining of hepatic tissue by immunohistochemistry of 12 months ND,  
478 CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed mice (ND n= 6 mice; CD-HFD n= 6  
479 mice; CD-HFD +  $\alpha$ -CD8 n= 5 mice). (d) Absolute and (e) relative quantification of hepatic  
480 leukocytes of 12 months CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed mice (CD-  
481 HFD n= 9 mice; CD-HFD +  $\alpha$ -CD8 n= 12 mice). (f) Cytokine expression for polarization of  
482 hepatic CD8+ T-cells of 12 months CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed  
483 mice (GzmB, IFN $\gamma$ , TNF: CD-HFD n= 13 mice;  $\alpha$ -CD8 + CD-HFD n= 17 mice; IL-10: CD-HFD  
484 n= 7 mice;  $\alpha$ -CD8 + CD-HFD n= 9 mice). (g) Expression of PD-1 of hepatic CD4+ and CD8+  
485 T-cells by flow cytometry of 12 months CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed  
486 mice (CD-HFD n= 11 mice;  $\alpha$ -CD8 + CD-HFD n= 17 mice). (h) Flow cytometry analysis for  
487 polarization of hepatic myeloid cells of 12 months CD-HFD or CD-HFD + 8 weeks treatment of  
488  $\alpha$ -CD8 fed mice (CD-HFD n= 8 mice;  $\alpha$ -CD8 + CD-HFD n= 12 mice). (i) Flow cytometric  
489 analysis for polarization of hepatic CD4+ T-cells of 12 months CD-HFD or CD-HFD + 8 weeks  
490 treatment of  $\alpha$ -CD8 fed mice (CD-HFD n= 12 mice;  $\alpha$ -CD8 + CD-HFD n= 17 mice). (j) Cytokine  
491 expression of hepatic CD4+ T-cells of 12 months CD-HFD or CD-HFD + 8 weeks treatment of  
492  $\alpha$ -CD8 fed mice (GzmB, IFN $\gamma$ , TNF: CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -CD8 n= 17 mice; IL-  
493 10, Foxp3: CD-HFD n= 7 mice; CD-HFD +  $\alpha$ -CD8 n= 9 mice). (k) Cytokine expression for  
494 polarization of hepatic NK and NKT-cells of 12 months CD-HFD or CD-HFD + 8 weeks  
495 treatment of  $\alpha$ -CD8 fed mice (CD-HFD n= 4 mice;  $\alpha$ -CD8 + CD-HFD n= 5 mice). (l) Gene set  
496 enrichment analysis of RNA sequencing data of hepatic tissue comparing CD-HFD with CD-  
497 HFD +  $\alpha$ -CD8 of 12 months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed mice  
498 (n= 5 mice/group).



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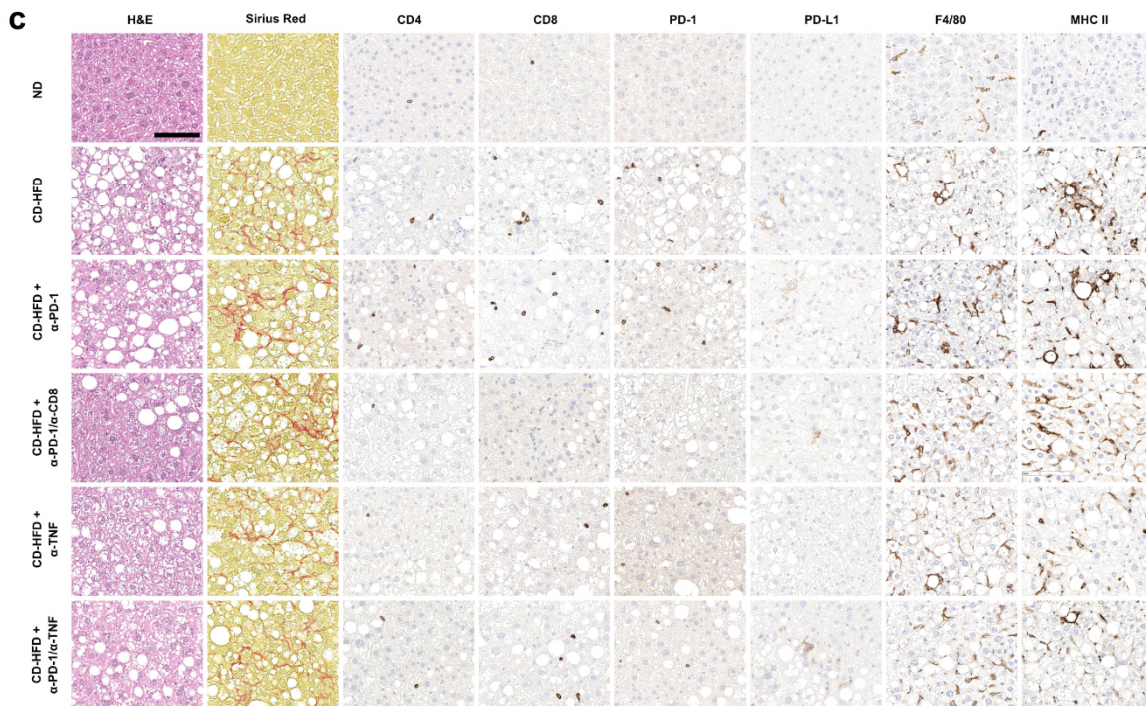
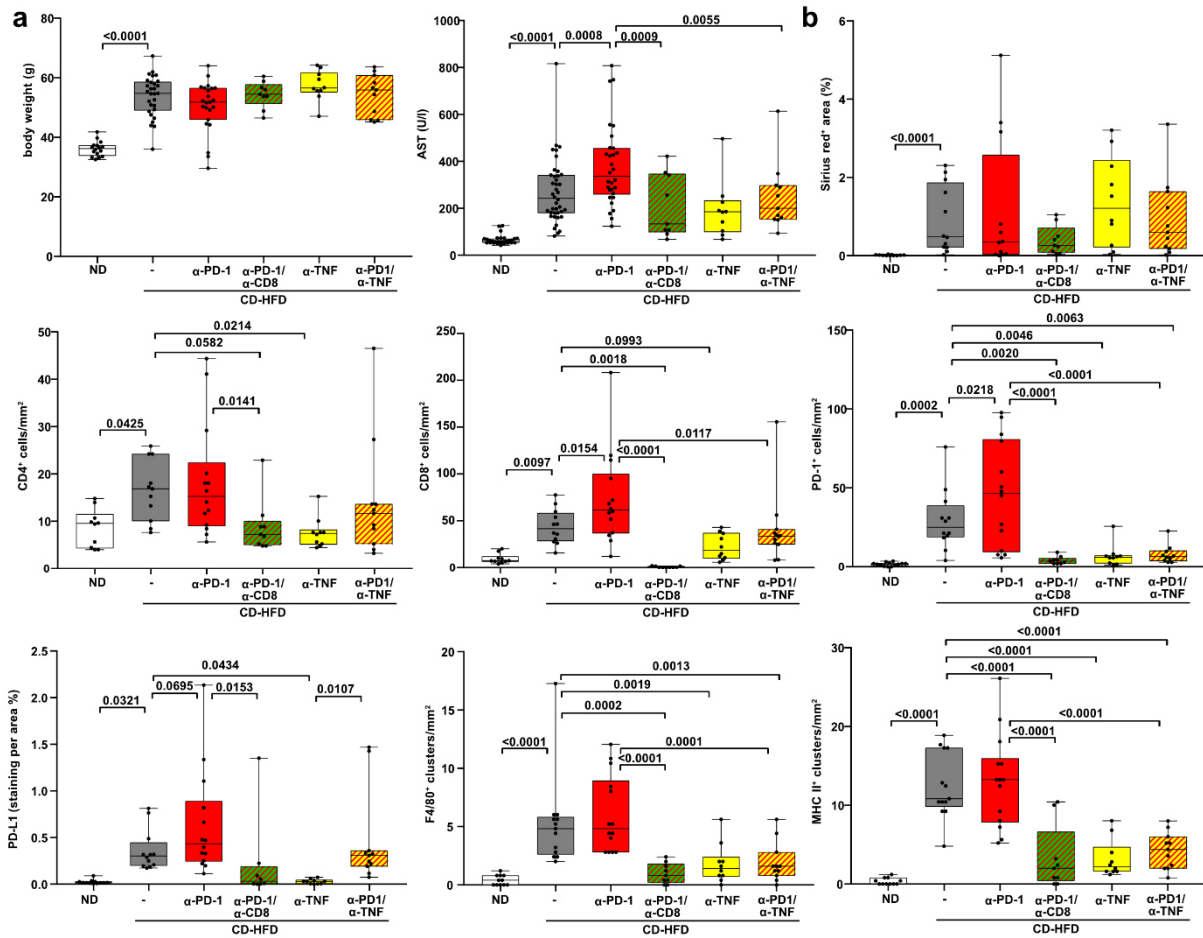
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## Rebuttal Figure 14

501 (a) H&E and Sirius Red staining, (b) body weight, (c) NAS evaluation by H&E, (d) fibrosis  
 502 evaluation of Sirius Red and (e) ALT levels of 12 months ND, CD-HFD, CD-HFD + 8 weeks  
 503 treatment of  $\alpha$ -CD8 or CD-HFD + 8 weeks co-depletion of  $\alpha$ -CD8/NK1.1 (body weight: ND n =  
 504 15 mice; CD-HFD n = 28 mice; CD-HFD +  $\alpha$ -CD8 n = 28 mice; fibrosis ND n = 19 mice; CD-HFD  
 505 n = 53 mice; CD-HFD +  $\alpha$ -CD8 n = 27 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n = 6 mice; NAS: ND n =  
 506 24 mice; CD-HFD n = 40 mice; CD-HFD +  $\alpha$ -CD8 n = 29 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n = 6;  
 507 ALT: ND n = 22 mice; CD-HFD n = 42 mice; CD-HFD +  $\alpha$ -CD8 n = 31 mice; CD-HFD +  $\alpha$ -  
 508 CD8/NK1.1 n = 6). Scale bar: 100  $\mu$ m. (f) Flow cytometry plots and (g) quantification of hepatic  
 509 NK1.1 abundance of 12 months ND, CD-HFD, CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 or CD-  
 510 HFD + 8 weeks co-depletion of  $\alpha$ -CD8/NK1.1 (ND n = 4 mice; CD-HFD n = 8 mice; CD-HFD +  
 511  $\alpha$ -CD8 n = 7 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n = 6 mice). (h) Gene set enrichment analysis of  
 512 RNA sequencing data of hepatic tissue comparing CD-HFD with CD-HFD + co-depletion of  $\alpha$ -  
 513 CD8/NK1.1 of 12 months ND, CD-HFD or CD-HFD + co-depletion of  $\alpha$ -CD8/NK1.1 (n = 5  
 514 mice/group). (i) Gene set enrichment analysis of RNA sequencing data of hepatic tissue



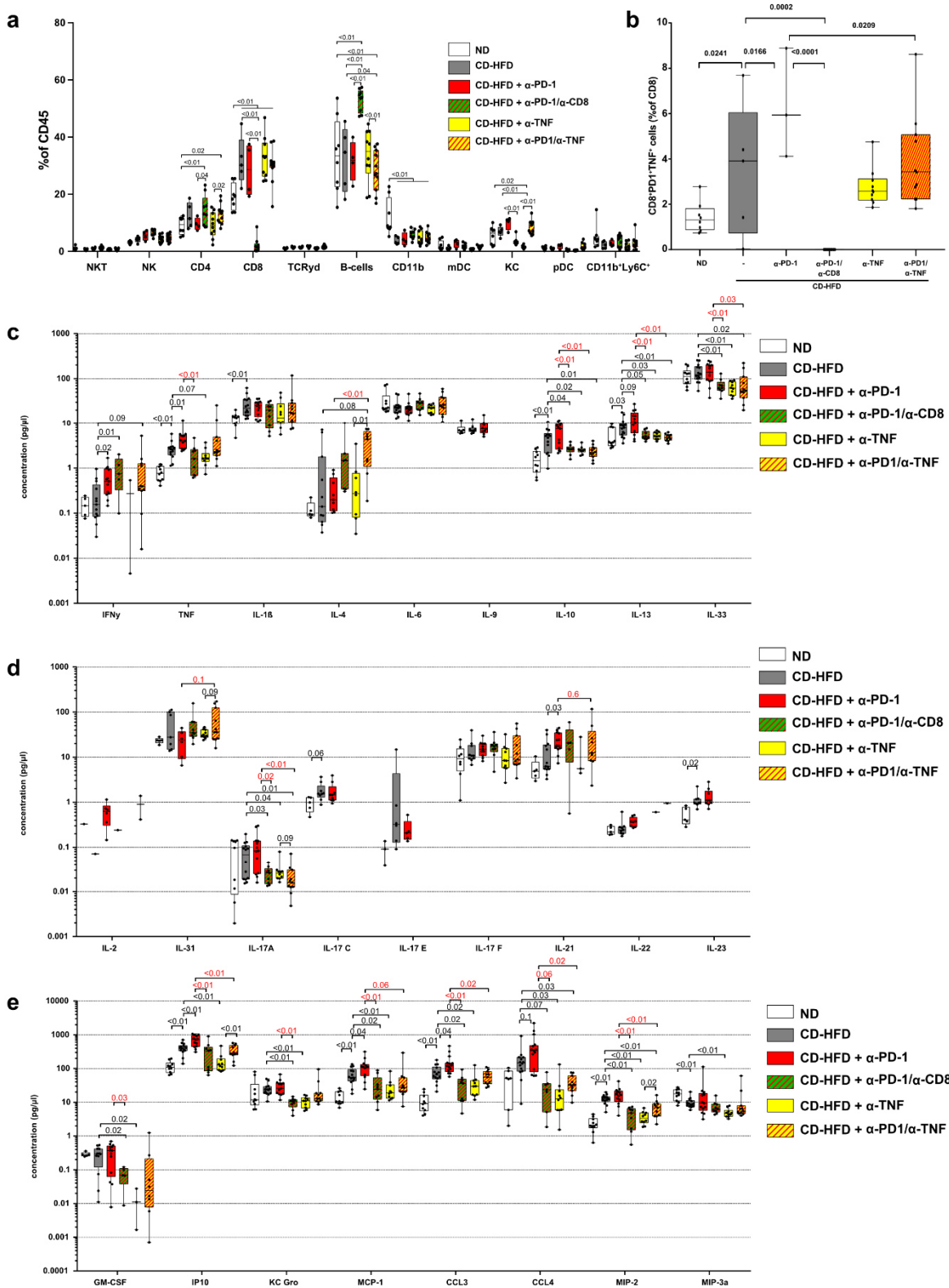
515 comparing or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed mice with CD-HFD + co-depletion of  
516  $\alpha$ -CD8/NK1.1 of 12 months ND, CD-HFD, CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed or CD-  
517 HFD + co-depletion of  $\alpha$ -CD8/NK1.1 (n= 5 mice/group)



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**521 Rebuttal Figure 15**

522 (a) Body weight, AST, and histological evaluation by (b) Sirius red, CD4, CD8, PD-1, PD-L1,  
523 F4/80, MHC-II and (c) staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  
524  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (body weight: ND n= 16 mice; CD-  
525 HFD n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-  
526 HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; AST: body weight: ND n= 30  
527 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
528 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; Sirius red: ND n=  
529 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
530 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD4: ND n= 10  
531 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
532 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8: ND n= 10  
533 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-  
534 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n=  
535 11 mice; PD-1: ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD  
536 +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 8 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 10  
537 mice; PD-L1: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  
538  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice;  
539 F4/80: ND n= 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-  
540 1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  
541  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1  
542 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  
543  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice).



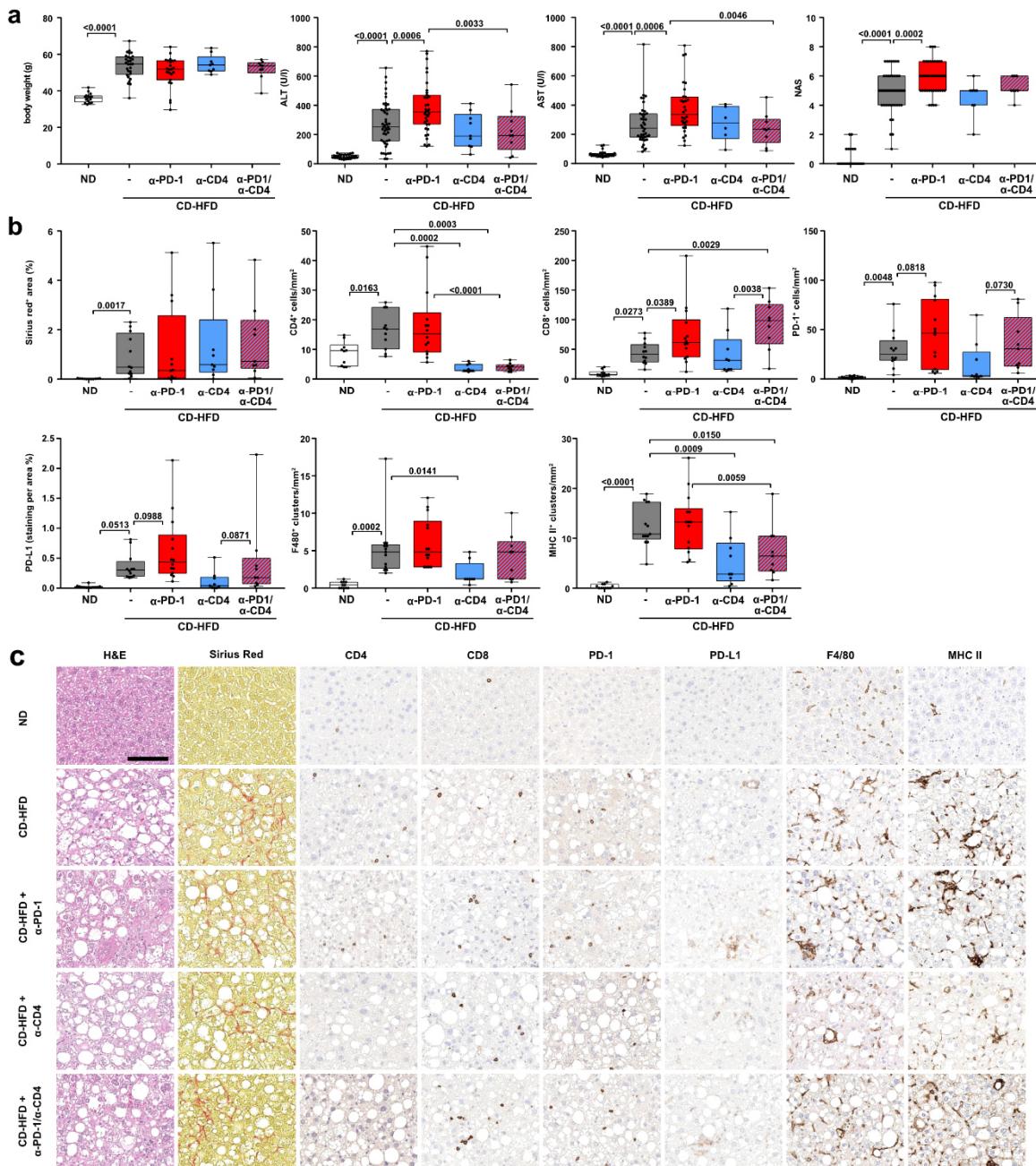
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**Rebuttal Figure 16**

(a) Quantification of hepatic immune cell composition and (b) CD8+PD-1+TNF+ T-cells by flow cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-PD-1/α-CD8, α-TNF, α-PD-1/α-TNF antibodies (Hepatic immune cell composition: ND n= 8



549 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice;  
 550 CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8+PD-1+TNF+: ND  
 551 n= 8 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 3 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
 552 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). (c) and (d)  
 553 multiplex ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12  
 554 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -  
 555 TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n=  
 556 13 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-  
 557 1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-  
 558 1/ $\alpha$ -TNF n= 11 mice).



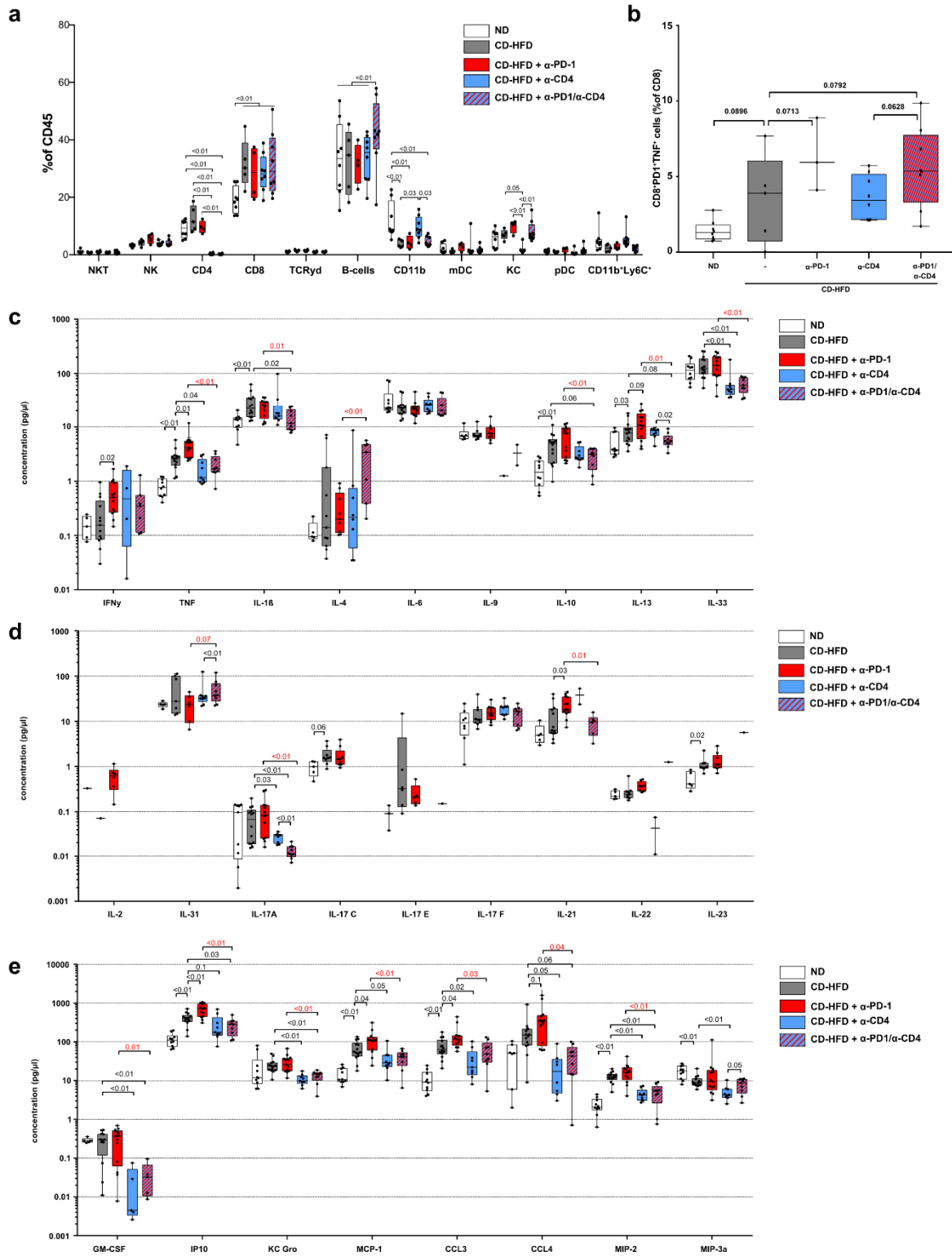
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### Rebuttal Figure 17

(a) Body weight, ALT, AST, NAS, and histological evaluation by (b) Sirius Red, CD4, CD8, PD-1, PD-L1, F4/80, MHC-II and (c) staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks



563 treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (body weight: ND n= 16 mice; CD-HFD  
564 n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-  
565 1/ $\alpha$ -CD4 n= 9 mice; ALT ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice;  
566 CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; AST: ND n= 30 mice; CD-  
567 HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -  
568 PD-1/ $\alpha$ -CD4 n= 9 mice; NAS: ND n= 31 mice; CD-HFD n= 46 mice; CD-HFD +  $\alpha$ -PD-1 n= 40  
569 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice; Sirius red: ND n= 11  
570 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-  
571 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; CD4: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-  
572 1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 11 mice; CD8: ND  
573 n= 10 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice;  
574 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-1: ND n= 13 mice; CD-HFD n= 12 mice; CD-HFD +  
575  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-L1:  
576 ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9  
577 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; F4/80: ND n= 11 mice; CD-HFD n= 13 mice; CD-  
578 HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice;  
579 MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -  
580 PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice). Scale  
581 bar: 100  $\mu$ m. All data are shown as mean  $\pm$  SEM.

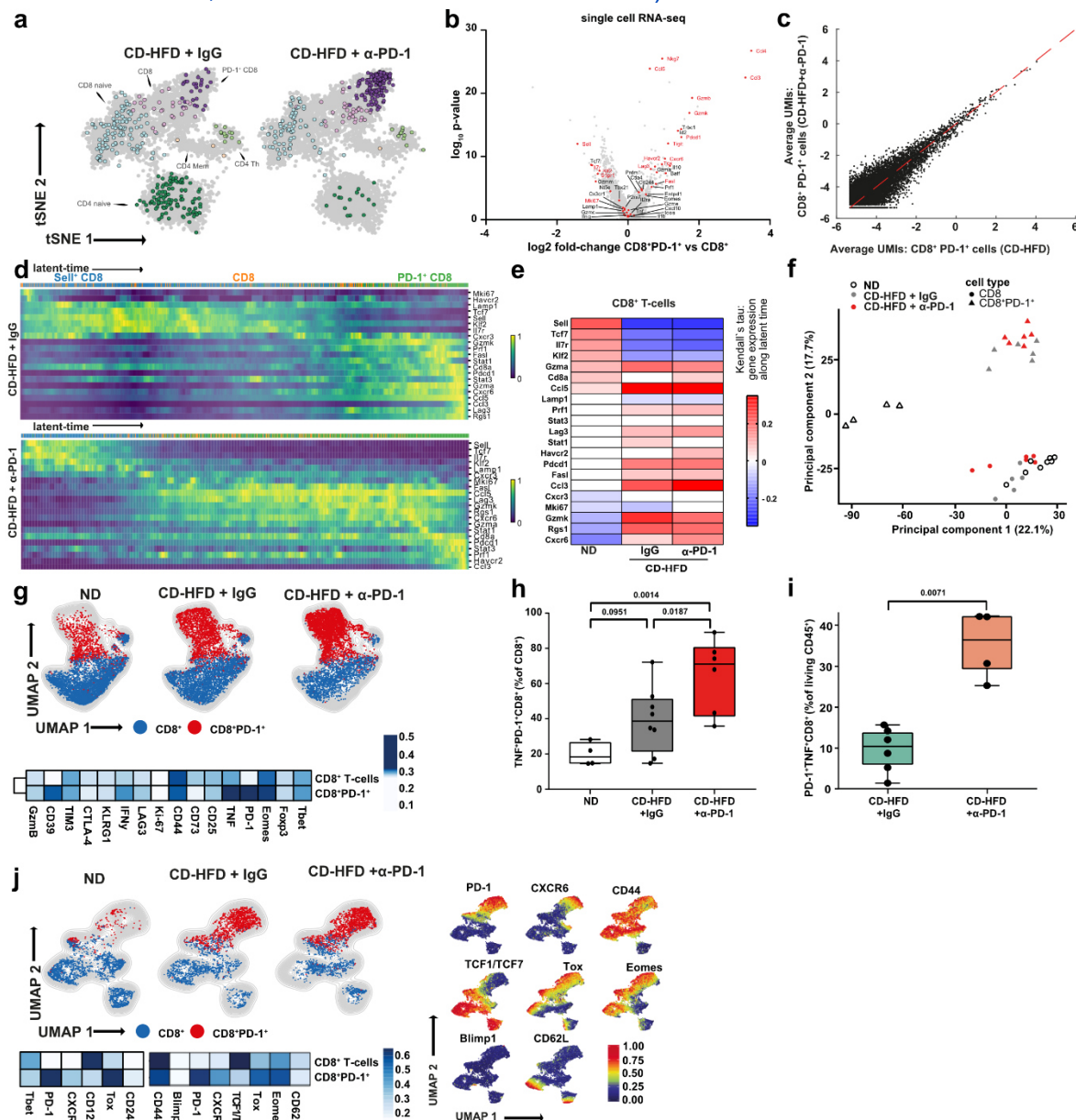


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### Rebuttal Figure 18

(a) Quantification of hepatic immune cell composition and (b) CD8+PD-1+TNF+ T-cells by flow cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (Hepatic immune cell composition: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice; CD8+PD-1+TNF+: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n=

589 3 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice). (c) and (d) multiplex  
 590 ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12 months ND,  
 591 CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4  
 592 antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 13 mice; CD-HFD +  
 593  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).



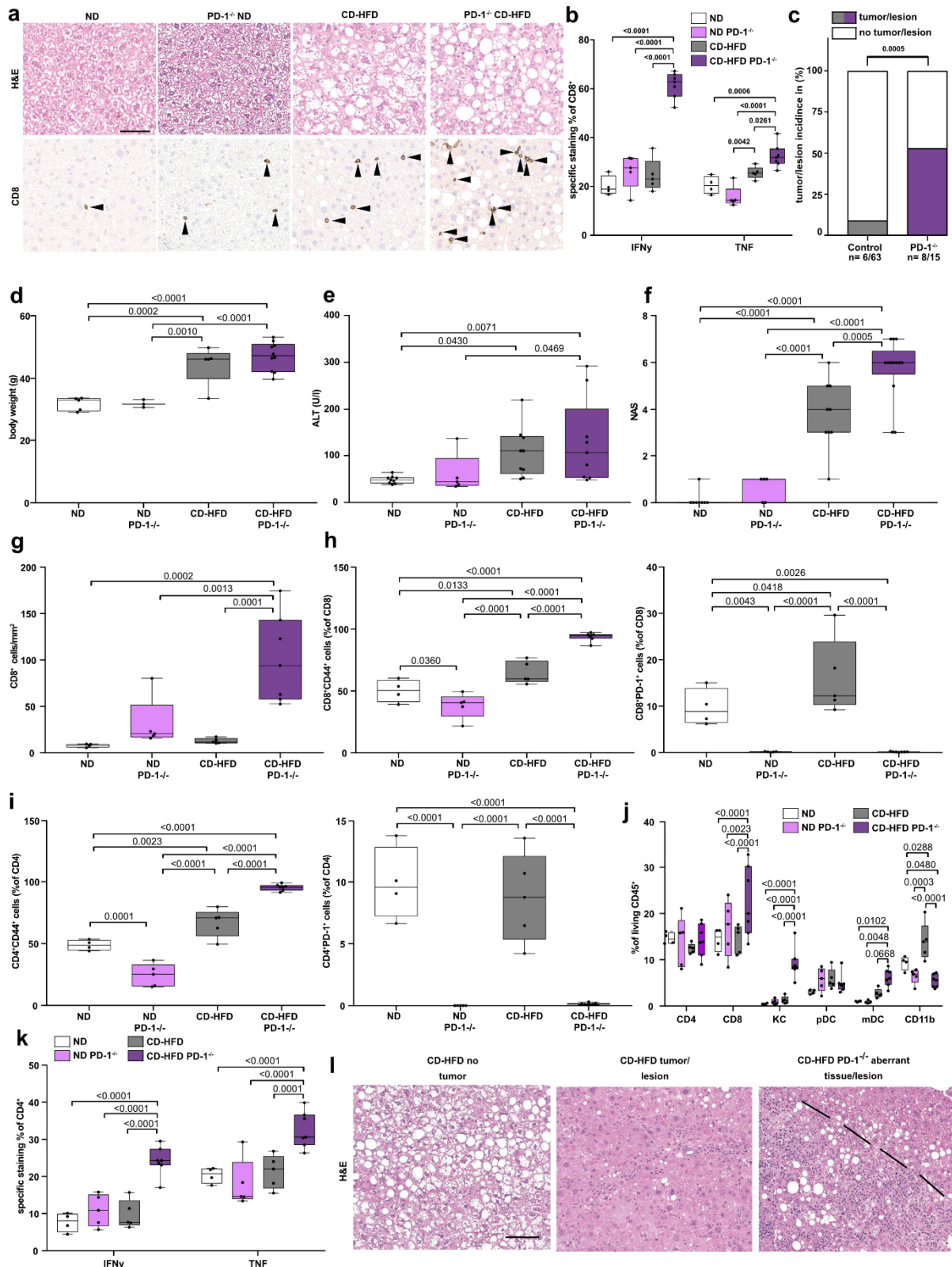
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### Rebuttal Figure 19

596 (a) scRNA-seq analysis of hepatic TCR $\beta$ <sup>+</sup> cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 or  $\alpha$ -CD8 antibodies (n= 3 mice/group). (b) Selected  
 597 marker expression in hepatic CD8<sup>+</sup> T-cells by scRNA-seq comparing CD8<sup>+</sup> with CD8<sup>+</sup>PD-1<sup>+</sup>  
 598 T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1  
 599 antibodies (n= 3 mice/group). (c) Average UMI comparison of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of  
 600 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n=  
 601 3 mice/group). (d) RNA velocity analyses of scRNA-seq data showing expression and (e)  
 602 correlation of expression along the latent-time of selected genes along the latent-time (n= 3  
 603 mice/group). Root cells: yellow cells indicate root cells, blue cells indicate cells farthest away  
 604 from root by RNA velocity. End points: yellow cells indicate end point cells, blue cells indicate  
 605



606 cells farthest away from defined end point cells by RNA velocity. Latent time: pseudo-time by  
607 RNA velocity, dark color indicate start of RNA velocity, yellow color indicate end point of latent  
608 time. RNA velocity flow: Blue cluster defined as start point, orange cluster as intermediate,  
609 green cluster as end point. Arrows indicate trajectory of cells. (f) PCA plot of hepatic CD8+ or  
610 CD8+PD-1+ T-cells sorted TCR $\beta$ + cells by mass spectrometry of 12 months ND, CD-HFD or  
611 CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD8+: ND n= 6 mice, CD-HFD  
612 + IgG n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice; CD8+PD-1+: ND n= 4 mice, CD-HFD + IgG n=  
613 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (g) UMAP representation showing the FlowSOM-guided  
614 clustering, heatmap showing the median marker expression, and (h) quantification of hepatic  
615 CD8+ T-cells of 12 months ND, CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  
616  $\alpha$ -PD-1 antibodies (ND n= 4 mice; CD-HFD + IgG n= 8 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (i)  
617 Quantification of CellCNN analyzed flow cytometry data of hepatic CD8+ T-cells of 12 months  
618 CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD +  
619 IgG n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice). (j) UMAP representation showing the FlowSOM-  
620 guided clustering, the expression intensity of the indicated marker and heatmap showing the  
621 median marker expression of flow cytometry data of hepatic CD8+PD-1+ T-cells of 12 months  
622 ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 6 mice;  
623 CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice).



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**Rebuttal Figure 20**

(a) Histological staining of hepatic tissue by H&E and CD8 of 6 months ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (H&E: ND n= 8 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 9 mice; PD-1<sup>-/-</sup> CD-HFD n= 13 mice; CD8: ND n= 4 mice; CD-HFD n= 5 mice; PD-1<sup>-/-</sup> CD-HFD n= 7 mice).

629 Arrowheads indicate CD8+ cells. Scale bar: 50  $\mu$ m. (b) Cytokine expression of hepatic CD8+  
630 T-cells of 6 months ND, PD-1-/- ND, CD-HFD or PD-1-/- CD-HFD-fed mice (ND n= 4 mice; PD-  
631 1-/- ND n= 5 mice; CD-HFD n= 5 mice; PD-1-/- CD-HFD n= 6 mice). (c) Tumor/lesion incidence  
632 of 6 months CD-HFD or PD-1-/- CD-HFD-fed mice (tumor incidence: CD-HFD n= 6  
633 tumors/lesions in 63 mice; PD-1-/- CD-HFD n= 6 tumors/lesions in 13 mice). (d) Body weight  
634 of 6 months ND, PD-1-/- ND, CD-HFD or PD-1-/- CD-HFD-fed mice (ND n= 5 mice; PD-1-/-  
635 ND n= 3 mice; CD-HFD n= 5 mice; PD-1-/- CD-HFD n= 10 mice). (e) ALT levels of ND, PD-1-/-  
636 ND, CD-HFD or PD-1-/- CD-HFD (ND n= 9 mice; PD-1-/- ND n= 5 mice; CD-HFD n= 9 mice;  
637 PD-1-/- CD-HFD n= 10 mice). (f) NAS evaluation by H&E of ND, PD-1-/- ND, CD-HFD or PD-  
638 1-/- CD-HFD fed mice (ND n= 8 mice; PD-1-/- ND n= 5 mice; CD-HFD n= 9 mice; PD-1-/- CD-  
639 HFD n= 13 mice). (g) CD8 staining of hepatic tissue by immunohistochemistry of 6 months ND,  
640 PD-1-/- ND, CD-HFD or PD-1-/- CD-HFD fed mice (ND n= 4 mice; PD-1-/- ND n= 5 mice; CD-  
641 HFD n= 5 mice; PD-1-/- CD-HFD n= 7 mice). (h) – (j) Characterization of hepatic T-cells by  
642 flow cytometry of 6 months ND, PD-1-/- ND, CD-HFD or PD-1-/- CD-HFD fed mice (ND n= 4  
643 mice; PD-1-/- ND n= 5 mice; CD-HFD n= 5 mice; PD-1-/- CD-HFD n= 6 mice). (k) Relative  
644 quantification of hepatic leukocytes of 6 months CD-HFD or PD-1-/- CD-HFD fed mice (ND n=  
645 4 mice; PD-1-/- ND n= 5 mice; CD-HFD n= 5 mice; PD-1-/- CD-HFD n= 6 mice). (l) Histological  
646 staining of hepatic tissue by H&E of CD-HFD or PD-1-/- CD-HFD fed mice (ND n= 8 mice; CD-  
647 HFD n= 9 mice; PD-1-/- CD-HFD n= 13 mice). Dotted line indicates tumor/lesion border. Scale  
648 bar: 100  $\mu$ m.  
649

650 3. The data on the NASH- and NASH-HCC-promoting role of CD8+ T-cells is similar to a  
651 previous study from the last author (Wolf et al, Cancer Cell). Hence a number of the findings  
652 presented in this manuscript are incremental with, adding PD1 into this context, with somewhat  
653 expected results, as well as novel techniques such as scRNA-seq.  
654

655 We thank Referee #1 for the opinion on the progress we tried to achieve with this manuscript  
656 as a follow-up study (Wolf et al., 2014). We politely disagree with the statement of Referee #1  
657 – that indicates “...are incremental with, adding PD1 into this context, with somewhat expected  
658 results, as well as novel techniques such as scRNA-seq.”, because:

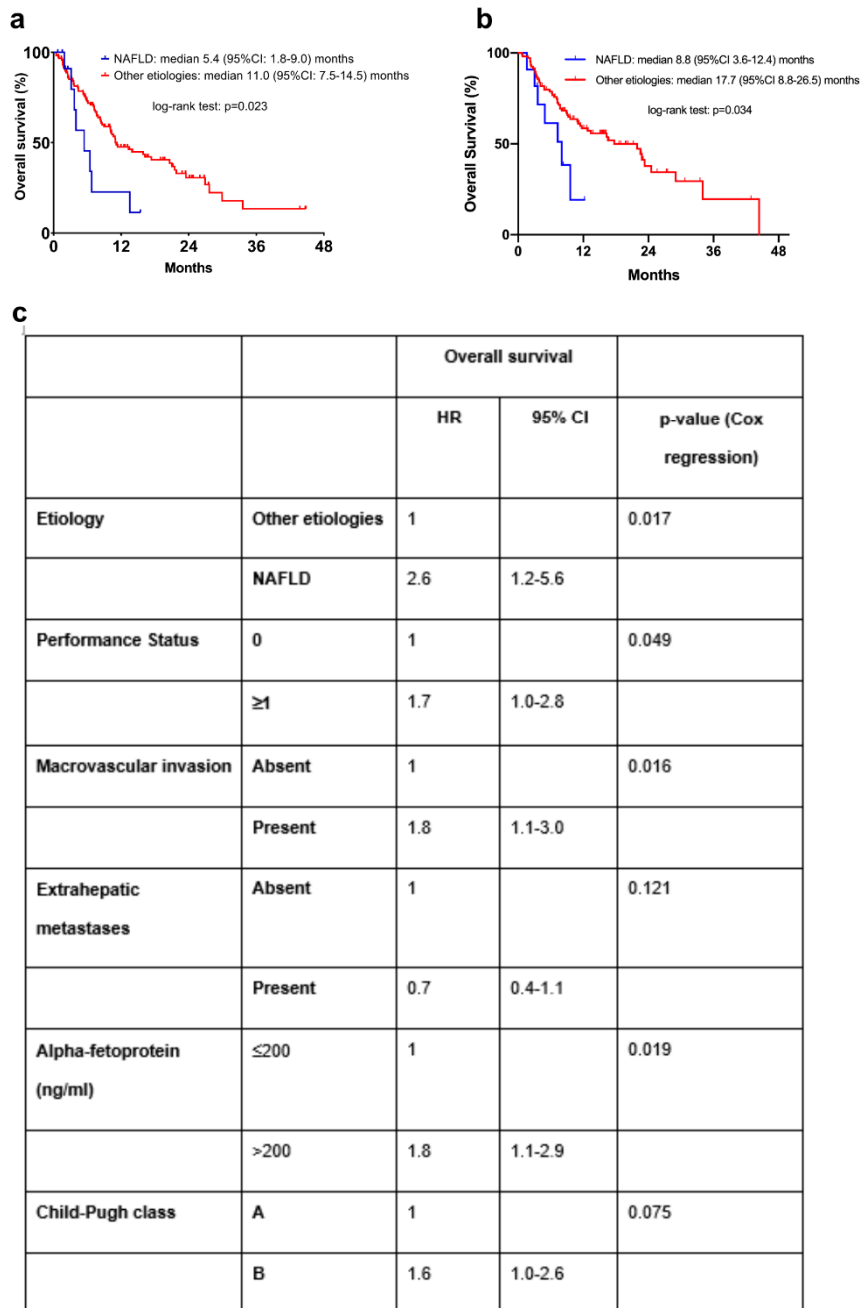
659 (i) Our presented data show for the first time that CD8+PD1+ T-cells and their behavior in  
660 the context of immunotherapy and metabolic syndrome affect liver cancer in an unexpected  
661 manner – CD8+PD1+ T cells are pro-tumorigenic in this context – which very likely has clinical  
662 implications.

663 Identification of increased hepatic abundance of unconventional activated resident-like  
664 CD8+PD-1+ (e.g. CXCR6+, TOX+, TNF+), but not a change of quality in these cells are the  
665 hepatocarcinogenesis-driver in the context of NASH is novel – and can be found also in the  
666 human situation (e.g. two IHC-cohorts across Europe comparing viral vs. NAFLD/NASH-HCC,  
667 one IHC cohort dissecting the abundance of cells depending on NASH pathology severity; also  
668 comparing control vs NAFLD/NASH patient samples by scRNA Seq, CYTOF and flow  
669 cytometry).

670 (ii) Our data expand current knowledge of NASH pathology-associated mechanisms (e.g.  
671 auto-aggression in a TCR-independent manner with the co-submitted manuscript Dudek et al.,  
672 corroborating the data in total 3x preclinical models of NASH). Furthermore, we tested this  
673 mechanism hypothesis on a functional level by various antibody-based treatments (PD-L1-  
674 targeted immunotherapy; combination therapy of anti-TNF/anti-PD-1, anti-CD4/anti-PD-1, anti-  
675 CD8/anti-PD1) and now identify that it indeed is TNF and CD8 T cells that promote liver cancer  
676 in the context of PD1-related immunotherapy.

677 (iii) Novel comparison/corroboration and in-depth analysis of T-cell populations in human  
678 and mouse NASH by scRNA, flow cytometry and CYTOF. We did not expect a link between  
679 resident-like CD8+PD1+ cells in the progression of NASH pathology and NASH-induced  
680 hepatocarcinogenesis, as well as the correlation of preclinical model to patient data, identifying  
681 NASH as an etiology of unfavorable predictor of response (e.g. the meta-analysis of 1656  
682 patients corroborates non-viral (NASH-related) HCC compared to viral-HCC as less  
683 responsive to immunotherapy (included in **Figure 6, Extended Data 30-32** and **Rebuttal**  
684 **Figure 1, 2**), as well as our own small retrospective NASH-HCC vs other-etiological-HCC  
685 cohort, which was validated in a second validation cohort of HCC-patients under  
686 immunotherapy (included in **Figure 6** and **Rebuttal Figure 21**).





687

688

### Rebuttal Figure 21

689 (a) Nonalcoholic fatty liver disease (NAFLD) is associated with a worse outcome in patients  
 690 with hepatocellular carcinoma (HCC) treated with PD-(L)1-targeted immunotherapy. A total of  
 691 130 patients with advanced HCC received PD-(L)1-targeted immunotherapy (Supplementary  
 692 Table 8). Kaplan-Meier curve display overall survival of patients with NAFLD vs. those with  
 693 any other etiology; all 130 patients were included in these survival analyses (NAFLD n=13, any  
 694 other etiology n=117). (b) Validation cohort of patients with HCC treated with PD-(L)1-targeted  
 695 immunotherapy. A total of 1180 patients with advanced HCC received PD-(L)1-targeted  
 696 immunotherapy (Supplementary Table 10). Kaplan-Meier curve display overall survival of  
 697 patients with NAFLD vs. those with any other etiology; all 118 patients were included in these



698 survival analyses (NAFLD n=11, any other etiology n=107). (c) Multivariate analysis of  
699 prognostic factors in HCC patients treated with anti-PD-(L)1-based immunotherapy.  
700

701 4. The human data are based on a very small and poorly analyzed cohort of patients with  
702 NASH-associated HCC (n=10-11). While the underlying question is important, pairing data  
703 from this small cohort with the data from the mouse model with its above-described limitations  
704 and confounders may send a wrong and potentially deleterious message to the community,  
705 and much more careful analysis as well as larger cohorts are needed to put the provided  
706 message on a solid scientific foundation: The authors should analyzed outcomes for NASH-  
707 HCC patients with or without cirrhosis to account for the possibility of worsened NASH in  
708 patients without cirrhosis (for which the cohort is much too small).

709  
710 We thank Referee #1 and fully agree, that the presented retrospective  
711 Nivolumab/Pembrolizumab-treated NAFLD/NASH-associated HCC cohort – although unique  
712 for Europe where treatment is not officially licensed - is too small for subgroup analysis for  
713 patients.

714 We have taken this point raised utmost seriously. Thus, we have strengthened our hypothesis  
715 of non-viral (NASH-related) HCC being less responsive to immunotherapy by a meta-analysis  
716 including patients of the three most important clinical trials (1656 patients, included in **Figure**  
717 **6, Extended Data 31-33** and **Rebuttal Figure 1, 2**).

718 Moreover, we have increased the number of patients in our initial clinical cohort from 65 to 130  
719 HCC patients under anti-PD(L)1-targeted immunotherapy and validated our results in a second  
720 cohort of 118 HCC patients under PD(L)1-targeted immunotherapy (included in **Figure 6** and  
721 **Rebuttal Figure 21**).

722 A disadvantage by nature of a retrospective analysis of cohort across multiple centers is, that  
723 clinical material that would have the potential to characterize in patient subgroups (e.g.  
724 worsened NASH) was not sampled. Furthermore, no paired biopsies or other biological  
725 materials (e.g. blood or serum) before/after immunotherapy were taken in this cohorts for HCC  
726 patients, making characterization of treatment response at the single patient resolution and  
727 thus subgroups impossible in this retrospective cohort. Therefore, we decided to investigate  
728 the outcomes for BCLC-C NAFLD/NASH-HCC vs other-etiological-HCC patients with cirrhosis  
729 and observed, that NAFLD/NASH-HCC have significantly reduced overall survival compared  
730 to other-etiological-HCC in this retrospective study. Of note, multivariate analyses identified  
731 NAFLD/NASH as an independent factor for treatment response (included in **Supplementary**  
732 **Table 9** and **Rebuttal Figure 21**). We validated these results in a second independent cohort  
733 of 118 under PD1-targeted immunotherapy based in North America, which included additional

734 n= 11 patients with NASH-HCC under immunotherapy, corroborating that NASH/NAFLD is a  
735 negative predictor to immunotherapy (main text).

736 We toned down the conclusions of our retrospective cohort in the manuscript and would like  
737 to point out, that larger cohorts and prospective clinical trials are of utmost importance for the  
738 scientific community and to investigate the points of Referee #1.

739

740 A. A cohort of n=10-11 NASH-associated HCC patients is unacceptable. Many of the  
741 parameters such as PFS are not significant and it cannot be excluded that inclusion of a larger  
742 number of NASH-HCC patients may change the data significantly.

743

744 We agree with Referee #1, however we would like to point out attention, that prominent trends  
745 or effects can also be seen in small retrospective cohorts as well. Although unique for Europe,  
746 where treatment is not officially licensed yet, the complete cohort we have gathered is too small  
747 for subgroup analysis for patients.

748 We decided to leave out the non-significant data of TTP and PFS in our manuscript. Moreover,  
749 upon recruiting the validation cohort of 118 HCC-patients under immunotherapy we decided  
750 to not show TTP and PFS, but instead the multivariate analysis (included in **Supplemental**  
751 **Table 9**). However, we are in line, that an increased patient cohort allows a more sophisticated  
752 analysis. Thus, as mentioned in the previous comment, we increased our patient cohort (from  
753 65 HCC-patients to 130 HCC-patients) and validated the results in the second cohort of 118  
754 HCC-patients under PD(L)1-targeted immunotherapy. Furthermore, we would like to highlight  
755 the message from the performed meta-analysis of 1656 patients, also pointing towards  
756 identifying NAFLD/NASH as a negative predictor of immunotherapy response in HCC. Still, the  
757 cohorts are small, and thus, we toned down the conclusions drawn from this retrospective  
758 cohort analyses (added in the main text, **Figure 6**).

759

760 B. The authors do not answer the question whether the differences in survival are due to failed  
761 checkpoint therapy or due to other differences between the two cohorts. Most likely, the  
762 differences in survival would persist if the authors removed all responders from the “other  
763 etiologies” group. Control groups that did not receive checkpoint inhibitors are missing to  
764 determine if survival is different between NASH and non-NASH HCC in patients who did not  
765 receive checkpoint inhibitors.

766

767 We thank Referee #1 for raising this important point of potential differences in survival due to  
768 potential confounders. To address these issues, we have submitted our data to multivariate

769 analyses, which we included in an updated **Supplementary Table 9**. When we excluded  
770 patients with a complete or partial response from the 112 patients with at least one follow-up  
771 imaging, 86 patients were available for analysis (NAFLD, n=9; other etiologies, n=77). Median  
772 OS was significantly shorter in the NAFLD group (5.4 (95%CI, 1.7-9.1) months vs. 10.3  
773 (95%CI, 8.2-12.4) months; p=0.006), as was median TTP (2.4 (95%CI, 2.1-2.7) months vs. 3.9  
774 (95%CI, 2.5-5.4) months; p=0.008), and median PFS (2.4 (95%CI, 1.9-3.0) months vs. 3.7  
775 (2.3-5.1) months; p=0.035). These data suggest that the improved outcome of non-NAFLD  
776 patients is not only driven by the better response rate observed in these patients. However,  
777 the interpretation of these data due to the size of the underlying cohorts needs to be taken with  
778 caution.

779 Like mentioned before, we have now included a meta-analysis with appropriate control  
780 cohorts, identifying immunotherapy vs control for viral HCC as favorable treatment (HR(viral)=  
781 0.64), in contrast, non-viral-HCC show less benefit (HR(non-viral)= 0.92). In this meta-analysis  
782 patients with NASH-HCC and Non-NASH HCC who did not receive checkpoint inhibitors are  
783 included as receiving either sorafenib (in RCT of front-line) or placebo (in RCT in second-line).  
784 We thank Referee #1 for pointing out the lack of appropriate control groups (e.g. NASH-HCC  
785 vs. different etiology-induced HCC under Sorafenib/different multi-kinase inhibitors as a  
786 second/third-line therapy). Although of extreme interest for public health and public knowledge,  
787 we described this important issue in our discussion and to the best of our knowledge there are  
788 no NASH-HCC treated cohorts available (apart from, possibly, inside of the big pharma-  
789 industry), which would allow an adequate control arm.

790 Available cohorts (El-Khoueiry et al., 2017; Finn et al., 2019, 2020) are only differentiating  
791 between viral vs. non-viral etiologies, which combine ASH and NASH-induced HCC.

792

793 C. Is there any indication of increase NASH activity in patients receiving Pembro or Nivo?

794

795 We thank Referee #1 for this important comment. We have added baseline AST and ALT in  
796 the pre-existing and novel cohorts (included in **Supplementary Table 8**). Like previously  
797 mentioned, the character of the retrospective studies did not allow to obtain paired biopsies  
798 before/after immunotherapy, and bigger cohorts of prospective clinical trials are needed.

799

800 D. There is no proper analysis of confounding factors.

801



Research for a Life without Cancer

802 We thank Referee #1 for pointing out this lack of analyses in our initial submission. We have  
803 now performed multivariate analyses, which we included in the main text and in an updated  
804 **Supplementary Tables 8 and 9.**

805 In short: Macrovascular invasion, a negative prognostic factor in HCC, was less frequent in  
806 NAFLD patients (23% vs 49%). NAFLD patients received immunotherapy more often as first-  
807 line therapy (46% vs. 23%), and the proportion of patients receiving the combination of  
808 atezolizumab plus bevacizumab, the only immunotherapy-based treatment that has  
809 succeeded in a phase III trial of advanced-stage HCC so far, was higher in the NAFLD cohort  
810 (23% vs. 5%). Despite these more favorable characteristics, immunotherapy was less effective  
811 in patients with NAFLD, which translated into a worse overall survival (OS) for the NAFLD  
812 cohort: 5.4 (95%CI, 1.8-9.0) months vs. 11.0 (95%CI, 7.5-14.5) months (p=0.023). Adjusting  
813 for other well-known prognostic factors (Child-Pugh class, macrovascular invasion,  
814 extrahepatic metastases, performance status, and alpha-fetoprotein (AFP)), NAFLD remained  
815 independently associated with worse survival (HR 2.6 (95%CI, 1.2-5.6; p=0.017). These data  
816 indicate that PD-1-targeted immunotherapy in HCC patients with concomitant NASH might  
817 lead to unfavorable effects.

818

819 E. Another problem is mixing Pembro and Nivo groups. Even though the target is the same,  
820 the authors need to provide subgroup analysis for this and increase the number far beyond  
821 what they have to make any meaningful conclusions in these subgroups.

822

823 We thank Referee#1 for this comment. Nivolumab and pembrolizumab are mostly considered  
824 comparable in solid tumors. Performing a subgroup analysis based on Nivolumab and  
825 pembrolizumab is simply not feasible nor realistic in HCC, even more so in NASH-HCC.

826 We would like to draw attention to other studies performed in solid tumors (NSCLC (Cui et al.,  
827 2020), and Melanoma (Moser et al., 2020)) that show a similar efficacy (although the overall  
828 level of evidence is low):

829 We agree with this point of Referee #1, which we so far have not been able to make clear.  
830 Similar to the previous point (4A.), our retrospective analyses of the patient cohorts is too small  
831 to address these concerns in an in-depth manner.

832 We agree with Referee #1, that both Nivolumab and Pembrolizumab are targeting the molecule  
833 PD-1, with similar response rates of 17-20% as monotherapy in HCC (El-Khoueiry et al., 2017;  
834 Zhu et al., 2018). The consensus in the literature is to combine both PD-1 targeting antibodies  
835 and pool their results. Moreover, we validated these results in the second cohort of 118 treated  
836 immunotherapy treated HCC-patients, including n= 11 NASH-HCC patients.

837

838 F. Characterization of patients is insufficient - how were other liver diseases excluded,  
839 including ALD, which is not trivial, and especially important in such small cohorts?

840

841 We thank Referee #1 for raising this important point and would like to draw the attention, that  
842 criteria for the retrospective patient cohort are described elsewhere (Scheiner et al., 2019).

843 We have especially analyzed the parameters to identify NAFLD/NASH from viral (e.g. patient  
844 history, liver histology, MRI, obesity). It should be indicated that the differences between NASH  
845 and BASH are indeed difficult to account for – less so when differentiating between NASH and  
846 ASH. Furthermore, we toned down our statement regarding the effects of immunotherapy in  
847 our patient cohorts/case reports in the revised manuscript.

848

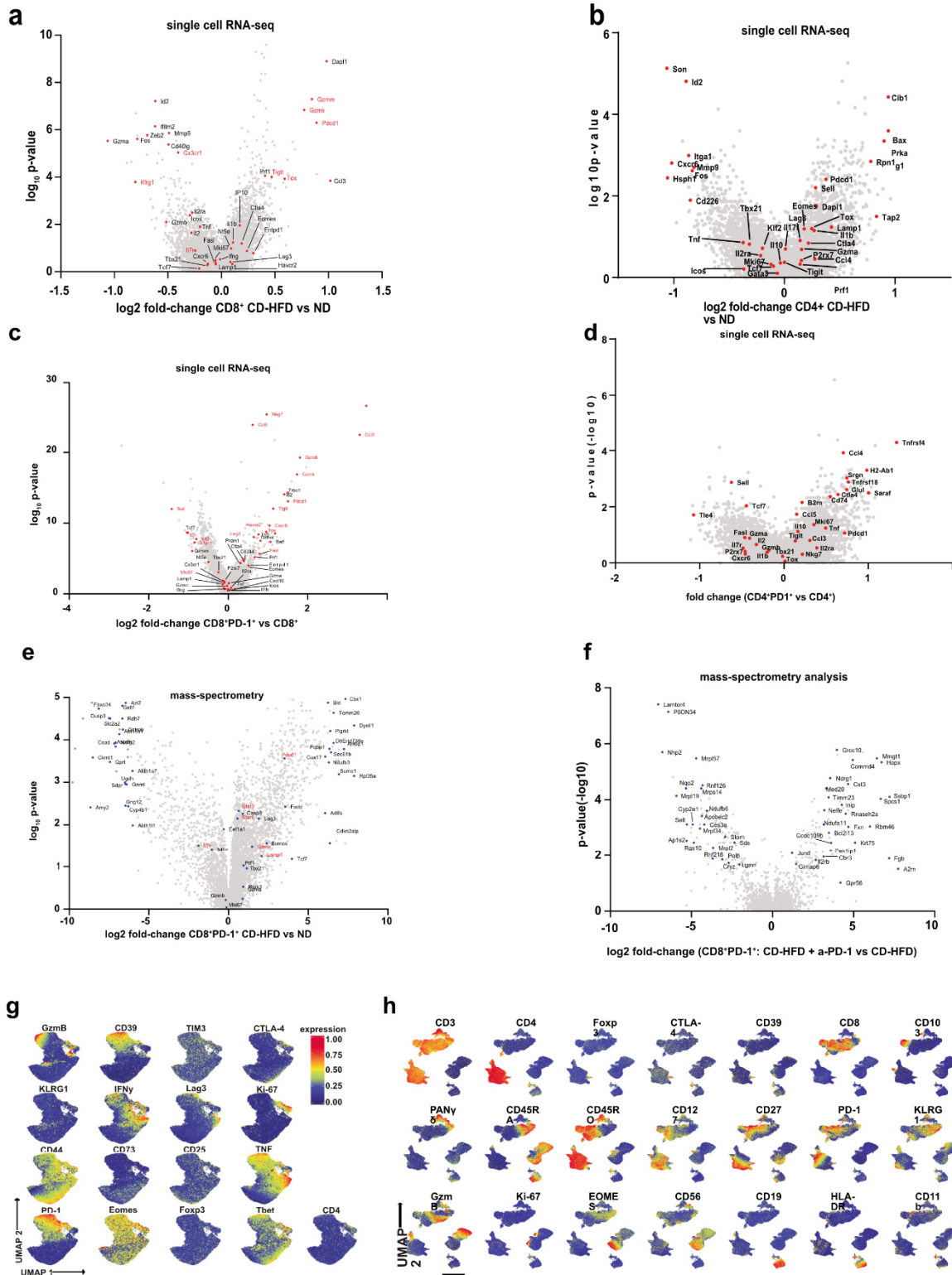
849 5. Do the authors get the same results when blocking CTLA-4 - which was, even though not  
850 approved for HCC - the first approach and published study to show efficacy of checkpoint  
851 inhibitors in HCC?

852

853 We thank Referee #1 for this important question and would like to draw the attention to a phase  
854 II trial combining TACE with Tremelimumab that did not differentiate between underlying  
855 etiology for the patient outcome or immune population (Agdashian et al., 2019; Duffy et al.,  
856 2016). This phase II trial showed a similar response rate (21-26%) compared to the 17-20%  
857 response rate for PD-1 targeted monotherapy (El-Khoueiry et al., 2017; Zhu et al., 2018).  
858 Clinical consensus for immunotherapy indicates increased hepatotoxicity of CTLA-4-  
859 compared to PD-1-targeting immunotherapy (Zen and Yeh, 2018), arguing in favor of PD-  
860 1/PD-L1-targeting immunotherapies for the future.

861 Although we observed in human Tregs cells CTLA-4 positivity by scRNA-seq and flow  
862 cytometry, in our manuscript CTLA-4 expression was not identified as significantly different  
863 between treatments as shown by scRNA-seq (**Figure 1**: CTLA-4 expression in CD8+ T-cells  
864 comparing ND vs CD-HFD: FC= 0.1894, p= 0.0642; **Extended Data 5**: CTLA-4 expression in  
865 CD4+ T-cells comparing ND vs CD-HFD: FC= 0.2173, p= 0.1431; **Figure 4 and Extended  
866 Data 18**). In our mass spectrometry-based data set, we found no significant change of CTLA-  
867 4 abundance (**Extended Data 5 and 18 and Rebuttal Figure 22**), corroborating our flow  
868 cytometry-based analysis, which had also low CTLA-4 expression in mouse or human  
869 (**Figures 4 and 5, Extended Data 18 and 25 and Rebuttal Figure 22**). Thus, we believe that  
870 the application of CTLA-4-targeted immunotherapy is unlikely to cause a positive effect in our  
871 preclinical model.

872 We have discussed the potential use of targeting rather T-cell activation (anti-CTLA-4) than  
 873 exhaustion (anti-PD-1 or anti-PD-L1) in combination, or together with a potential generation of  
 874 tumor antigens by ablation strategies (e.g. TACE).  
 875



876

**877 Rebuttal Figure 22**

878 (a) Selected average marker expression in T-cell subsets of CD8+ and (b) CD4+ sorted TCRβ+  
879 by scRNA-seq of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). (c) Selected marker  
880 expression in hepatic CD8+ T-cells by scRNA-seq comparing CD8+ with CD8+PD-1+ T-cells  
881 of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment of α-PD-1 (n= 3  
882 mice/group). (d) Selected marker expression in hepatic CD4+ T-cells by scRNA-seq comparing  
883 CD4+ with CD4+PD-1+ T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks  
884 treatment of α-PD-1 fed mice (n= 3 mice/group). (e) Selected marker expression in hepatic  
885 CD8+PD-1+ T-cells by mass- spectrometry of 12 months ND or CD-HFD-fed mice (ND n= 4  
886 mice, CD-HFD n= 6 mice). (f) Selected marker expression in hepatic CD8+PD-1+ T-cells  
887 sorted TCRβ+ cells by mass- spectrometry of 12 months CD-HFD or CD-HFD-fed + 8 weeks  
888 treatment of α-PD-1 fed mice (n= 6 mice/group). Candidates developing steady in-/decrease  
889 from ND to CD-HFD to CD-HFD-fed mice + 8 weeks treatment of α-PD-1 are indicated in red.  
890 (n= 6 mice/group). (g) Analysis of 5000 randomly chosen TCRβ+ CD8+ cells of flow cytometry  
891 data to define distinct marker expression of 12 months ND, CD-HFD + IgG, CD-HFD-fed mice  
892 + 8 weeks treatment of α-PD-1 (ND n= 4 mice; CD-HFD n= 8 mice; CD-HFD + α-PD-1 n= 6  
893 mice). (h) Analysis of CD45+ cells by flow cytometry derived from hepatic biopsies of control  
894 and NAFLD/NASH patients to define distinct marker expression (Supplementary Table 2:  
895 control n= 6 patients; NAFLD/NASH n= 11 patients).  
896



897 **Referee #2 (Remarks to the Author):**

898 In their manuscript, Pfister and colleagues aim to show that CD8+PD-1+ T-cells expand during  
899 progressing, diet-induced NAFLD and, upon treatment with anti-PD-1 antibodies, that these  
900 cells can promote carcinogenesis by establishing an inflammatory tumor microenvironment in  
901 a diet-induced, murine model of advanced NAFLD. Additionally, the authors observe a similar,  
902 intratumoral CD8+CD103+PD-1+ T-cell subset in NASH-induced human HCC patients and  
903 claim that patients with NASH-induced HCC respond worse to anti-PD-1 therapy compared to  
904 HCC of other origin.

905 While the seminal observation in this paper is intriguing, namely that anti-PD-1 treatment can  
906 exacerbate tumorigenesis in a murine model of NASH-induced HCC, the authors fail to  
907 demonstrate clear causal relationships between the implicated cell types, liver inflammation  
908 and tumor development in the vast amount of the data they present, which therefore remain  
909 largely correlative. I will highlight my major concerns below.

910

911 [We thank Referee #2 for the concise and detailed comments and understanding of our aimed](#)  
912 [key points to be delivered in the manuscript. Also, we thank Referee #2 for pointing out the](#)  
913 [limitations of our study of correlative data interpretation rather than functional dissection. We](#)  
914 [appreciate Referee's #2 opinion, that our human cohort results lead to indications of a worse](#)  
915 [response rate of NAFLD/NASH-induced HCC compared to non-NAFLD/NASH-HCC upon PD-](#)  
916 [1 targeted immunotherapy. We would like to address the referee's concerns in the following](#)  
917 [section point-by-point by new experimental data, rephrasing of the text, and re-analysis of the](#)  
918 [underlying as well as novel data-sets.](#)

919

920 1. In the reporting summary, the authors state that "Exclusion criteria was pre-established and  
921 the CD-HFD fed mice which did not show the NASH phenotype, high ALT, AST and body  
922 weight, were excluded from the analysis". I fail to understand why this decision was taken as  
923 these mice offer valuable insight in the author's proposed mechanism. Do CD-HFD mice  
924 without overt signs of NASH have reduced CD8+PD-1+ T-cells? Do these mice also less  
925 frequently grow tumors upon anti-PD-1 blockade? Do the T-cells in the livers of these mice fail  
926 display an enhanced effector phenotype? Aside from the valuable experimental insights that  
927 could be gained from these mice, the decision to exclude these CD-HFD but non-NASH mice  
928 from analysis also invalidates any claim that links a given diet to a given phenotype since mice  
929 that did not fit the authors' desired phenotype were excluded.

930

931 We thank Referee #2 for the above questions. All mice were included in the respective  
932 treatment – as stated in the paper, indicated by the large mouse data sets in **Figure 1-4** in  
933 NAS, ALT, AST, and body weight. Thus, the statement “Exclusion criteria ...” is inappropriate  
934 and a mistake made on our side and is corrected in an updated Reporting Summary. We fully  
935 agree with Referee #2 that these mice “offer valuable insight in the proposed mechanism” and  
936 this is actually why we have included all of them in our analyses.

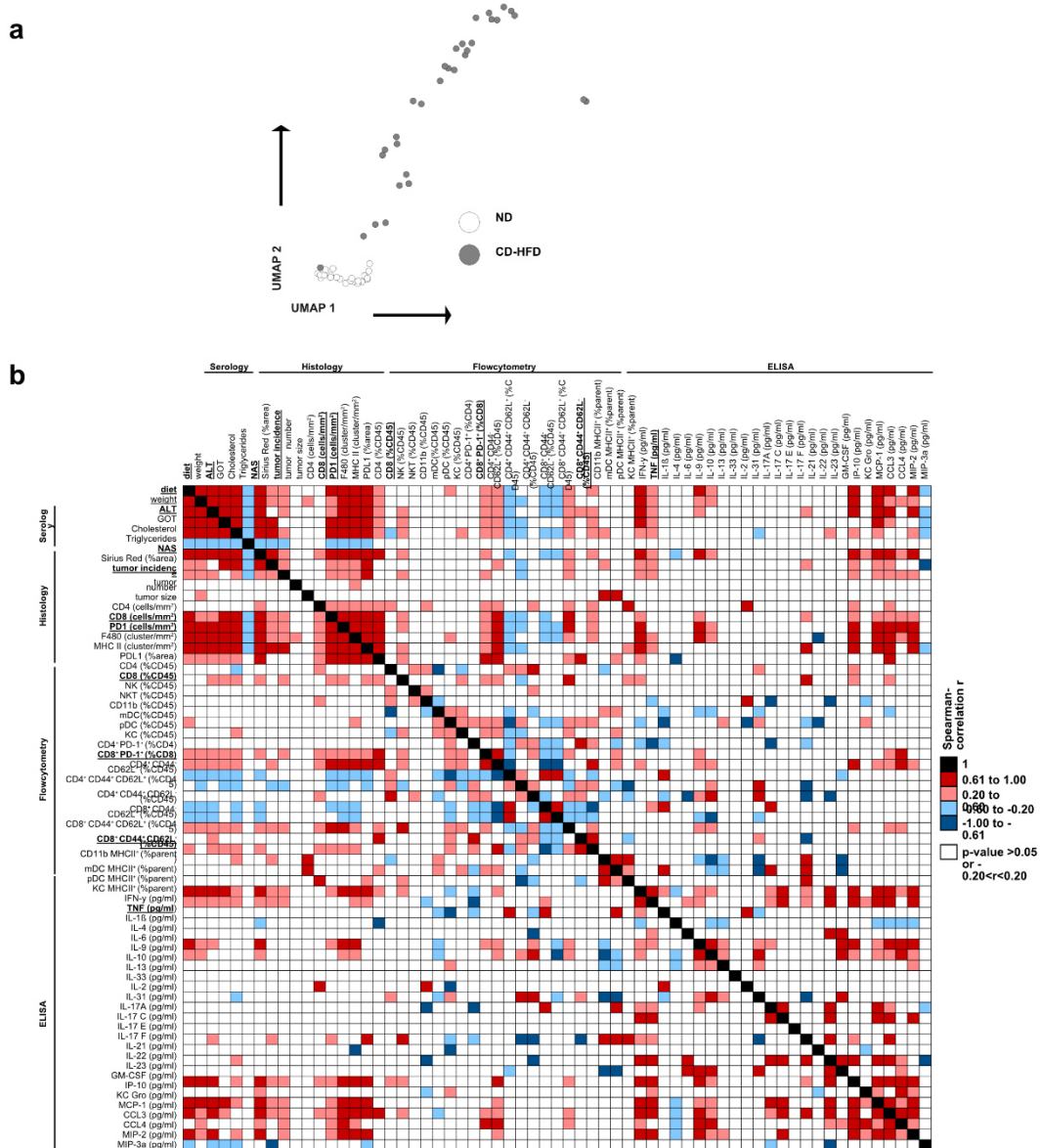
937 To display the experimental range of mice fed 12 months CD-HFD, we have now performed  
938 correlations of a large number of integrated parameters of each mouse (e.g. tumor incidence,  
939 tumor size, tumor nodule number, immune-histochemistry, serology, flow cytometry data;  
940 included now in **Figures 1 and 4, Extended Data 4 and 24 and Rebuttal Figure 23, 24**): In  
941 more detail, we have - for example - re-analyzed our data sets to dissect the potential  
942 correlations of CD8+ T-cells, PD-1+ T-cells, ALT, fibrosis, and NAS, as well as tumor  
943 incidence, tumor nodule size, and effector phenotype – by artificial intelligence and machine  
944 learning clustering. We have now included these analyses in our revised manuscript.

945 We did not analyze the hepatic environment at time points 10, but after 12 months under diet,  
946 after treatment finished, thus a paired analysis of mice with reduced CD8+PD-1+ T-cells and  
947 their reaction to PD-1-targeted immunotherapy is not possible. In 12 months, CD-HFD-fed mice  
948 CD8 (%CD45) and effector CD8 cells (CD8+CD44+CD62L-) correlate positively with markers  
949 of severity of NASH pathology (e.g. ALT, AST, NAS), as well as tumor incidence (included in  
950 **Extended Data 4 and Rebuttal Figure 23**). In 12 months CD-HFD-fed mice polarization by  
951 PD-1 of these CD8+ T-cells (CD8+PD-1+(%CD8)) correlate positively with ALT, AST, but not  
952 significantly with NAS or tumor incidence, indicating that the hepatic abundance of CD8+PD-  
953 1+ cells is important for NASH (e.g. CD8+PD-1+ (%CD45) correlates (Spearman correlation  
954  $r= 0.3844$ ,  $p= 0.0058$ ) with NAS, not reported in the paper).

955 Correlation data included in **Extended Data 24 and Rebuttal Figure 24** shows, that PD-1-  
956 targeted immunotherapy correlates positively with markers of severity of NASH pathology (e.g.  
957 ALT, AST, NAS), with tumor incidence and tumor numbers per liver, and hepatic CD8 T-cells  
958 (e.g. by histology and flow cytometry), effector CD8 cells (CD8+CD44+CD62L-), as well as the  
959 polarization of CD8+PD-1+(%CD8). These data indicate similar to the Referee’s comment,  
960 that mice with reduced hepatic CD8 T-cells and thus also less effector CD8 cells  
961 (CD8+CD44+CD62L-) develop fewer tumors, and that in our data set reduced numbers of  
962 hepatic CD8+PD1+ T-cells result in lower NAS and lower tumor incidence upon PD-1-targeted  
963 immunotherapy (included in **Extended Data 24 and Rebuttal Figure 24**).

964 We agree with Referee #2, that these data allowed us to gain valuable insights understanding  
965 the phenotype, why some mice develop milder NAFLD/NASH when compared to experimental

966 controls submitted to similar times of diet feeding, and how this affected PD-1 blockade. We  
 967 would like to point out that mice develop NAFLD/NASH at 12 months post-diet start with an  
 968 incidence of 100% (please also see **Figures 1** and **Rebuttal Figure 25**).

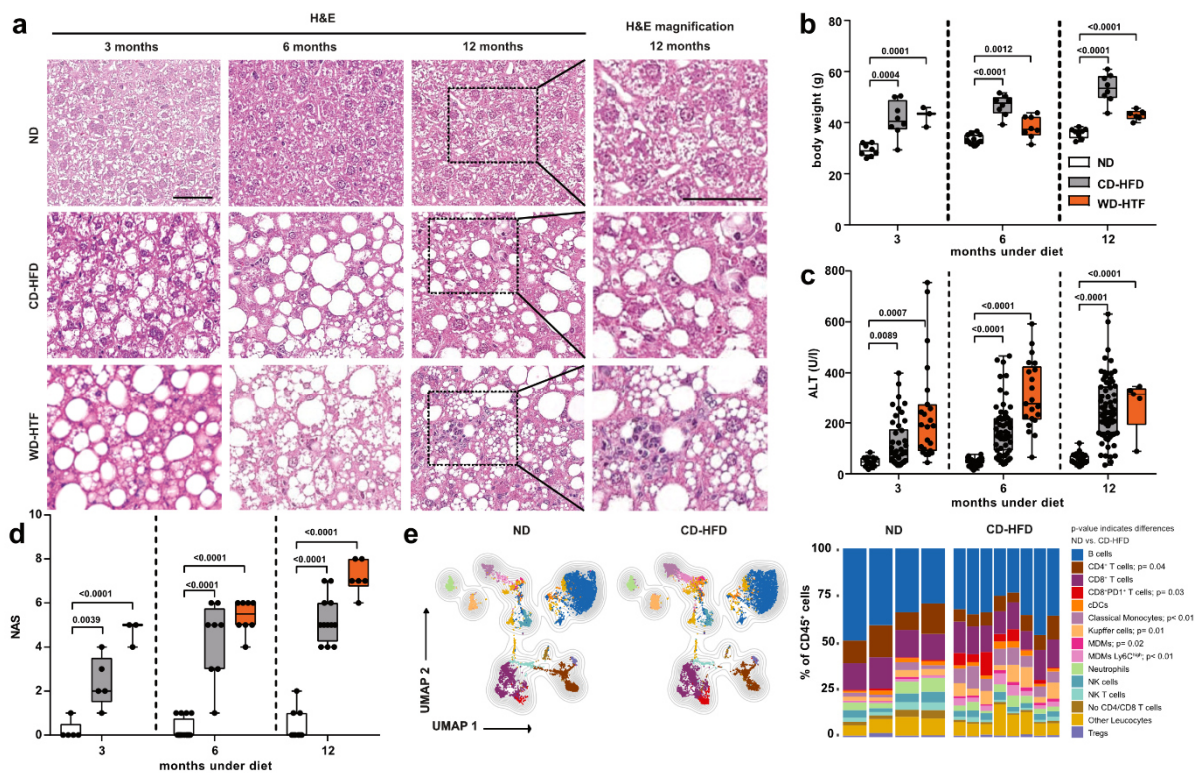


969  
 970 **Rebuttal Figure 23**  
 971 (a) UMAP representation of 63 parameters (serology, flow cytometry, histology) indicating  
 972 NASH pathology severity measured of 12 months ND or CD-HFD fed mice (ND n= 22 mice;  
 973 CD-HFD n= 31 mice). (b) Data gathered from hepatic tissue analyses was binary correlated  
 974 with each other of 6- or 12-months ND or CD-HFD-fed mice (ND n= 47 mice; CD-HFD n= 72  
 975 mice). (c) H&E, CD8, and PD-1 staining, evaluation by NAS and quantification of CD8+ cells  
 976 and PD-1+ expressing cells by immunohistochemistry of 32-weeks old hURI-tetOFFhep and  
 977 non-transgenic litter control mice (n=6 mice/group). Arrowheads indicate specific staining  
 978 positive cells. Scale bar: 100  $\mu$ m.



981 **Rebuttal Figure 24**

982 (a) UMAP representation of 63 parameters (serology, flow cytometry, histology) and (b)  
 983 selected display of analyzed parameters indicating NASH pathology severity measured of 12  
 984 months ND or CD-HFD fed mice (ND n= 22 mice; CD-HFD n= 31 mice; CD-HFD +  $\alpha$ -PD-1 n=  
 985 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; CD-HFD +  $\alpha$ -CD8 n= 24 mice; CD-HFD +  $\alpha$ -  
 986 CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice;  
 987 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4  
 988 n= 9 mice). (c) Data gathered from hepatic tissue analyses was binary correlated with each  
 989 other of 6- or 12-months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8,  $\alpha$ -CD8/ $\alpha$ -  
 990 NK1.1;  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or  $\alpha$ -PD-1/ $\alpha$ -CD4 fed mice (ND  
 991 n= 47 mice; CD-HFD n= 72 mice; CD-HFD +  $\alpha$ -PD-1 n= 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6  
 992 mice; CD-HFD +  $\alpha$ -CD8 n= 29 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -  
 993 CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD  
 994 +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).


 996  
 997

**Rebuttal Figure 25**

998 (a) Histological staining of hepatic tissue by H&E of 3, 6 or 12 months ND, CD-HFD or WD-  
 999 WD-HTF fed mice (H&E: 3 months: ND n= 5 mice; CD-HFD n= 5 mice; WD-HTF n= 3 mice; 6  
 1000 months: ND n= 16 mice; CD-HFD n= 8 mice; WD-HTF n= 8 mice; 12 months: ND n= 9 mice;  
 1001 CD-HFD n= 12 mice; WD-HTF n= 6 mice). Scale bar: 50  $\mu$ m. (b) Body weight of 3, 6 or 12  
 1002 months ND, CD-HFD or WD-HTF mice (3 months: ND n= 8 mice; CD-HFD n= 8 mice; WD-  
 1003 HTF n= 3 mice; 6 months: ND n= 14 mice; CD-HFD n= 8 mice; WD-HTF n= 8 mice; 12 months:  
 1004 ND n= 8 mice; CD-HFD n= 8 mice; WD-HTF n= 6 mice). (c) ALT levels of 3, 6 or 12 months  
 1005 ND, CD-HFD or WD-HTF mice (3 months: ND n= 15 mice; CD-HFD n= 46 mice; WD-HTF n=  
 1006 23 mice; 6 months: ND n= 46 mice; CD-HFD n= 59 mice; WD-HTF n= 21 mice; 12 months:  
 1007 ND n= 25 mice; CD-HFD n= 69 mice; WD-HTF n= 5 mice). (d) NAS evaluation by H&E of 3, 6  
 1008 or 12 months ND, CD-HFD or WD-HTF mice (3 months: ND n= 5 mice; CD-HFD n= 5 mice;  
 1009 WD-HTF n= 3 mice; 6 months: ND n= 16 mice; CD-HFD n= 8 mice; WD-HTF n= 8 mice; 12

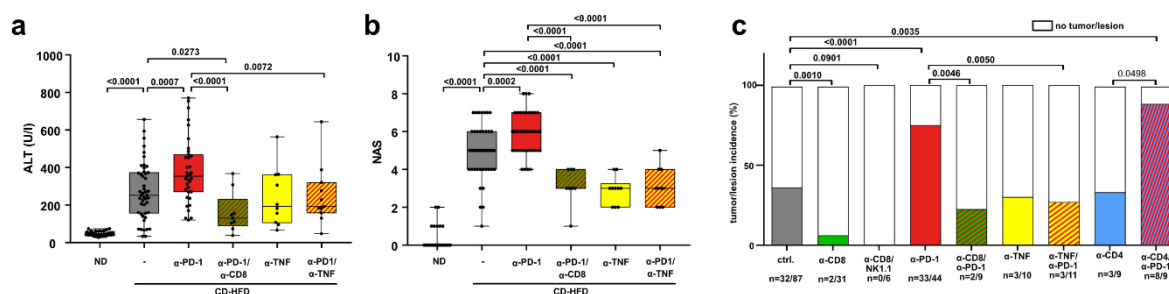
1010 months: ND n= 9 mice; CD-HFD n= 12 mice; WD-HTF n= 6 mice). (e) UMAP representation  
1011 of 5000 randomly chosen CD45+ cells and quantification of hepatic immune cell composition  
1012 by flow cytometry of 12 months ND or CD-HFD fed mice (ND n= 4 mice; CD-HFD n= 8 mice).  
1013

1014 2. The data presented by the authors fail to demonstrate clear causal relationships. As an  
1015 example, the authors note in lines 341-343 that a pro-inflammatory hepatic environment is  
1016 created by TNF upon anti-PD-1 treatment, yet fail to show supporting evidence that this indeed  
1017 drives “necro-inflammation” and accelerated hepatocarcinogenesis. The authors should  
1018 neutralize TNF in their in vivo models to determine whether this molecule is indeed required  
1019 for their phenotype, i.e., inflammatory microenvironment, liver damage and increased  
1020 tumorigenicity.

1021  
1022 We thank Referee #2 for this very important point. We agree with the comment of Referee #2  
1023 and therefore have performed anti-TNF treatment in NASH mice with/without PD-1 targeted  
1024 immunotherapy (included in **Figure 4, Extended Data 20 and 21** and **Rebuttal Figure 26-28**).  
1025 Of note, data from these experiments demonstrate that TNF, derived from CD8+ T-cells is the  
1026 main driver of the pro-tumorigenic effects of T-cells in the context of immunotherapy in NASH  
1027 (included in **Figure 3** and **Rebuttal Figure 29**).

1028 Furthermore, we would like to highlight, that our manuscript correlates increased hepatic  
1029 abundance of CD8+PD-1+ T-cells upon PD-1-targeted immunotherapy as crucial for driving  
1030 hepatocarcinogenesis. Besides, we have now performed additional scRNA-seq and velocity  
1031 blot analyses for human patients with NAFLD/NASH or steatosis and compared those with  
1032 mouse immune cells. These data demonstrate high similarities between CD8+ PD1+ T-cells  
1033 derived from human and mouse NASH livers.

1034 Moreover, we would like to draw the attention of this Referee to the improved cross-referencing  
1035 to the co-submitted manuscript Dudek et al., in which the authors also show that TNF is one  
1036 key molecule driving increased CD8-dependent hepatic pathogenesis.



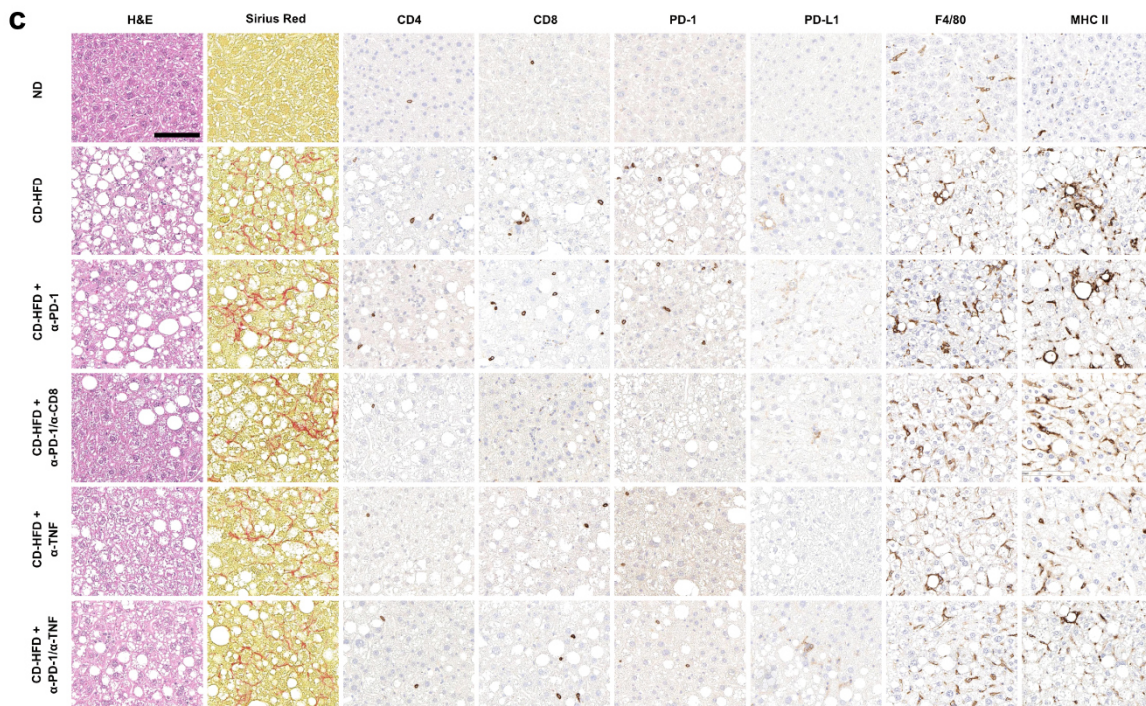
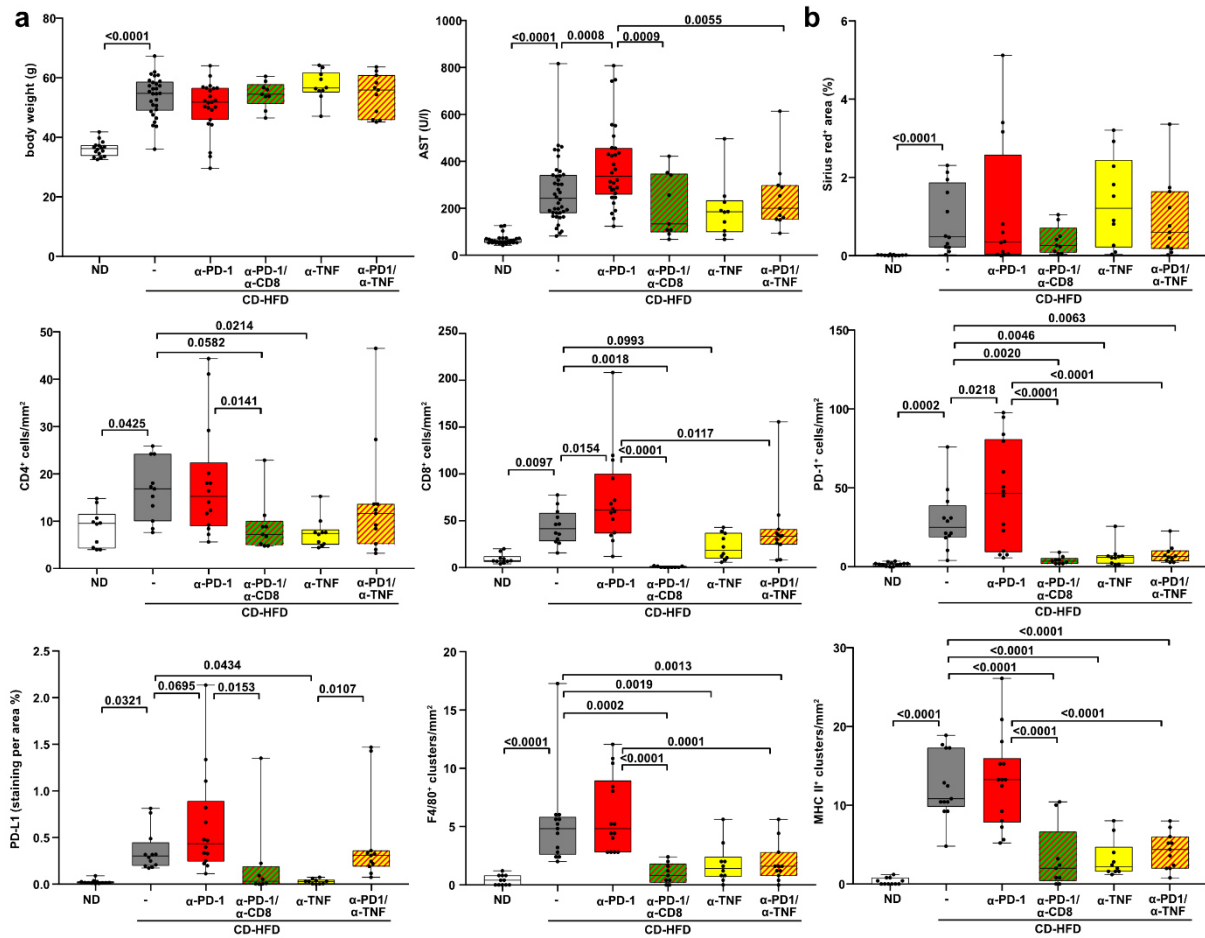
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### 1038 **Rebuttal Figure 26**

1039 (a) ALT and (b) NAS evaluation of 12 months ND, CD-HFD, CD-HFD-fed mice + 8 weeks  
1040 treatment of  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF, or  $\alpha$ -PD-1/ $\alpha$ -TNF (ND n= 30 mice; CD-HFD n= 47  
1041 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF



1042 n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). (c) Quantification of tumor incidence of 12  
1043 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -CD8,  $\alpha$ -CD8/NK1.1,  $\alpha$ -PD-1,  
1044  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or  $\alpha$ -PD-1/ $\alpha$ -CD4 (tumor incidence: CD-HFD n=  
1045 32 tumors/lesions in 87 mice; CD-HFD +  $\alpha$ -CD8 n= 2 tumors/lesions in 31 mice; CD-HFD +  $\alpha$ -  
1046 CD8/NK1.1 n= 0 tumors/lesions in 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 33 tumors/lesions in 44 mice;  
1047 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 2 tumors/lesions in 9 mice; CD-HFD +  $\alpha$ -TNF n= 3 tumors/lesions  
1048 in 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 3 tumors/lesions in 11 mice); CD-HFD +  $\alpha$ -CD4 n= 3  
1049 tumors/lesions in 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 tumors/lesions in 9 mice).

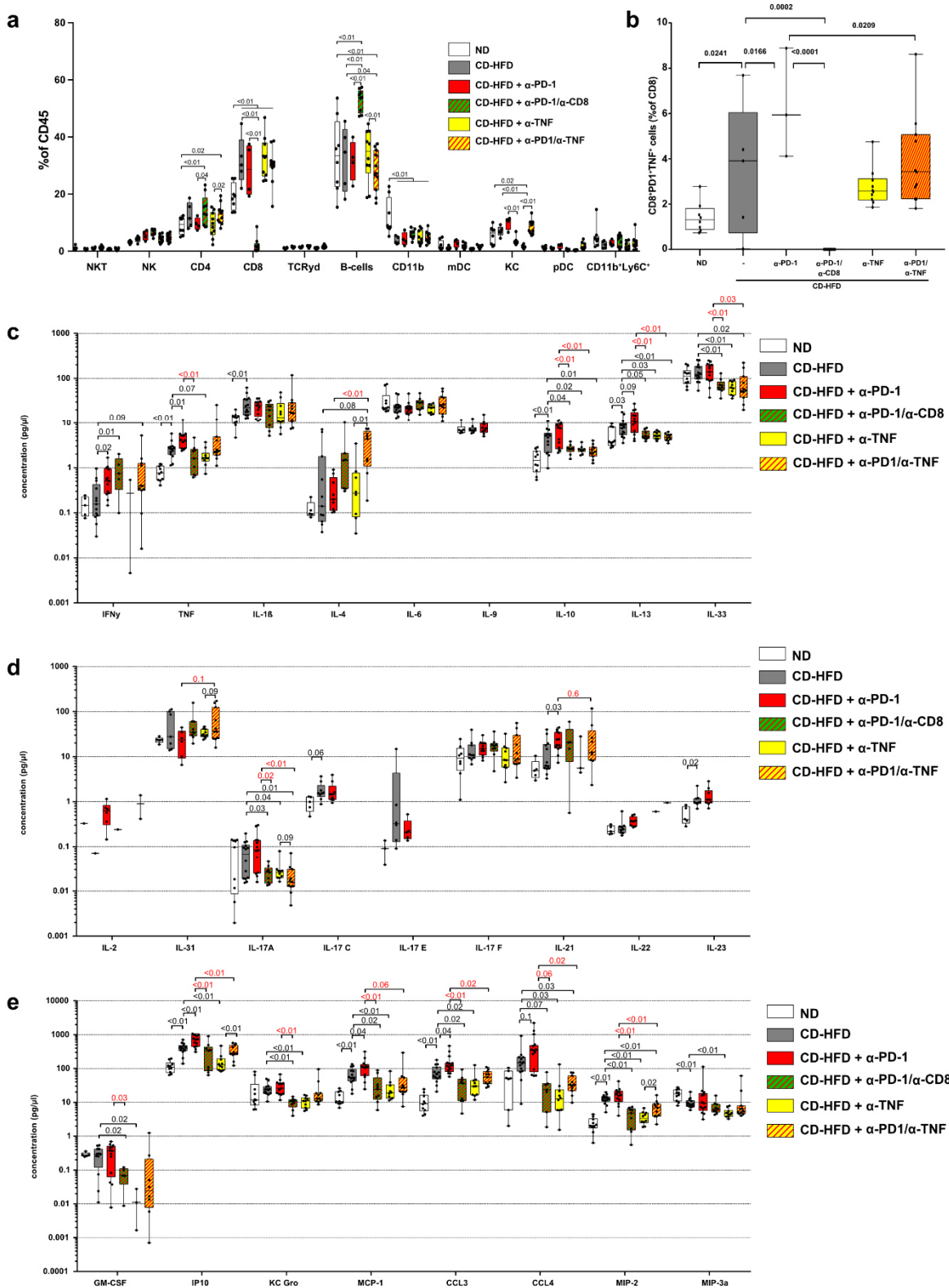


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1053 **Rebuttal Figure 27**

1054 (a) Body weight, AST, and histological evaluation by (b) Sirius red, CD4, CD8, PD-1, PD-L1,  
1055 F4/80, MHC-II and (c) staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  
1056  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (body weight: ND n= 16 mice; CD-  
1057 HFD n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-  
1058 HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; AST: body weight: ND n= 30  
1059 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
1060 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; Sirius red: ND n=  
1061 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
1062 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD4: ND n= 10  
1063 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
1064 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8: ND n= 10  
1065 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-  
1066 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n=  
1067 11 mice; PD-1: ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD  
1068 +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 8 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 10  
1069 mice; PD-L1: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  
1070  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice;  
1071 F4/80: ND n= 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-  
1072 1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  
1073  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1  
1074 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  
1075  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). Scale bar: 100  $\mu$ m.

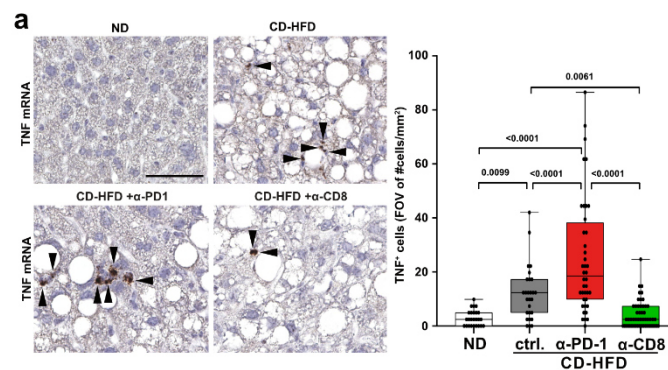


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**Rebuttal Figure 28**

(a) Quantification of hepatic immune cell composition and (b) CD8+PD-1+TNF+ T-cells by flow cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-PD-1/α-CD8, α-TNF, α-PD-1/α-TNF antibodies (Hepatic immune cell composition: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD + α-PD-1 n= 4 mice; CD-HFD + α-PD-1/α-CD8 n= 9 mice;

1082 CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8+PD-1+TNF+: ND  
1083 n= 8 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 3 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
1084 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). (c) and (d)  
1085 multiplex ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12  
1086 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -  
1087 TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n=  
1088 13 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-  
1089 1/ $\alpha$ -TNF n= 11 mice).



1090  
1091 **Rebuttal Figure 29**

1092 (a) Quantification of RNA in situ hybridization for hepatic TNF+ cells of 12 months ND, CD-  
1093 HFD or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -CD8 or  $\alpha$ -PD-1 (ND n= 25 FOV in 3 mice;  
1094 CD-HFD n= 27 FOV in 3 mice; CD-HFD +  $\alpha$ -PD-1 n= 40 FOV in 3 mice; CD-HFD +  $\alpha$ -CD8 n=  
1095 55 FOV in 3 mice). Arrowheads indicate TNF+ cells. Scale bar: 20  $\mu$ m.  
1096

1097 3. Based on the authors' presented data, this problem can be further expanded. In Figure S9d  
1098 and S9m, the authors show an increase in the number of antigen-presenting cells and  
1099 increased MHC-II expression. Are these recruited upon liver inflammation? Are they required  
1100 for liver inflammation?

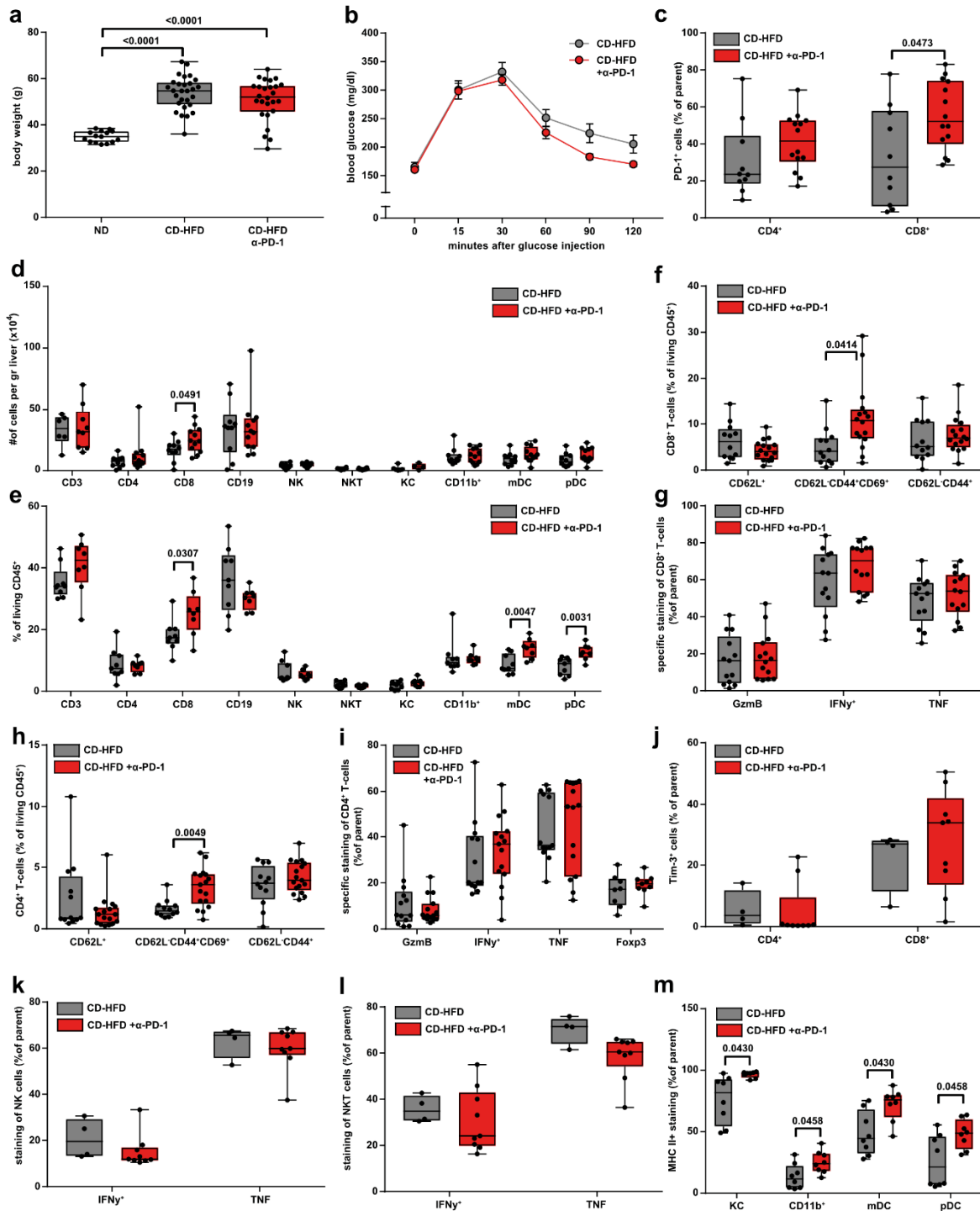
1101  
1102 We thank Referee #2 for raising the point about myeloid cells in the context of chronic  
1103 inflammation and would like to interpret the data shown in **Extended Data 11** and **Rebuttal**  
1104 **Figure 30** in comparison to **Extended Data 8** and **Rebuttal Figure 31**, which now indicates,  
1105 that antigen-presenting cells and increased MHC-II expression are a result of increased liver  
1106 inflammation upon PD-1 targeted immunotherapy.

1107 We would like to highlight our previous study (Malehmir et al., 2019), which demonstrated, that  
1108 myeloid cells are correlated with liver inflammation and are recruited as a consequence of  
1109 NASH development. Moreover, we have shown by depletion of antigen-presenting cells,  
1110 including Kupffer cells (by chlodronate encapsulating liposomes) abrogates or prevents NASH  
1111 development.

1112 To address the point raised by Referee #2 more experimentally, we analyzed our mouse  
1113 cohorts in total by AI, which indicates that hepatic MHCII+ cells correlate positively with NASH  
1114 pathology (weight, NAS, ALT, AST, cholesterol, fibrosis by Sirius Red staining, hepatic

1115 concentrations of MCP-1, CCL3, MIP-2, and IL-21) and MHCII+ as a marker of myeloid  
1116 activation on different subsets correlated predominantly in CD11b+CD11c+ (myeloid dendritic  
1117 cells (CD11b+CD11c+) with ALT, GOT, NAS in 12 months CD-HFD-fed mice (included in  
1118 **Extended Data 4** and **Rebuttal Figure 23**). To dissect the Referees question in our  
1119 experimental functional antibody-treatment experiments (included in **Extended Data 24** and  
1120 **Rebuttal Figure 24**). MHCII+ cells correlate positively with CD-HFD and CD-HFD+PD-1-  
1121 targeted immunotherapy, as well as NASH pathology (weight, NAS, ALT, AST, cholesterol,  
1122 fibrosis by Sirius Red staining, hepatic concentrations of MCP-1, CCL3, CCL4, MIP-2, and IL-  
1123 21) in 12 months old mice. Moreover, MHCII+ as a marker of myeloid activation on different  
1124 subsets correlated for CD11b+MHCII+ and mDC+MHCII+ positive with PD-1-targeted  
1125 immunotherapy, ALT, AST, NAS CCL4, and MIP-2. pDC+MHCII+ and KC+MHCII+ cells  
1126 correlated negatively in CD8-depleted and CD8+NK1.1 co-depleted animals. The latter  
1127 myeloid subset correlates positively with fibrosis and tumor incidence when pooling the data  
1128 of all treatments.

1129 We would like to highlight our previous study (Malehmir et al., 2019), which showed, that  
1130 myeloid cells are correlated with liver inflammation and are recruited as a consequence of  
1131 NASH development. However, a genetic study using CCR2-/- mice (impaired myeloid  
1132 recruitment upon inflammation) developed NASH and NASH-induced tumors; in contrast,  
1133 Rag1-/- mice with functional myeloid but impaired adaptive immune compartments were  
1134 protected from NASH and NASH-induced tumors (Wolf et al., 2014). These data argue, that  
1135 myeloid cells are recruited to the liver, extend, and fine-tune liver inflammation.



1136

1137

### Rebuttal Figure 30

1138 (a) Body weight of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 15 mice; CD-HFD n= 28 mice; CD-HFD +  $\alpha$ -PD-1 n= 26 mice).

1139 (b) Assessment of metabolic tolerance by intra peritoneal glucose tolerance test of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n= 9 mice/group).

1140 (c) Expression of PD-1 of hepatic CD4<sup>+</sup> and PD-1<sup>+</sup> T-cells by flow cytometry of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD n= 10 mice;  $\alpha$ -PD-1 + CD-HFD n= 13 mice).

1141 (d) Absolute and (e) relative quantification of hepatic leukocytes

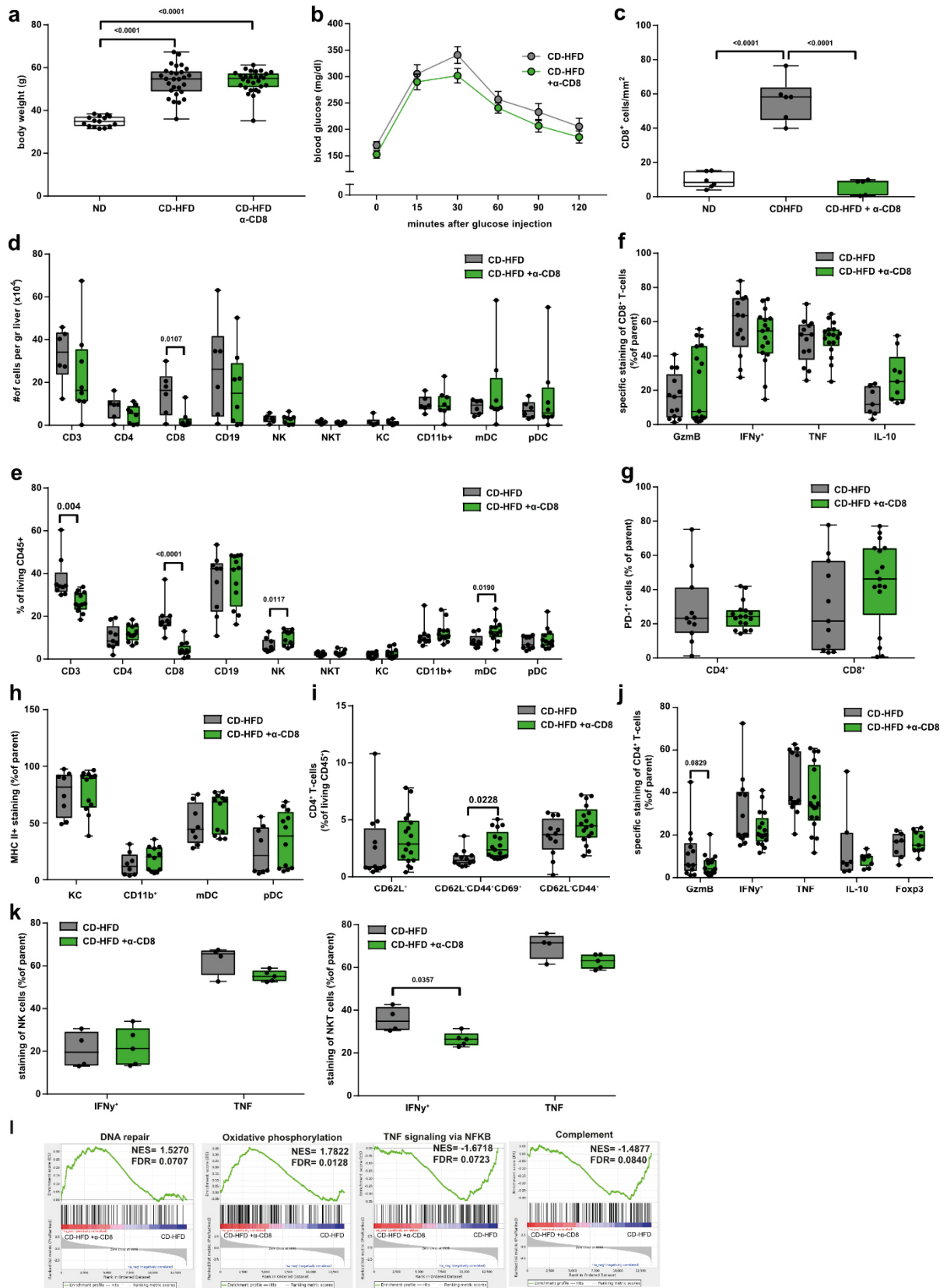
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1145 of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD3:  
1146 CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 10 mice; CD4, CD8, CD19, NK, NKT, CD11b+,  
1147 mDC, pDC: CD-HFD n= 10 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice, KC: CD-HFD n= 6 mice; CD-  
1148 HFD +  $\alpha$ -PD-1 n= 4 mice). (f) Flow cytometric analysis for polarization of hepatic CD8+ T-cells  
1149 of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-  
1150 HFD n= 10 mice;  $\alpha$ -PD-1 + CD-HFD n= 14 mice). (g) Cytokine expression of hepatic CD4+ T-  
1151 cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies  
1152 (CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice). (h) Flow cytometry analysis for  
1153 polarization of hepatic CD4+ T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks  
1154 treatment by  $\alpha$ -PD-1 antibodies (CD-HFD n= 12 mice;  $\alpha$ -PD-1 + CD-HFD n= 17 mice). (i)  
1155 Cytokine expression of hepatic CD4+ T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8  
1156 weeks treatment by  $\alpha$ -PD-1 antibodies (GzmB, IFN $\gamma$ , TNF: CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -  
1157 PD-1 n= 14 mice; IL-10, Foxp3: CD-HFD n= 7 mice; CD-HFD +  $\alpha$ -PD-1 n= 9 mice). (j)  
1158 Expression of Tim-3 of hepatic CD4+ and CD8+ T-cells by flow cytometry of 12 months CD-  
1159 HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD n= 4 mice;  $\alpha$ -  
1160 PD-1 + CD-HFD n= 9 mice). (k) Cytokine expression for polarization of hepatic NK and (l) NKT-  
1161 cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies  
1162 (n= 5 mice/group). (m) Flow cytometric analysis for polarization of hepatic myeloid cells of 12  
1163 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD n=  
1164 8 mice;  $\alpha$ -PD-1 + CD-HFD n= 12 mice).



1167 **Rebuttal Figure 31**

1168 (a) Body weight of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -  
1169 CD8 antibodies (ND n= 15 mice; CD-HFD n= 28 mice; CD-HFD +  $\alpha$ -CD8 n= 28 mice). (b)  
1170 Assessment of metabolic tolerance by intra peritoneal glucose tolerance test of 12 months CD-  
1171 HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 8 mice; CD-  
1172 HFD +  $\alpha$ -CD8 n= 10 mice). (c) Quantification of CD8 staining of hepatic tissue by  
1173 immunohistochemistry of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment  
1174 by  $\alpha$ -CD8 antibodies (ND n= 6 mice; CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -CD8 n= 5 mice). (d)  
1175 Absolute and (e) relative quantification of hepatic leukocytes of 12 months CD-HFD or CD-  
1176 HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 9 mice; CD-HFD +  $\alpha$ -  
1177 CD8 n= 12 mice). (f) Analyses of cytokine expression for polarization of hepatic CD8+ T-cells  
1178 of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (GzmB,  
1179 IFN $\gamma$ , TNF: CD-HFD n= 13 mice;  $\alpha$ -CD8 + CD-HFD n= 17 mice; IL-10: CD-HFD n= 7 mice;  $\alpha$ -  
1180 CD8 + CD-HFD n= 9 mice). (g) Expression of PD-1 of hepatic CD4+ and CD8+ T-cells by flow  
1181 cytometry of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8  
1182 antibodies (CD-HFD n= 11 mice;  $\alpha$ -CD8 + CD-HFD n= 17 mice). (h) Flow cytometry analysis  
1183 for polarization of hepatic myeloid cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks  
1184 treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 8 mice;  $\alpha$ -CD8 + CD-HFD n= 12 mice). (i) Flow  
1185 cytometric analysis for polarization of hepatic CD4+ T-cells of 12 months CD-HFD or CD-HFD-  
1186 fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 12 mice;  $\alpha$ -CD8 + CD-HFD  
1187 n= 17 mice). (j) Cytokine expression of hepatic CD4+ T-cells of 12 months CD-HFD or CD-  
1188 HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (GzmB, IFN $\gamma$ , TNF: CD-HFD n= 13  
1189 mice; CD-HFD +  $\alpha$ -CD8 n= 17 mice; IL-10, Foxp3: CD-HFD n= 7 mice; CD-HFD +  $\alpha$ -CD8 n=  
1190 9 mice). (k) Cytokine expression for polarization of hepatic NK and NKT-cells of 12 months  
1191 CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 4 mice;  
1192  $\alpha$ -CD8 + CD-HFD n= 5 mice). (l) Gene set enrichment analysis of RNA sequencing data of  
1193 hepatic tissue comparing CD-HFD with CD-HFD-fed mice +  $\alpha$ -CD8 of 12 months ND, CD-HFD  
1194 or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (n= 5 mice/group).

1195

1196 4. In Figure S11 the authors show an increase in many inflammatory mediators upon anti-PD-  
1197 1 therapy; which of these are required for the accelerated carcinogenesis? While the authors  
1198 propose a mechanism based on liver inflammation leading to increased hepatocarcinogenesis  
1199 upon anti-PD-1 blockade, they provide little if any conclusive evidence for this hypothesis.

1200

1201 We thank Referee #2 for asking this important question. We believe that the inflammatory  
1202 mediators for increased hepatocarcinogenesis stem from the increase of CD8+ T-cells upon  
1203 anti-PD1 immunotherapy. Importantly, by performing depletion experiments of different T-cell  
1204 subsets – anti-CD8 or anti-CD4, we can demonstrate that the CD8+ T-cells but not CD4+ T-  
1205 cells are needed for driving hepatocarcinogenesis and driving the pro-tumorigenic effect of  
1206 anti-PD1-related immunotherapy (included in **Figure 4, Extended Data 20-23 and Rebuttal**  
1207 **Figure 32-34**).

1208 Of note, PD-1-targeted immunotherapy increases the hepatic abundance of CD8+PD1+ T-  
1209 cells in vivo (included in e.g. **Extended Data 11 and Rebuttal Figure 35a, b**), as well as  
1210 increases the number of CD8+PD1+ cells in vitro (included **Extended Data 18 and Rebuttal**



1211 **Figure 35c**). To understand the nuances of the observed necro-inflammation, anti-PD1-related  
1212 immunotherapy, and liver cancer formation, we perform correlations analysis of fibrosis, tumor  
1213 nodule number, tumor size, ALT, NAS, CD8, and PD-1 expression by machine learning and  
1214 neuronal networking (included in **Figures 1 and 4, Extended Data 4 and 24 and Rebuttal**  
1215 **Figure 23, 24**).

1216 We have analyzed the inflammatory environment looking into a specific signature (ICF) on the  
1217 transcriptional level in NASH mice with and without anti-PD1-related immunotherapy (included  
1218 in **Figure 3 and Rebuttal Figure 35d**). This transcriptional ICF signature is a predictor of liver  
1219 cancer formation triggered through inflammation in humans. It can be stated that the altered  
1220 inflammatory signature of NASH livers in the context of anti-PD1-related immunotherapy  
1221 overlaps with a signature that from human patients is known to have a bad prognosis and high  
1222 correlation with inflammation triggered liver cancer. Importantly, upon CD8+ T cell depletion  
1223 the intrahepatic ICF signature is downregulated – demonstrating that CD8+ T cell-derived  
1224 inflammatory mediators might be linked with liver cancer formation.

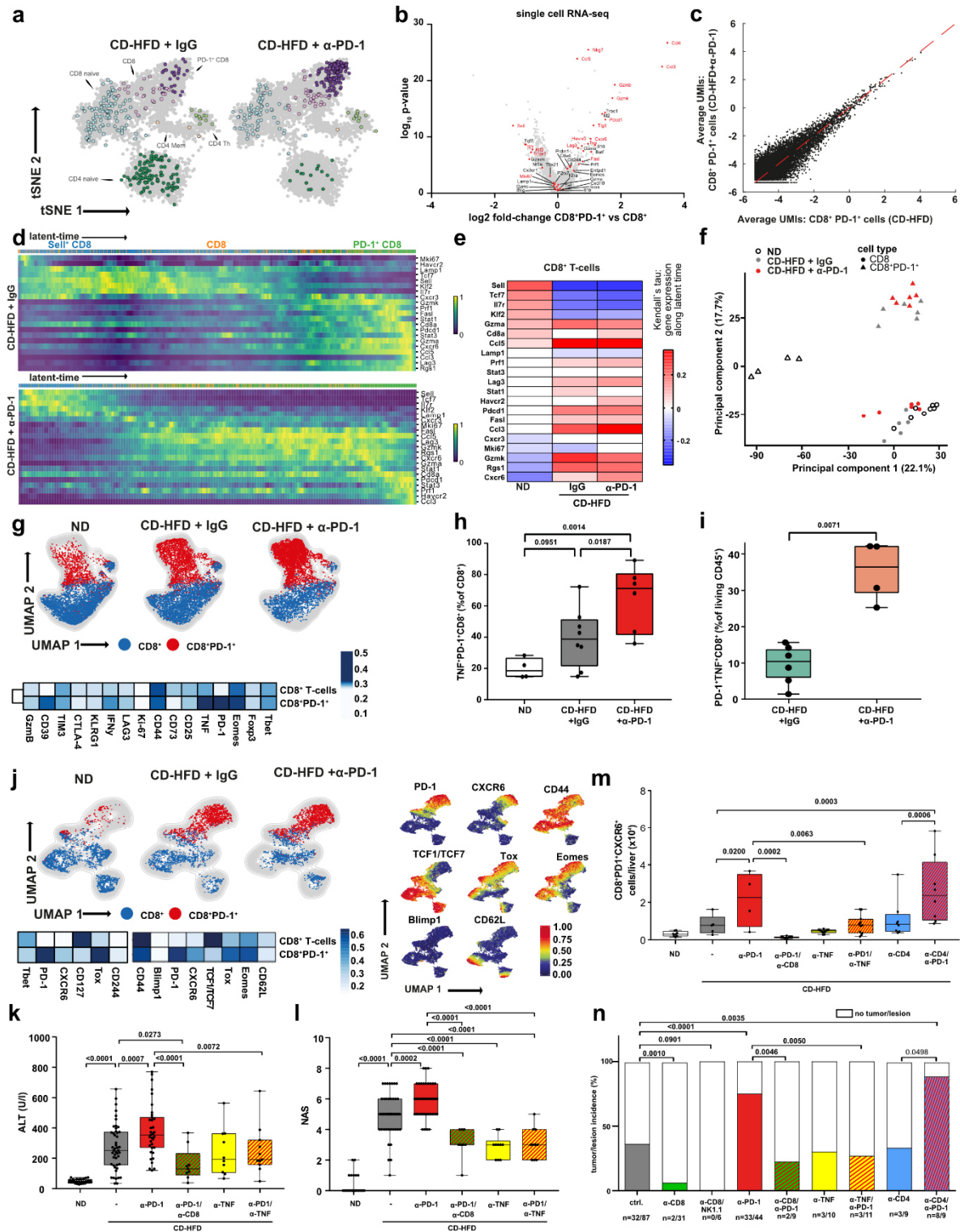
1225 Moreover, to identify factors secreted in relation to CD8+ T-cells in NASH livers (as identified  
1226 by their reduction upon anti-CD8 treatment) we have performed *in situ* RNA hybridization  
1227 analyses for several cytokines. Further, we have performed flow cytometry and RNA-seq of  
1228 hepatic tissues as well as scRNA-seq from human and mouse immune cells. Doing so, we  
1229 have identified T-cell derived TNF as a possible, important candidate for increased  
1230 hepatocarcinogenesis upon PD1-targeted immunotherapy.

1231 To test this hypothesis on a functional level, we performed an anti-PD1/anti-TNF as well as an  
1232 anti-TNF treatment alone. These experiments demonstrate that TNF is a functionally important  
1233 cytokine contributing to the anti-PD1 antibody treatment mediated pro-carcinogenic effect.

1234 Besides, we would like to draw attention to the improved cross-referencing to the co-submitted  
1235 manuscript Dudek et al., which shows that TNF and IL-15, a target downstream of IL-21 - both  
1236 upregulated upon anti-PD-1 therapy - are crucial mediators of CD8-mediated hepatic cell  
1237 death.

1238 In line, literature highlight the crucial role of TNF for hepatocarcinogenesis (Nakagawa et al.,  
1239 2014; Park et al., 2011; Pikarsky et al., 2004) and that anti-TNF treatment uncouples the  
1240 toxicity of CTLA-4/PD-1-targeted immunotherapy (Perez-Ruiz et al., 2019).

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### Rebuttal Figure 32

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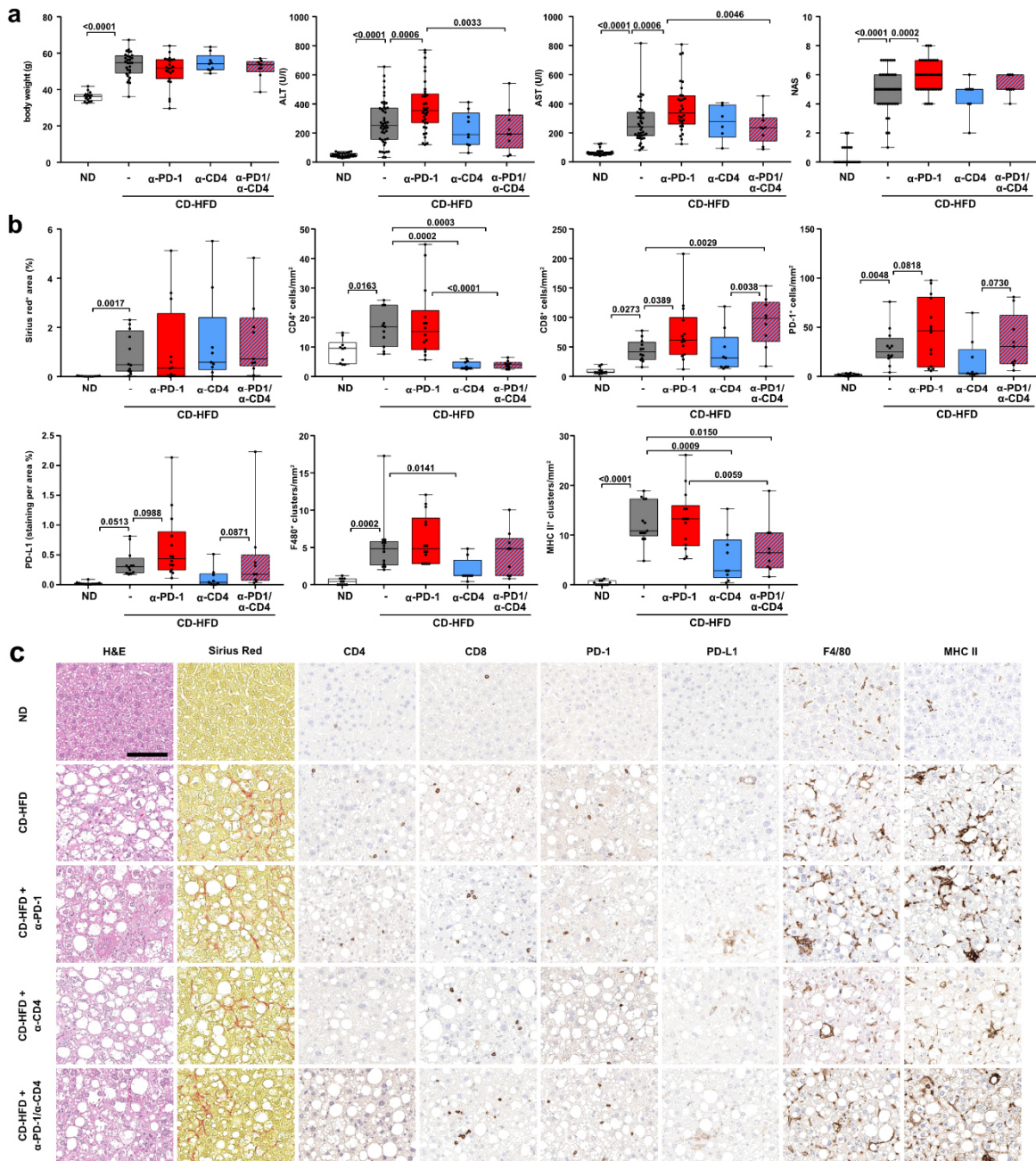
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(a) ScRNA-seq analysis of hepatic TCRβ<sup>+</sup> cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 or α-CD8 antibodies (n= 3 mice/group). (b) Selected marker expression in hepatic CD8<sup>+</sup> T-cells by scRNA-seq comparing CD8<sup>+</sup> with CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (n= 3 mice/group). (c) Average UMI comparison of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of

1249 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n=
 1250 3 mice/group). (d) RNA velocity analyses of scRNA-seq data showing expression and (e)
 1251 correlation of expression along the latent-time of selected genes along the latent-time (n= 3
 1252 mice/group). Root cells: yellow cells indicate root cells, blue cells indicate cells farthest away
 1253 from root by RNA velocity. End points: yellow cells indicate end point cells, blue cells indicate
 1254 cells farthest away from defined end point cells by RNA velocity. Latent time: pseudo-time by
 1255 RNA velocity, dark color indicate start of RNA velocity, yellow color indicate end point of latent
 1256 time. RNA velocity flow: Blue cluster defined as start point, orange cluster as intermediate,
 1257 green cluster as end point. Arrows indicate trajectory of cells. (f) PCA plot of hepatic CD8+ or
 1258 CD8+PD-1+ T-cells sorted TCR $\beta$ + cells by mass spectrometry of 12 months ND, CD-HFD or
 1259 CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD8+: ND n= 6 mice, CD-HFD
 1260 + IgG n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice; CD8+PD-1+: ND n= 4 mice, CD-HFD + IgG n=
 1261 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (g) UMAP representation showing the FlowSOM-guided
 1262 clustering, heatmap showing the median marker expression, and (h) quantification of hepatic
 1263 CD8+ T-cells of 12 months ND, CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by
 1264  $\alpha$ -PD-1 antibodies (ND n= 4 mice; CD-HFD + IgG n= 8 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (i)
 1265 Quantification of CellCNN analyzed flow cytometry data of hepatic CD8+ T-cells of 12 months
 1266 CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD +
 1267 IgG n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice). (j) UMAP representation showing the FlowSOM-
 1268 guided clustering, the expression intensity of the indicated marker and heatmap showing the
 1269 median marker expression of flow cytometry data of hepatic CD8+PD-1+ T-cells of 12 months
 1270 ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 6 mice;
 1271 CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (k) ALT and (l) NAS evaluation of 12 months
 1272 ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF, or  $\alpha$ -
 1273 PD-1/ $\alpha$ -TNF antibodies (ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice;
 1274 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF
 1275 n= 11 mice). (m) Quantification of hepatic CD8+PD-1+CXCR6+ T-cells ND, CD-HFD, CD-
 1276 HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4,
 1277 or  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35
 1278 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-
 1279 1/ $\alpha$ -TNF n= 11 mice); CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice). (n)
 1280 Quantification of tumor incidence of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks
 1281 treatment by  $\alpha$ -CD8,  $\alpha$ -CD8/NK1.1,  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or
 1282  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (tumor incidence: CD-HFD n= 32 tumors/lesions in 87 mice; CD-
 1283 HFD +  $\alpha$ -CD8 n= 2 tumors/lesions in 31 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 0 tumors/lesions in
 1284 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 33 tumors/lesions in 44 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 2
 1285 tumors/lesions in 9 mice; CD-HFD +  $\alpha$ -TNF n= 3 tumors/lesions in 10 mice; CD-HFD +  $\alpha$ -PD-
 1286 1/ $\alpha$ -TNF n= 3 tumors/lesions in 11 mice); CD-HFD +  $\alpha$ -CD4 n= 3 tumors/lesions in 9 mice; CD-
 1287 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 tumors/lesions in 9 mice).



### Rebuttal Figure 33

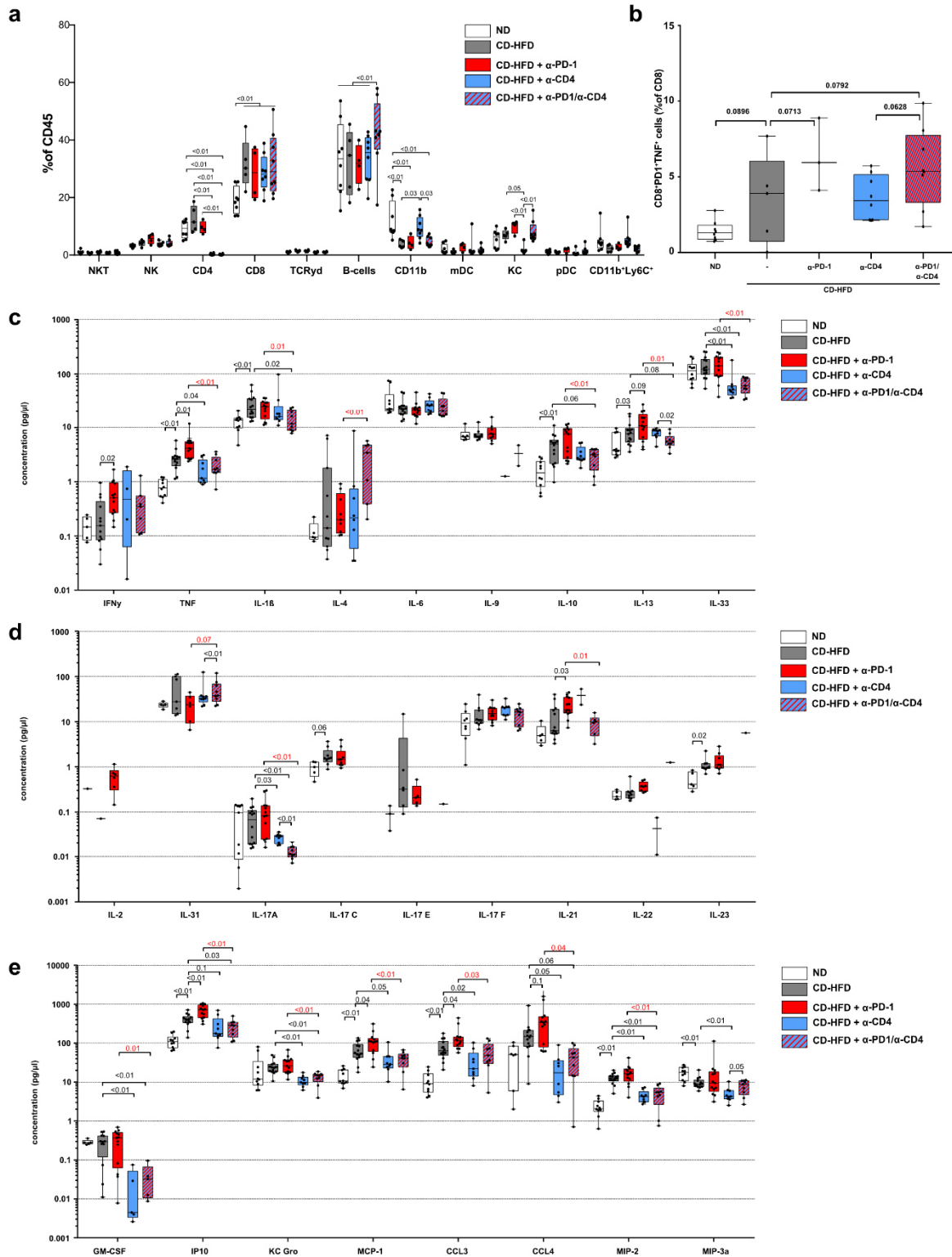
(a) Body weight, ALT, AST, NAS, and histological evaluation by (b) Sirius Red, CD4, CD8, PD-1, PD-L1, F4/80, MHC-II and (c) staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (body weight: ND n= 16 mice; CD-HFD n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; ALT ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; AST: ND n= 30 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; NAS: ND n= 31 mice; CD-HFD n= 46 mice; CD-HFD +  $\alpha$ -PD-1 n= 40 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice; Sirius red: ND n= 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; CD4: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 11 mice; CD8: ND n= 10

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Research for a Life without Cancer

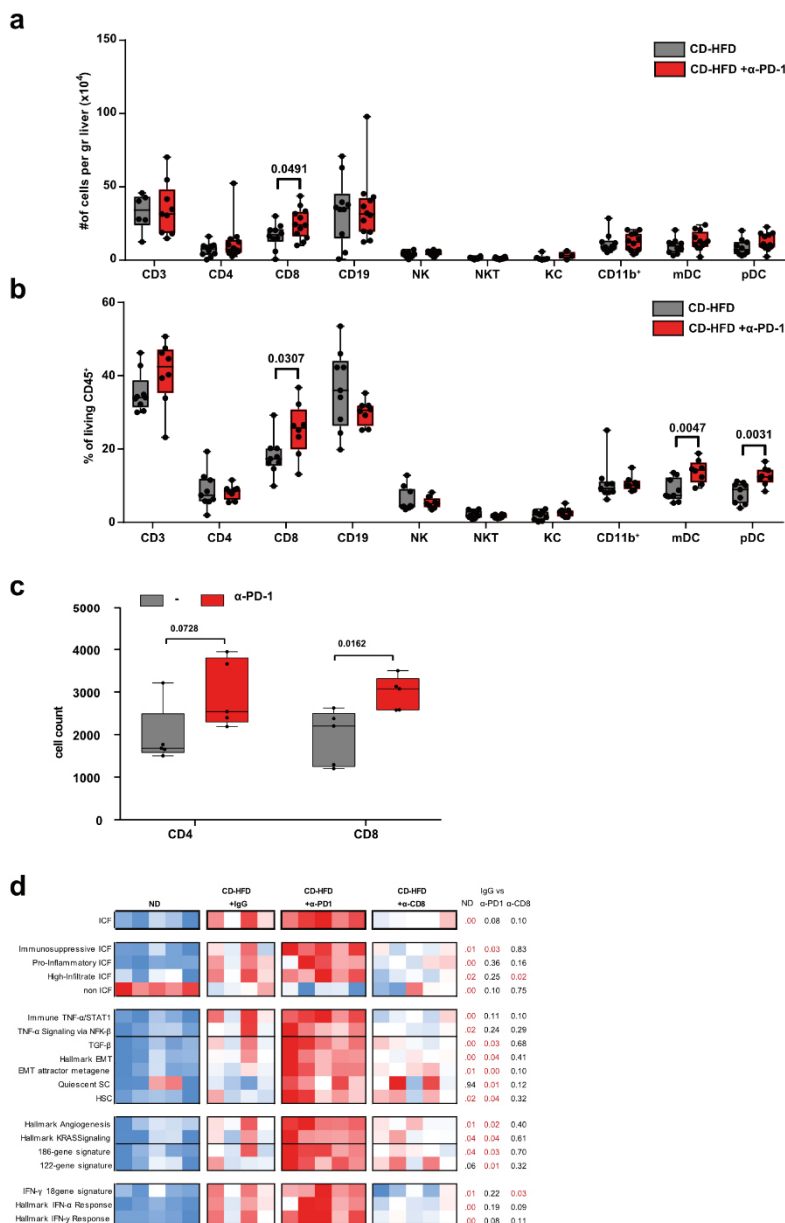
1302 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-  
1303 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-1: ND n= 13 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -  
1304 PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-L1:  
1305 ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9  
1306 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; F4/80: ND n= 11 mice; CD-HFD n= 13 mice; CD-  
1307 HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice;  
1308 MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -  
1309 PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice). Scale  
1310 bar: 100  $\mu$ m.



1311  
1312 **Rebuttal Figure 34**

1313 (a) Quantification of hepatic immune cell composition and (b) CD8+PD-1+TNF+ T-cells by flow  
1314 cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by α-PD-1,  
1315 α-CD4, α-PD-1/α-CD4 antibodies (Hepatic immune cell composition: ND n= 8 mice; CD-HFD  
1316 n= 5 mice; CD-HFD + α-PD-1 n= 4 mice; CD-HFD + α-CD4 n= 8 mice; CD-HFD + α-PD-1/α-  
1317 CD4 n= 8 mice; CD8+PD-1+TNF+: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD + α-PD-1 n=

1318 3 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice). (c) and (d) multiplex  
 1319 ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12 months ND,  
 1320 CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4  
 1321 antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 13 mice; CD-HFD +  
 1322  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).



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### Rebuttal Figure 35

(a) Absolute and (b) relative quantification of hepatic leukocytes of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-1 (CD3: CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 10 mice; CD4, CD8, CD19, NK, NKT, CD11b+, mDC, pDC: CD-HFD n= 10 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice, KC: CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice). (c) In vitro stimulated splenic CD8 T cells from C57Bl/6 mice were treated with  $\alpha$ -PD-1 antibody for 72 hours (cell count: n= 5 experiments/group; Ki-67: n= 4 experiments/group). (d) Immune-related gene expression patterns of RNA sequencing data of hepatic tissue of 12 months ND, CD-HFD or CD-HFD—fed mice + 8 weeks treatment of  $\alpha$ -PD-1 or  $\alpha$ -CD8 (ND, CD-HFD +  $\alpha$ -PD-1, CD-HFD +  $\alpha$ -CD8 n= 5 mice/group; CD-HFD n= 4 mice).

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1335 5. Some of the data the authors present seems internally inconsistent. As an example, the  
1336 authors postulate that the pro-inflammatory hepatic environment is responsible for the increase  
1337 in liver cancer incidence in anti-PD-1-treated mice, which they underscore by an increase in  
1338 inflammatory cytokines in the liver microenvironment (Figure S11). However, they also show  
1339 that upon CD8 depletion, which reduces cancer incidence, the inflammatory cytokines do not  
1340 significantly reduce compared to the CD-HFD diet mice alone. This implies that the  
1341 inflammatory microenvironment is not actually responsible for increased cancer incidence.  
1342 How do the authors harmonize these findings?

1343

1344 We thank Referee #2 for his comment on the bivalence of cellular and micro-environmental  
1345 induced cell death, inflammation, and liver cancer formation. However, we firmly state, that our  
1346 data is not internally inconsistent, and have added several experiments that clarify the  
1347 mechanisms of action.

1348 We state, that anti-PD-1 therapy induces an increased hepatic inflammatory  
1349 microenvironment, indicated by a) increased abundance of hepatic immune cells (mainly CD8+  
1350 and CD8+PD-1+ cells) (included in **Figure 2 and Extended Data 11 and Rebuttal Figure 30,**  
1351 **36**); b) by increased inflammation-associated cytokines (e.g. IFN $\gamma$ , TNF, IL-21, IP10, MCP-1,  
1352 CCL3) (included in **Extended Data 13 and Rebuttal Figure 37**); c) on mRNA expression levels  
1353 we actually clearly see the increase in all pathways relevant for inflammation induced liver  
1354 cancer – as analyzed by the ICF-signature (included in **Figure 3 and Rebuttal Figure 35d**).  
1355 Thus, we think, that there are 2 components (first cells, like CD8+ T-cells and second, the  
1356 inflammatory liver environment) responsible for (increased) liver cancer incidence.

1357 We agree with Referee #2 that initially this appears not logic – but we believe that a liver tissue  
1358 homogenate analysis cannot uncover the CD8+-T cell restricted cytokine changes, as other  
1359 immune cells will still produce inflammatory immune cells. This is indicated for example in  
1360 **Figure 3 and Rebuttal Figure 29**, which shows, that upon CD8 depletion TNF+ cells are  
1361 significantly reduced by *in situ* hybridization. Again, effects of the CD8 depletion manifests  
1362 strongly on mRNA expression level as pathways relevant for inflammation induced liver cancer  
1363 are strongly reduced– as analyzed by the ICF-signature (included in **Figure 3 and Rebuttal**  
1364 **Figure 35d**).

1365 Moreover, as stated by the Referee it appears that anti-CD8 treatment alone did not, but anti-  
1366 CD8/anti-PD-1 did reduce several chemokines indicative of a hepatic inflammatory  
1367 environment on protein level, that are responsible for myeloid cell attraction like MCP-1, CCL2,



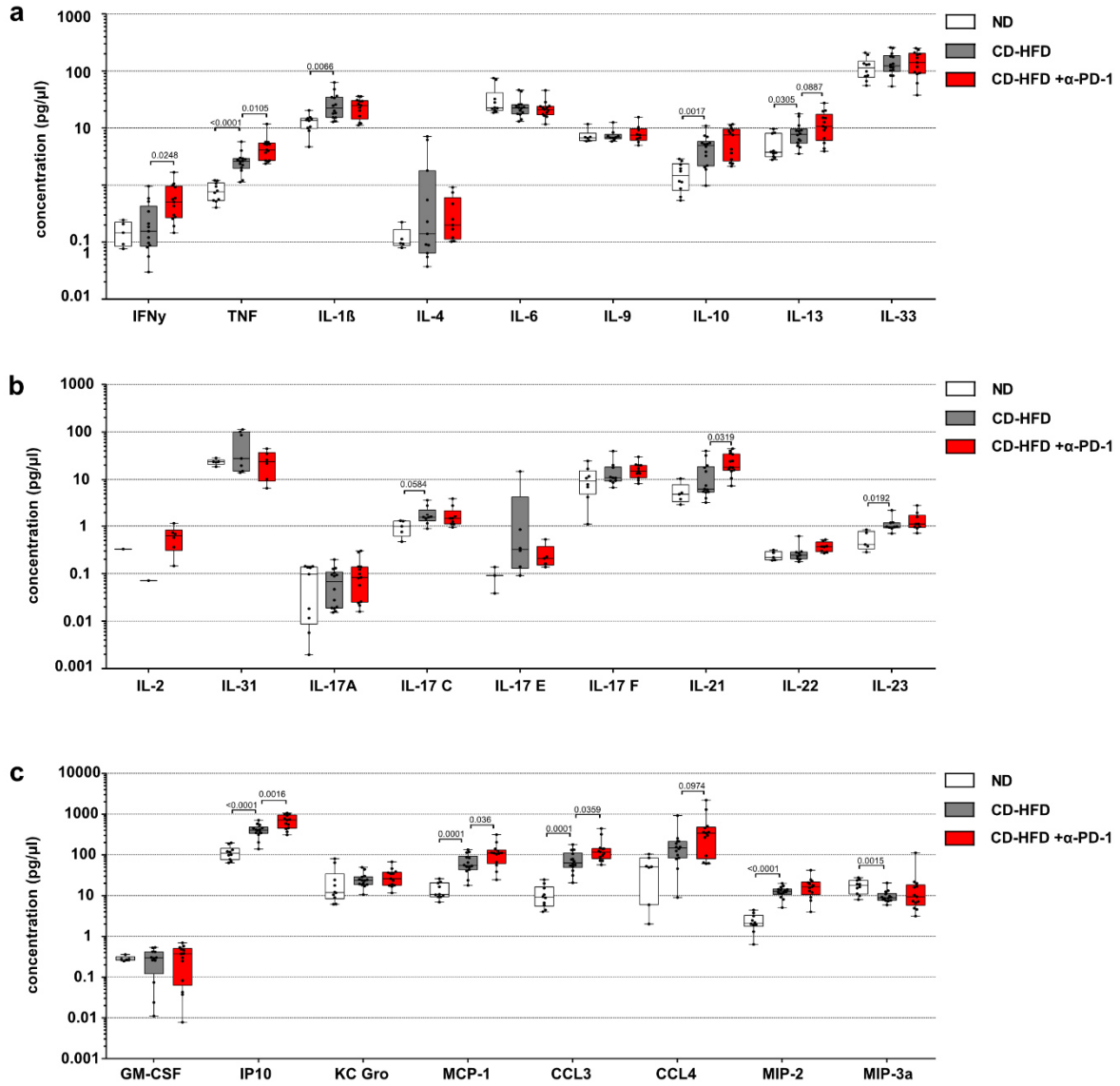
1368 CCL3, MIP-3a, or alarmins like IL-33 (included in **Extended Data 10+21** and **Rebuttal Figure**  
1369 **28c-d, 31**).

1370 Moreover, we want to point out that our data are also confirmed by the co-submitted manuscript  
1371 Dudek et al., revealing that the mechanisms of CD8+ T-cell mediated cell death is 1) CD8+ T-  
1372 cell dependent, 2) TCR independent, and 3) TNF is a crucial cytokine sensitizing the CD8+ T-  
1373 cell to get auto-aggressive and thus starts to mediate cell death.

1374 We demonstrate that TNF is a marker of a pro-inflammatory, pro-carcinogenic hepatic  
1375 environment and that it is increased upon PD-1-targeted immunotherapy and remains high in  
1376 CD8+ depleted mice (included in **Extended Data 10** and **Rebuttal Figure 31**). However, CD8  
1377 depleted mice lack tumor development (included in **Figure 2** and **Rebuttal Figure 36j**). In line  
1378 with Referee #2 and the co-submitted manuscript Dudek et al., we think, that the presence of  
1379 CD8+ T-cells is essential to drive hepatocarcinogenesis. We thus have performed the above  
1380 mentioned CD8 depletion combined with PD-1 targeted immunotherapy to underline that CD8+  
1381 T-cells are essential for increased hepatocarcinogenesis upon PD-1-targeted immunotherapy  
1382 compared to control mice under CDHFD diet (included in **Figure 4** and **Extended Data 20+21**  
1383 and **Rebuttal Figure 27, 28, 32**).

1384 We have functionally strengthened data shown by Dudek et al. that TNF - as a marker of the  
1385 inflammatory environment - is crucial for sensitizing the hepatic microenvironment to CD8 T-  
1386 cell -mediated cell death by performing anti-TNF with/without PD-1-targeted immunotherapy.  
1387 This has allowed the interpretation and has been experimentally demonstrated that only an  
1388 inflammatory environment combined with the presence of CD8 T-cells drives increased  
1389 hepatocarcinogenesis upon PD-1-targeted immunotherapy (included in **Figure 4, Extended**  
1390 **Data 20+21** and **Rebuttal Figure 27, 28, 32**).

1391 Furthermore, to shed new light on potential compensatory immunological mechanisms of  
1392 CD4+PD-1+ T-cells in the context of PD-1-targeted immunotherapy, we have performed CD4  
1393 depletion with/without PD-1-targeted immunotherapy (included in **Extended Data 22 and 23**  
1394 and **Rebuttal Figure 33, 34**). Notably, these experiments indicate that in contrast to CD8+ T-  
1395 cells CD4+ T-cells do not play a major effector role in comparison to CD8+ T-cells in anti-PD1  
1396 related liver cancer formation in the context of NASH and anti-PD1 treatment (included in  
1397 **Figure 32n**).



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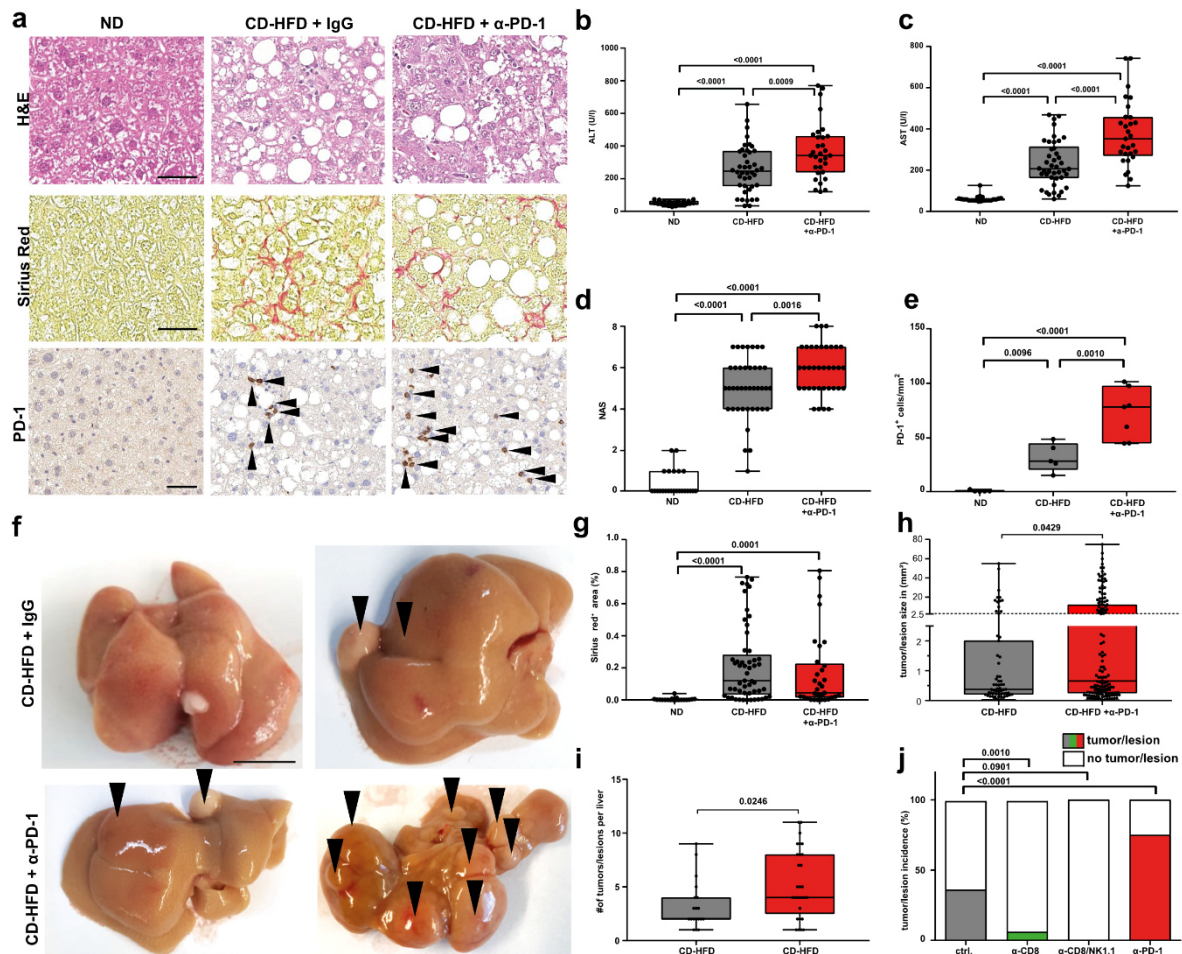
**Rebuttal Figure 36**

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(a) and (b) multiplex ELISA concentrations of hepatic inflammation-associated cytokines and (c) chemokines of 12 months ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-1 (ND n = 10 mice; CD-HFD n = 14 mice; CD-HFD +  $\alpha$ -PD-1 n = 13 mice).

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**Rebuttal Figure 37**

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(a) Histological staining of hepatic tissue by H&E, Sirius Red, PD-1 and CD8 of 12 months ND,

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CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1 (H&E: ND n= 24 mice; CD-HFD n= 40

1407

mice; CD-HFD +  $\alpha$ -PD-1 n= 36 mice; Sirius Red: ND n= 19 mice; CD-HFD n= 31 mice; CD-

1408

HFD +  $\alpha$ -PD-1 n= 27 mice; PD-1: ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 7

1409

mice). Arrowheads indicate PD-1<sup>+</sup> cells. Scale bar: 50  $\mu$ m. (i) ALT and (j) AST levels of 12

1410

months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1 (ALT: ND n= 22 mice; CD-

1411

HFD n= 42 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice). (k) NAS evaluation by H&E of 12 months

1412

ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1 (ND n= 24 mice; CD-HFD n= 40 mice;

1413

CD-HFD +  $\alpha$ -PD-1 n= 36 mice). (l) Quantification of PD-1 staining of hepatic tissue by

1414

immunohistochemistry of 12 months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1

1415

(ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -CD8 n= 7 mice). (m) Macroscopy of liver of

1416

12 months CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1. Arrowheads indicate

1417

tumor/lesions. Scale bar: 10 mm. (n) Fibrosis evaluation of Sirius Red staining of 12 months

1418

ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1 (ND n= 19 mice; CD-HFD n= 53 mice;

1419

CD-HFD +  $\alpha$ -PD-1 n= 33 mice). (o) Quantification of tumor/lesion size and (p) tumor load of

1420

12 months CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -PD-1 (tumor/lesion size, tumor load:

1421

CD-HFD n= 19 mice; CD-HFD +  $\alpha$ -PD-1 n= 29 mice). (q) Quantification of tumor incidence of

1422

12 months CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8, co-depletion of  $\alpha$ -CD8/NK1, or

1423

$\alpha$ -PD-1 (tumor incidence: CD-HFD n= 32 tumors/lesions in 87 mice; CD-HFD +  $\alpha$ -CD8 n= 2

1424

tumors/lesions in 31 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 0 tumors/lesions in 6 mice; CD-HFD

1425

+  $\alpha$ -PD-1 n= 33 tumors/lesions in 44 mice).

1426

1427 6. Crucially, and related to my previous point, the authors also did not perform CD8 depletion  
1428 in the context of anti-PD-1 treatment to show that CD8 cells are indeed the cells that are  
1429 responsible for increased carcinogenesis upon anti-PD-1 therapy.

1430

1431 We thank Referee #2 for this important comment and fully agree that anti-PD-1 treatment in  
1432 the context of CD8 depletion is crucial for data interpretation and we included this experiment  
1433 in a revised manuscript (included in **Figure 4, Extended Data 20 and 21 and Rebuttal Figure**  
1434 **27, 28, 32**).

1435 The combined anti-CD8/anti-PD-1 treatment has allowed an understanding on a functional  
1436 level, that indeed increased the hepatic abundance of CD8+PD-1+ T-cells upon PD-1-targeted  
1437 immunotherapy is crucial for driving hepato-carcinogenesis. Notably, this treatment reduced  
1438 NAS, liver damage and some cytokines (e.g. MCP-1, CCL2, CCL3, MIP-3a) that affect the  
1439 pathway of CD8+ T-cell activation by the liver environment (e.g. IL33, IL21).

1440

1441 7. At times, the authors are (highly) selective in the data they choose to discuss and interpret.  
1442 As an example, regarding Figure 1i, the authors describe the CD8+ T-cells in CD-HFD mice to  
1443 demonstrate profiles of cytotoxicity and effector function because of increased expression of  
1444 GzmK/M and Pdcd1. However, in the same plot shows that these cells have reduced  
1445 expression of GzmA/B, Klrg1, Il2ra, TNF and Il2; all markers of effector/cytotoxicity. How do  
1446 the authors harmonize these observations?

1447

1448 We thank Referee #2 for asking this important question. As Referee #2 highlighted in the  
1449 example of **Figure 1**, we think it is of vital importance to display the observed profile of CD8 T-  
1450 cells on a broad scale. We believe that this particular character of T cells – that initially appears  
1451 to be exhausted (e.g. TOX expression) is actually hyperactivated with a particular pattern of  
1452 expression.

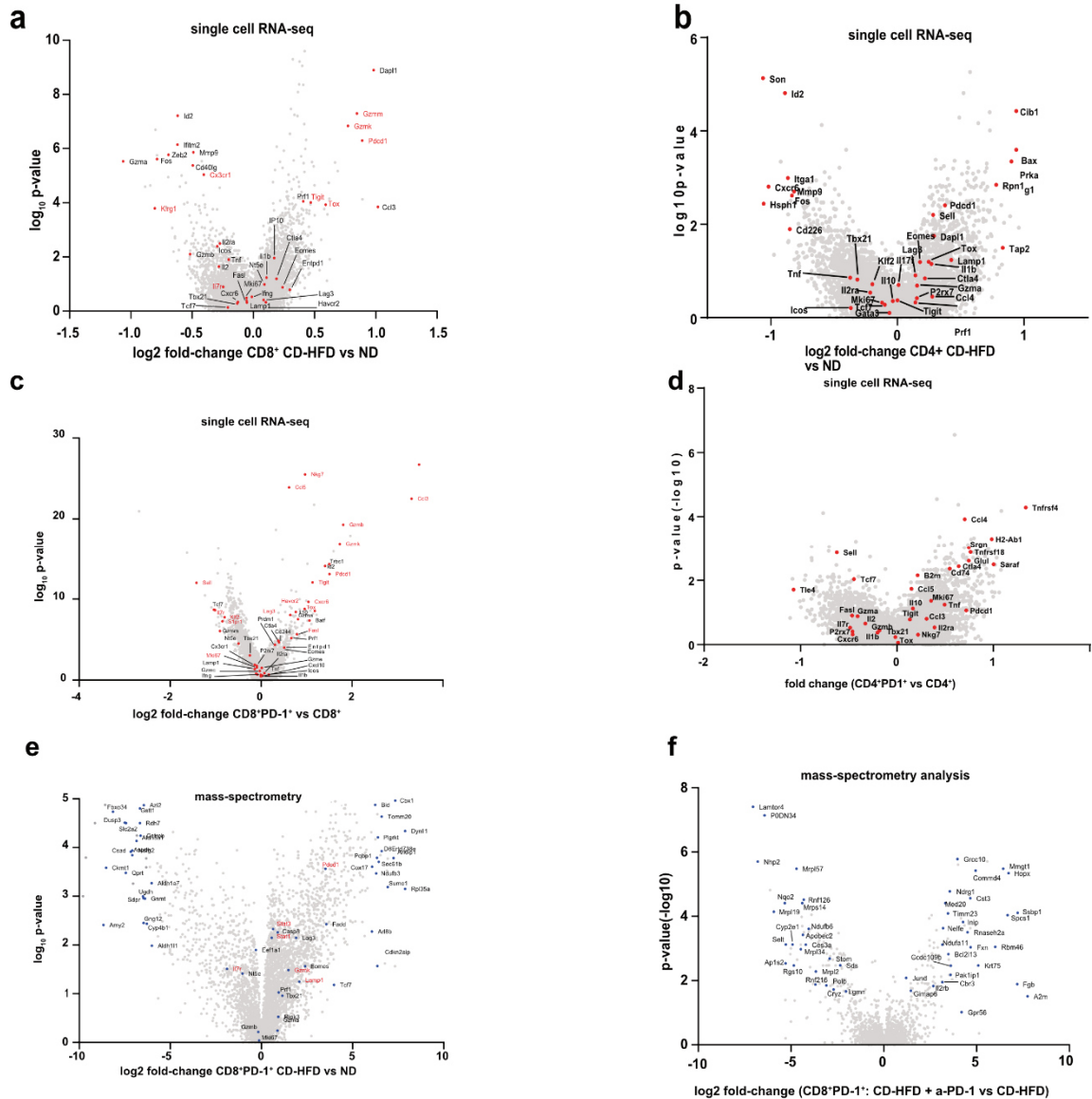
1453 Thus, the single-cell technology allows dissecting the expression profile of CD-HFD-fed CD8+  
1454 T-cells into a combination of cytotoxicity/exhaustion expression, indicative of a unconventional  
1455 activation/effector. To not lose single-cell resolution and how the data translates into proteins,  
1456 we have corroborated these data by mass-spectrometry. These data corroborated the scRNA-  
1457 data of **Figure 1** with enrichment for effector function (e.g. T-cell activation, T-cell  
1458 differentiation, and NK mediated cytotoxicity) in CD-HFD-fed CD8+PD-1+ T-cells (included in  
1459 **Extended Data 5 and Rebuttal Figure 38**). Thus, we decided to display a wide variety of  
1460 markers of effector function/cytotoxicity allowing the reader a more sophisticated view into the

1461 phenotype. Moreover, we have compared this pattern with human NASH and indeed could  
1462 find that patients with NASH do resemble a similar pattern.

1463 To test this unconventional activation/exhaustion phenotype on a functional level, we  
1464 performed all the treatments described in **Figures 2-4** in the absence or in the presence of  
1465 anti-PD1-related immunotherapy (anti-CD8, anti-CD8/anti-NK1.1, anti-CD8/anti-PD1, anti-  
1466 PD1, anti-PDL1, anti-TNF, anti-TNF/anti-PD1, and as control experiment anti-CD4 and anti-  
1467 CD4/anti-PD1), as well as the corroboration with the human data.

1468 For example, an increased anti-inflammatory role by IL-10 expressing CD8+ T-cells upon PD1-  
1469 targeted immunotherapy could not be corroborated (included in **Extended Data 19** and  
1470 **Rebuttal Figure 39**) (Breuer et al., 2020). Of note, in this publication diet-based NAFLD  
1471 induction was achieved by feeding either WD or CD-HFD for 8-10 weeks. This is in strong  
1472 contrast to our experimental regime of applying diet for 3, 6, or 12 months as we show, that  
1473 the preclinical model presents different stages of NASH pathology severity including  
1474 hepatocarcinogenesis (included in **Figure 1** and **Rebuttal Figure 25**).

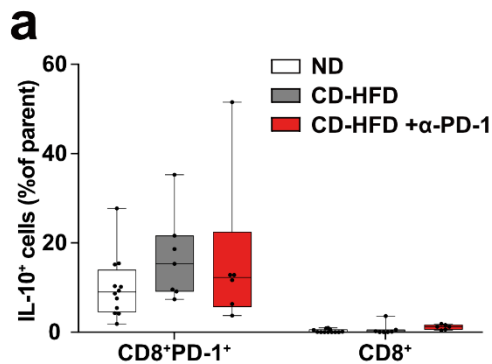
1475 Furthermore, we would like to draw attention to the improved cross-referencing to the co-  
1476 submitted manuscript Dudek et al., which confirmed a CD8 profile of effector  
1477 function/exhaustion/cytotoxicity on a functional level (e.g. TNF sensitizing, high Granzyme  
1478 expression, TCR-independent mediated cell death). Moreover, we tried to improve the  
1479 discussion on recent literature on the role of CD8 T-cells in metabolic diseases.



1480  
1481

### Rebuttal Figure 38

1482 (a) Selected average marker expression in T-cell subsets of CD8<sup>+</sup> and (b) CD4<sup>+</sup> sorted TCRβ<sup>+</sup>  
1483 by scRNA-seq of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). (c) Selected marker  
1484 expression in hepatic CD8<sup>+</sup> T-cells by scRNA-seq comparing CD8<sup>+</sup> with CD8<sup>+</sup>PD-1<sup>+</sup> T-cells  
1485 of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment of α-PD-1 (n= 3  
1486 mice/group). (d) Selected marker expression in hepatic CD4<sup>+</sup> T-cells by scRNA-seq comparing  
1487 CD4<sup>+</sup> with CD4<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks  
1488 treatment of α-PD-1 fed mice (n= 3 mice/group). (e) Selected marker expression in hepatic  
1489 CD8<sup>+</sup>PD-1<sup>+</sup> T-cells by mass- spectrometry of 12 months ND or CD-HFD-fed mice (ND n= 4  
1490 mice, CD-HFD n= 6 mice). (f) Selected marker expression in hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells  
1491 sorted TCRβ<sup>+</sup> cells by mass- spectrometry of 12 months CD-HFD or CD-HFD-fed + 8 weeks  
1492 treatment of α-PD-1 fed mice (n= 6 mice/group). Candidates developing steady in-/decrease  
1493 from ND to CD-HFD to CD-HFD-fed mice + 8 weeks treatment of α-PD-1 are indicated in red.  
1494 (n= 6 mice/group).



1495  
1496

### Rebuttal Figure 39

1497 (a) Polarization by flowcytometry of hepatic CD8+PD-1+ T-cells of 12 months ND, CD-HFD or  
1498 CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-1 (ND n= 12 mice; CD-HFD n= 7 mice; CD-  
1499 HFD +  $\alpha$ -PD-1 n= 6 mice).  
1500

1501 8. Regarding Figure 1e, the authors state that CD-HFD contain a significantly altered immune  
1502 composition that mainly affects the CD8+ T-cell compartment. However, this finding was not  
1503 significant ( $p=0.09$  for CD8+PD-1+ T-cells and ns for CD8+ T-cells). In this plot, the authors  
1504 do show significant differences in frequency of CD4+ T-cells ( $p<0.01$ ), classical monocytes  
1505 ( $p<0.01$ ) and MDMs Ly6CHigh ( $p=0.01$ ). Why are these cell types not regarded as interesting?  
1506 Are these cells responsible for the authors' proposed phenotype? In line 259 the authors state  
1507 that there are only minor differences in the CD4 compartment, yet when looking at the data  
1508 (Figure S9h and Figure S9f) the difference in the CD4 subset of CD62L-CD44+CD69+ upon  
1509 anti-PD-1 blockade is as strong as, if not stronger than, in the same subset of CD8 T-cells,  
1510 which the authors do deem interesting.

1511  
1512 We thank Referee #2 pointing out these details in our analysis. We agree with Referee #2, that  
1513 immunological subsets represented in our data set are well described in the literature (e.g.  
1514 reduction of CD4+ T-cells (Ma et al., 2016) and changes in the myeloid compartment, including  
1515 classical monocytes and MDMs Ly6CHigh (Malehmir et al., 2019; Nakagawa et al., 2014),  
1516 therefore the respective citations are included in our introduction and discussion.

1517 We added new data and have re-analyzed the data displayed in **Figure 1e** according to  
1518 Referee's #4 comments also by highlighting NKT cells. These results, in CD8+PD1+ ( $p= 0.03$ ),  
1519 significantly changed. Other changed cellular subsets after 12 months of CD-HFD feeding are  
1520 CD4+ T-cells ( $p= 0.04$ ), classical monocytes ( $p< 0.01$ ), KC ( $p= 0.01$ ), MDMs ( $p=0.02$ ), MDMs  
1521 Ly6C+ ( $p< 0.01$ ). We agree with Referee #2, that CD4 T-cells and their expression of PD-1  
1522 might play a crucial role in shaping the liver micro-environment and in the observed phenotype

1523 and thus included analysis of CD4 T-cells to the majority of our experiments (e.g. **Extended**  
1524 **Data 3** and **Rebuttal Figure 40**).

1525 However, the magnitude of effects observed in CD4+ T-cells is minor when compared to CD8+  
1526 T-cells (e.g. **Extended Data 11** mean (CD8+CD62L-CD44+CD69+) ~12% (%of CD45+) vs  
1527 mean (CD4+CD62L-CD44+CD69+) ~4% (%of CD45+) upon PD-1 targeted immunotherapy).  
1528 Data obtained from CD4 depletion with/without PD1-targeted immunotherapy indicate, that the  
1529 increased hepatocarcinogenesis in the context of immunotherapy is independent of hepatic  
1530 abundance of CD4+ T-cells in the preclinical NASH model (included in **Figure 4**, **Extended**  
1531 **Data 22 and 23** and **Rebuttal Figure 32n, 33, 34**).

1532 However, CD4+ T-cells might have a diverse set of effector functions (e.g. interpreting tumor  
1533 incidence in anti-CD8/anti-PD1 treated animals: although CD4 cells show trends for  
1534 decreasing, CD4 are relatively increased in the absence of CD8+ T-cells but immunotherapy,  
1535 thus CD4+ T-cells might be responsible for baseline tumor incidence in the context of  
1536 immunotherapy (included in **Extended Data 22 and 23** and **Rebuttal Figure 33, 34**); or CD4  
1537 might have a tumor controlling role, as there are the trends of increased tumor incidence upon  
1538 anti-CD4/anti-PD1 co-treatment (tumor incidence (anti-PD-1 mono-treatment)= 75% vs tumor  
1539 incidence (anti-CD4/anti-PD1 co-treatment)= 88%) (included in **Figure 4** and **Rebuttal Figure**  
1540 **32n**)).

1541 Of note, CD4+ T-cells might also significantly changed in the human situation, and have also  
1542 analyzed human CD4+ cells a by scRNA-Seq (included in **Extended Data 25c** and **Rebuttal**  
1543 **Figure 41a**). In addition, we have performed RNA velocity analyses of the scRNA Seq data of  
1544 mouse and human CD4 T cells. In mouse, no significant velocity flow was detected in 12  
1545 months CD-HFD-fed mice, indicating, that CD4 cells are not transcriptionally activated and  
1546 driven by NASH-conditions or PD-1-targeted immunotherapy in NASH. However, we want to  
1547 point out, that in the mouse NASH model CD8 T-cells increase statistically significant, and thus  
1548 CD4 are relatively fewer cells compared to CD8. Therefore, the velocity analysis of mouse  
1549 CD4 T-cells need to be taken with caution, because we included 300-500 cells only per  
1550 described subset. As a consequence, we included the negative CD4 T-cell data not in the  
1551 manuscript but in the Rebuttal letter as **Rebuttal Figure 42**. Velocity analyses on human CD4  
1552 lead to comparable problems like seen in mouse. As a consequence, we included the negative  
1553 CD4 T-cell data not in the manuscript but in the Rebuttal letter as **Rebuttal Figure 42**.

1554 Like previously mentioned in point 3 raised by Referee #2 concerning the myeloid cells, our  
1555 presented data argue, that myeloid cells are recruited to the liver, extend and fine-tune liver  
1556 inflammation. While we see MDMs Ly6C+ cells increased comparing 12 months ND vs CD-  
1557 HFD-fed mice, our functional treatments (anti-PD-1, anti-CD8/anti-PD-1, anti-TNF, anti-





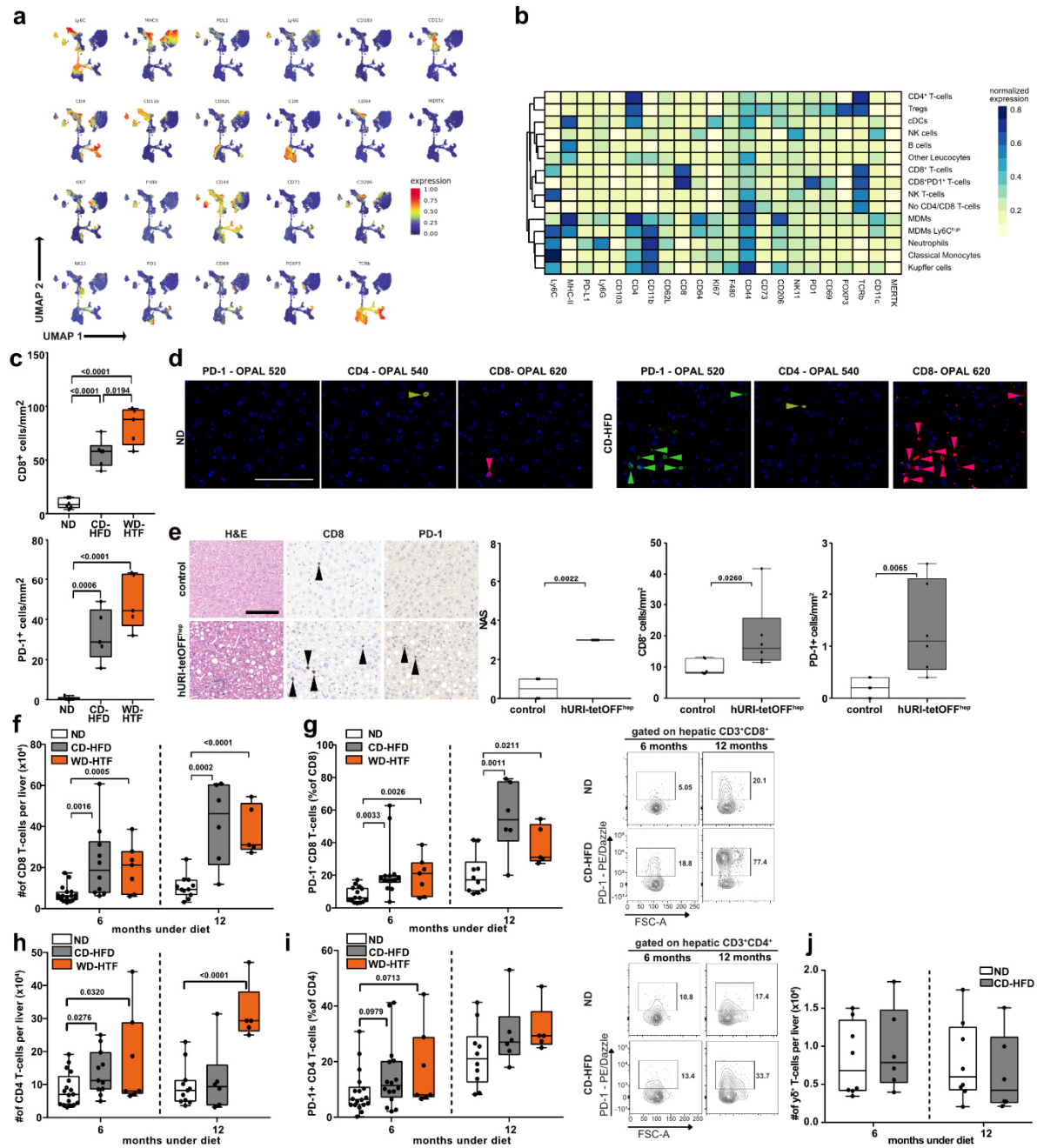
1558 TNF/anti-PD-1, anti-CD4 and anti-CD4/anti-PD-1) did not result in significant changes in  
1559 CD11b+Ly6C+ cells, indicating a rather minor role in comparison to the changes we observed  
1560 in the CD8 compartment (included in **Extended Data 4, 21, 23 and 24** and **Rebuttal Figure**  
1561 **23, 24, 28, 34**).

1562 Furthermore, we discuss the myeloid changes and potential role of CD4+ T-cells in greater  
1563 detail in the main text.

1564 Finally, we performed an anti-CD4 antibody treatment with or without the combination of anti-  
1565 PD1-related immunotherapy. Anti-CD4 antibody treatment successfully depleted or strongly  
1566 reduced intrahepatic CD4+ T cells in NASH. However, depletion of CD T cells did not reduce  
1567 liver cancer incidence – which is in contrast to CD8+ T cell depletion. Rather, in contrast, CD4  
1568 T cell depletion showed a trend in increase of tumor incidence – in line with published data by  
1569 Ma et al., 2016 (Nature).

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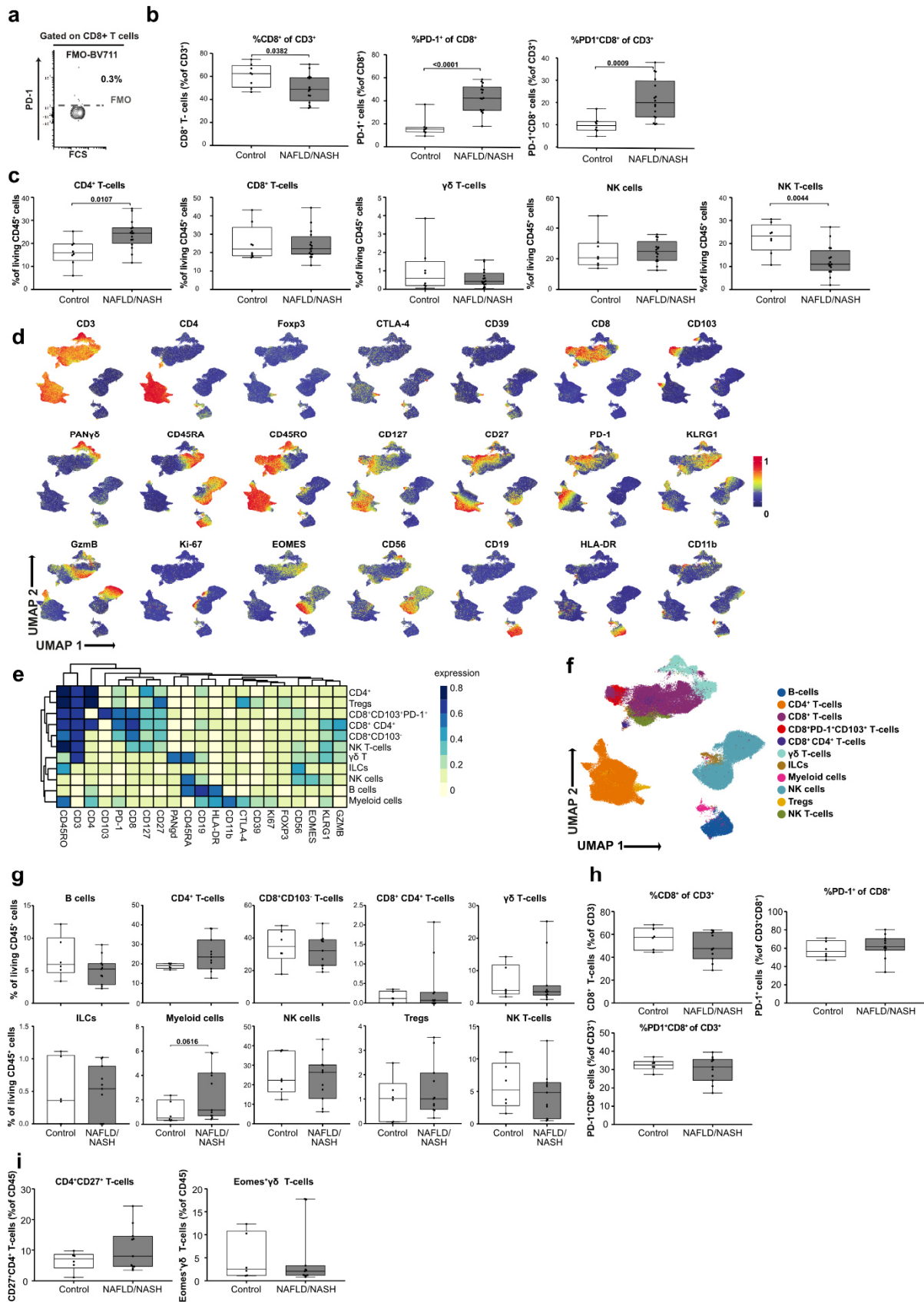
1572

### Rebuttal Figure 40

1573 (a) Analysis of 5000 randomly chosen CD45+ cells by flow cytometry to define distinct marker  
 1574 expression of 12 months ND or CD-HFD-fed mice (ND n= 4 mice; CD-HFD n= 8 mice). (b)  
 1575 Average marker expression of defined CD45+ subsets of 5000 randomly chosen CD45+ cells  
 1576 by flow cytometry of 12 months ND or CD-HFD-fed mice (ND n= 4 mice; CD-HFD n= 8 mice).  
 1577 (c) Quantification of hepatic CD8+ cells and PD-1+ expressing cells by immunohistochemistry  
 1578 of 12 months ND, CD-HFD or WD-HTF-fed mice (PD-1: n= 5 mice/group; CD8: ND n= 6 mice;  
 1579 CD-HFD n= 6 mice; WD-HTF n= 5 mice). (d) Immunofluorescence staining of single channel-  
 1580 staining PD-1, CD8 and CD4 (ocher) of 12 months ND or CD-HFD-fed mice (n= 3 mice/group).  
 1581 Arrowheads indicate CD8+ (red), PD-1+ (green) or CD4+ (ocher) cells. Scale bar: 100  $\mu$ m. (e)  
 1582 H&E, CD8 and PD-1 staining, evaluation by NAS and quantification of CD8+ cells and PD-1+  
 1583 expressing cells by immunohistochemistry of 32-weeks old hURI-tetOFF<sup>hep</sup> and non-



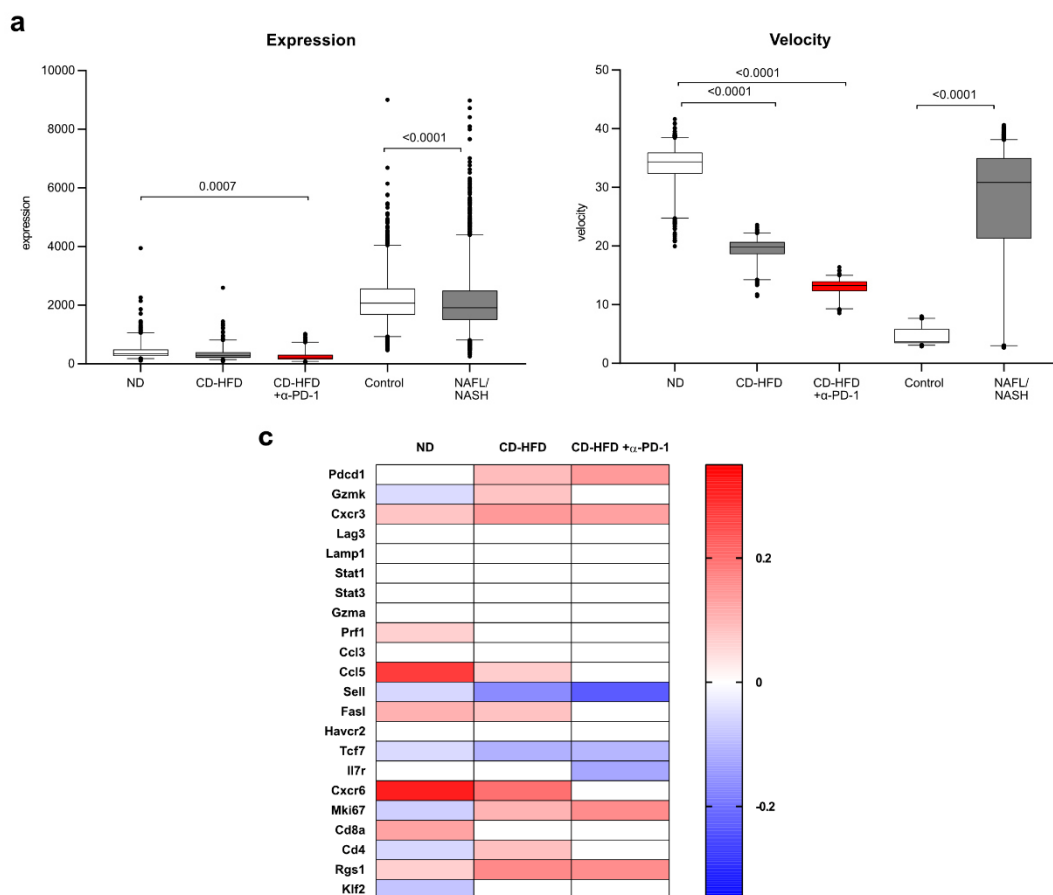
1584 transgenic litter control mice (n=6 mice/group). Arrowheads indicate specific staining positive  
1585 cells. Scale bar: 100  $\mu$ m. (f) Quantification of abundance, (g) PD-1 expression and flow  
1586 cytometry plots of hepatic CD8<sup>+</sup> T-cells by flow cytometry of 6 or 12 months ND or CD-HFD-  
1587 fed mice (abundance of CD8: 6 months: ND n= 17 mice; CD-HFD n= 10 mice; WD-HTF n= 7  
1588 mice; 12 months: ND n= 11 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice; PD-1 expression  
1589 in CD8<sup>+</sup> T-cells: 6 months: ND n= 15 mice; CD-HFD n= 14 mice; WD-HTF n= 7 mice; 12  
1590 months: ND n= 10 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice). (h) Quantification of  
1591 abundance, (i) PD-1 expression and flow cytometry plots of hepatic CD4<sup>+</sup> T-cells by flow  
1592 cytometry of 6 or 12 months ND or CD-HFD fed mice (abundance of CD4: 6 months: ND n=  
1593 17 mice; CD-HFD n= 10 mice; WD-HTF n= 7 mice; 12 months: ND n= 11 mice; CD-HFD n= 6  
1594 mice; WD-HTF n= 5 mice; PD-1 expression in CD4<sup>+</sup> T-cells: 6 months: ND n= 15 mice; CD-  
1595 HFD n= 14 mice; WD-HTF n= 7 mice; 12 months: ND n= 10 mice; CD-HFD n= 6 mice; WD-  
1596 HTF n= 5 mice). (j) Hepatic abundance of TCR $\gamma\delta$  T-cells of 6 or 12 months ND or CD-HFD fed  
1597 mice (6 months ND n= 8 mice; CD-HFD n= 6 mice; 12 months ND n= 8 mice; CD-HFD n= 6  
1598 mice).  
1599



1600  
1601

1602 **Rebuttal Figure 41**

1603 (a) Flow cytometry plot of FMO control, (b) quantification of patient-liver-derived PD-1+CD8+  
 1604 T-cells, and (c) quantification of CD4, CD8,  $\gamma\delta$ , NK and NKT cells healthy or NAFLD/NASH  
 1605 patients (Supplementary Table 1: healthy n= 8 patients; NAFLD/NASH n= 16 patients). (d)  
 1606 Analysis of randomly chosen CD45+ cells and (e) average marker expression of defined  
 1607 CD45+ subsets by flow cytometry derived from hepatic biopsies of control and NAFLD/NASH  
 1608 patients to define distinct marker expression (Supplementary Table 2: control n= 6 patients;  
 1609 NAFLD/NASH n= 11 patients). (f) Definition of cellular subsets, (g) relative quantification of  
 1610 defined cellular subsets of randomly chosen CD45+ cells, (h) polarization of CD8+ T-cells and  
 1611 (i) quantification of CD4+CD27+, or  $\gamma\delta$  TCR+Eomes+ T-cells by flow cytometry derived from  
 1612 hepatic biopsies of healthy and NAFLD/NASH patients (Supplementary Table 2: control n= 6  
 1613 patients; NAFLD/NASH n= 11 patients).



1614

 1615 **Rebuttal Figure 42**

1616 (a) RNA Velocity analyses of scRNA-seq data showing expression, and (b) velocity of patient-  
 1617 liver-derived CD4+ T-cells of control, or NAFLD/NASH patients in comparison to mouse-liver-  
 1618 derived CD4+ T-cells (patients: NAFLD/NASH n= 3 patients; mouse: n= 3 mice/group).  
 1619 (c) Correlation of expression along the latent-time of selected genes along the latent-time  
 1620 (mouse: n= 3 mice/group).  
 1621

1622 9. Along these lines, in line 387 the authors state that consistent with previous results, effects  
 1623 on the CD4+PD-1+ T-cell compartment remained minor, yet the differences observed for

1624 matching analyses (i.e. S17a vs S17g, S17b vs S17f, S17i vs S17j) of CD4 and CD8  
1625 populations show similar, if not stronger, effects for the CD4 T-cell population. Why are these  
1626 differences disregarded by the authors?

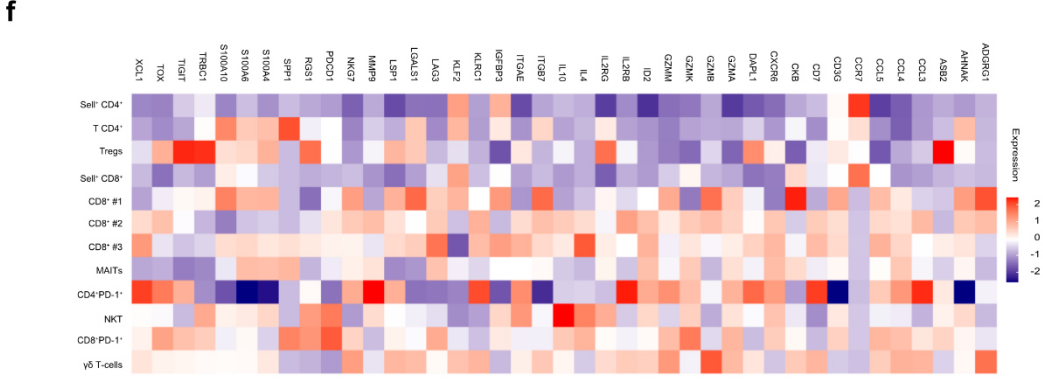
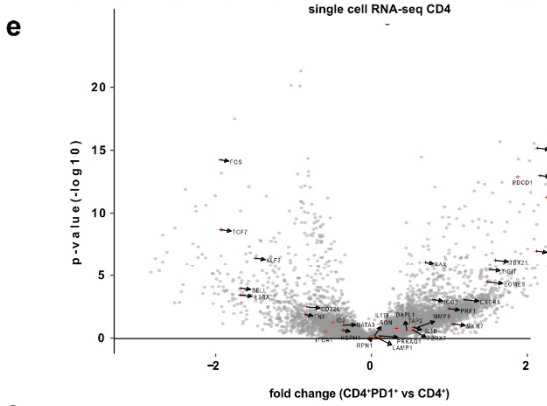
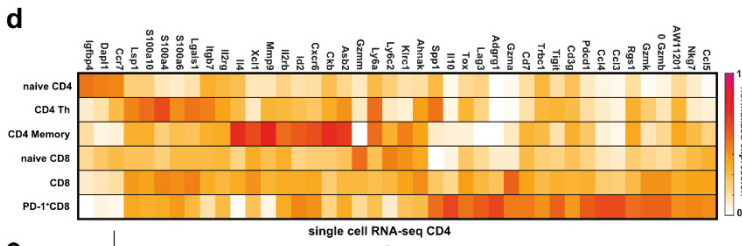
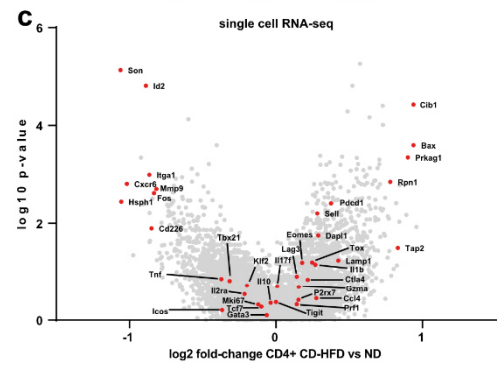
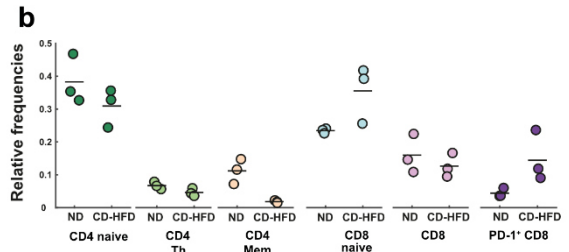
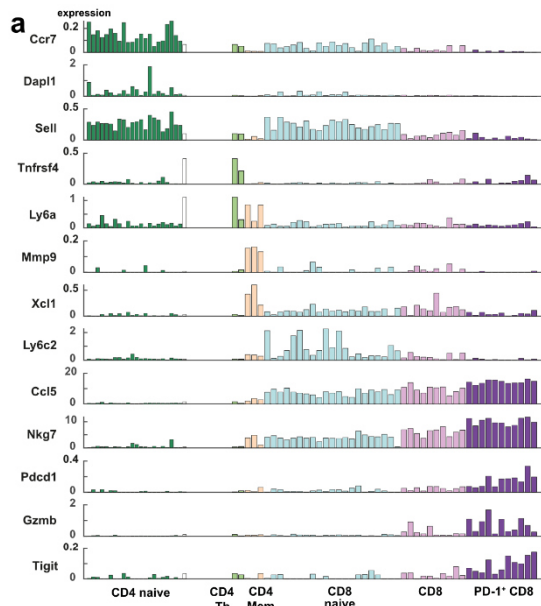
1627

1628 We believe that the comment of Referee #2 is important and we are in line that the context of  
1629 highlighting potential CD4-mediated effects in the context of PD-1-targeted therapy had to be  
1630 investigated in detail (e.g. in **Extended data 5, 18** and **Rebuttal Figure 43**) In line with the  
1631 comment of Referee#2, we set out to investigate the character and function of CD4+ T-cells  
1632 by scRNA-seq analyses in human and mouse NASH livers, but like raised in point 8 of Referee  
1633 #2 strongly suggest to take the velocity analysis of mouse CD4 T-cells with caution, because  
1634 we included 300-500 cells only per described subset. Thus, we included these analyses in only  
1635 in the **Rebuttal Figure 42**. Moreover, our experiments using an anti-CD4 depleting antibody  
1636 alone or in the context of anti-PD1-related immunotherapy indicate a minor role of the CD4  
1637 compartment in our model as well (included in **Extended Data 22, 23** and **Rebuttal Figure**  
1638 **33, 34**).

1639 As mentioned in point 8 raised by Referee #2, we agree with Referee #2, that similar  
1640 phenotypes can be observed when comparing effects in CD4+ and CD8+ T-cell subsets upon  
1641 PD-1 targeting immunotherapy. We do not disregard the changes in the CD4 compartment but  
1642 would like to draw attention to the magnitude of changes in the setting of chronic hepatic  
1643 inflammation – and the functional experiments with anti-CD8, anti-CD8/anti-PD-1, anti-CD4,  
1644 and anti-CD4/anti-PD1 antibodies.

1645 We have also discussed the relevant literature as well as our data on CD4+ T cells in the  
1646 discussion in detail. We, in addition, believe that the CD4+ T-cell depletion experiments  
1647 with/without PD-1 targeted immunotherapy in mice have enabled us to strengthen our  
1648 hypothesis on a more functional level: CD4 depletion alone or in the context of anti-PD1-related  
1649 immunotherapy in NASH-induced HCC failed to revert/prevent liver cancer formation. In  
1650 contrast, anti-CD8 depleting antibody treatment alone reverted/prevented liver cancer  
1651 formation.

1652 The role of CD4+ T-cells in the context of immunotherapy remains to be defined in more detail,  
1653 as CD4-depletion did not lead to a reversal of the pro-tumorigenic effects of anti-PD1 therapy  
1654 in the context of NASH induced HCC. However, CD4+ T-cells might exert a  
1655 protective/controlling role in the context of PD1-targeted immunotherapy and presence of  
1656 CD8+ T-cells, as combinatorial treatment of anti-CD4 depletion and PD1-targeted  
1657 immunotherapy led to an increase of tumor incidence compared to anti-PD1 treatment alone  
1658 (included in **Figure 4, Extended Data 22 and 23** and **Rebuttal Figure 32-34**).



1659  
1660

1661 **Rebuttal Figure 43**

1662 (a) Marker expression of CD4+ and CD8+ sorted TCR $\beta$ + cells defining T-cell subsets by single  
1663 cell RNA-sequencing of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). (b) Relative  
1664 frequency of CD4+ and CD8+ sorted TCR $\beta$ + cells by single cell RNA-sequencing of 12 months  
1665 ND or CD-HFD fed mice (n= 3 mice/group). (c) Selected marker expression in CD4+ T-cells  
1666 sorted TCR $\beta$ + cells by single cell RNA-sequencing of 12 months ND or CD-HFD fed mice (n=  
1667 3 mice/group). (d) Selected average marker expression in T-cell subsets of CD4+ and CD8+  
1668 sorted TCR $\beta$ + by scRNA-seq of 12 months ND or CD-HFD-fed mice (n= 3 mice/group).(e)  
1669 Differential gene expression of CD4+PD-1+ vs CD4+ T-cells and (f) selected average marker  
1670 expression in CD4+ and CD8+ T-cell subsets of by scRNA-seq of control and NAFLD/NASH  
1671 patients (control n= 4 patients; NAFLD/NASH n= 7 patients).  
1672

1673 10. Similarly, in Figure 5a, the authors claim that a CD8+PD-1+ T-cell population arises upon  
1674 NASH. However, there is a, perhaps even stronger, depletion of an Eomes+ gamma-delta T-  
1675 cell subset. Additionally, a very strong induction of a CD4+CD27+ population is observed in  
1676 NASH samples. Why are these not discussed? Can these populations also be identified in the  
1677 authors' murine models? Do these contribute to the authors' described phenotype? The  
1678 authors should deplete CD4 T-cells and gamma-delta T-cells in their murine models, as these  
1679 cell types may, at the very least, contribute to what occurs in patients.

1680  
1681 We thank Referee #2 for raising this important concern. Indeed, we have so far not discussed  
1682 the loss of gamma-delta T-cell subsets or a potential increase of CD4+ T-cells and included  
1683 this now thoroughly in the revised version of the manuscript (included in **Extended Data 3, 21,**  
1684 **23, 25 and 26** and **Rebuttal Figure 28a, 34a, 41, 44**). In line with the comments of Referee#2,  
1685 we have now described and discussed these populations in detail, by scRNA-seq and  
1686 multicolor flow cytometry in mouse and three distinct human cohorts recruited from 3 different  
1687 centers across Europe.

1688 As mentioned in points 8 and 9 raised by Referee #2, we have depleted CD4 T-cells  
1689 with/without PD-1 targeted immunotherapy. Of note, CD27 could not be detected in our  
1690 scRNA-seq data set obtained from the preclinical mouse model as significantly changed. In  
1691 human bulk RNA-seq CD27 expression increased, but CD4 expression decreases with the  
1692 severity of pathology. CD27+CD4+ T cells did not reach statistical significance in our cohorts  
1693 by flow cytometry (included in **Extended Data 25** and **Rebuttal Figure 41**). Of note, in our  
1694 second cohort, CD4+ T-cells are significantly enriched in NAFLD/NASH patients by flow  
1695 cytometry, however as this cohort was analyzed retrospectively, we could not analyze CD27  
1696 expression (included in **Extended Data 25**). Furthermore, the abundance of CD4+CD27+ cells  
1697 was not increased in our human scRNA cohorts (included in **Extended Data 27** and **Rebuttal**  
1698 **Figure 44**).

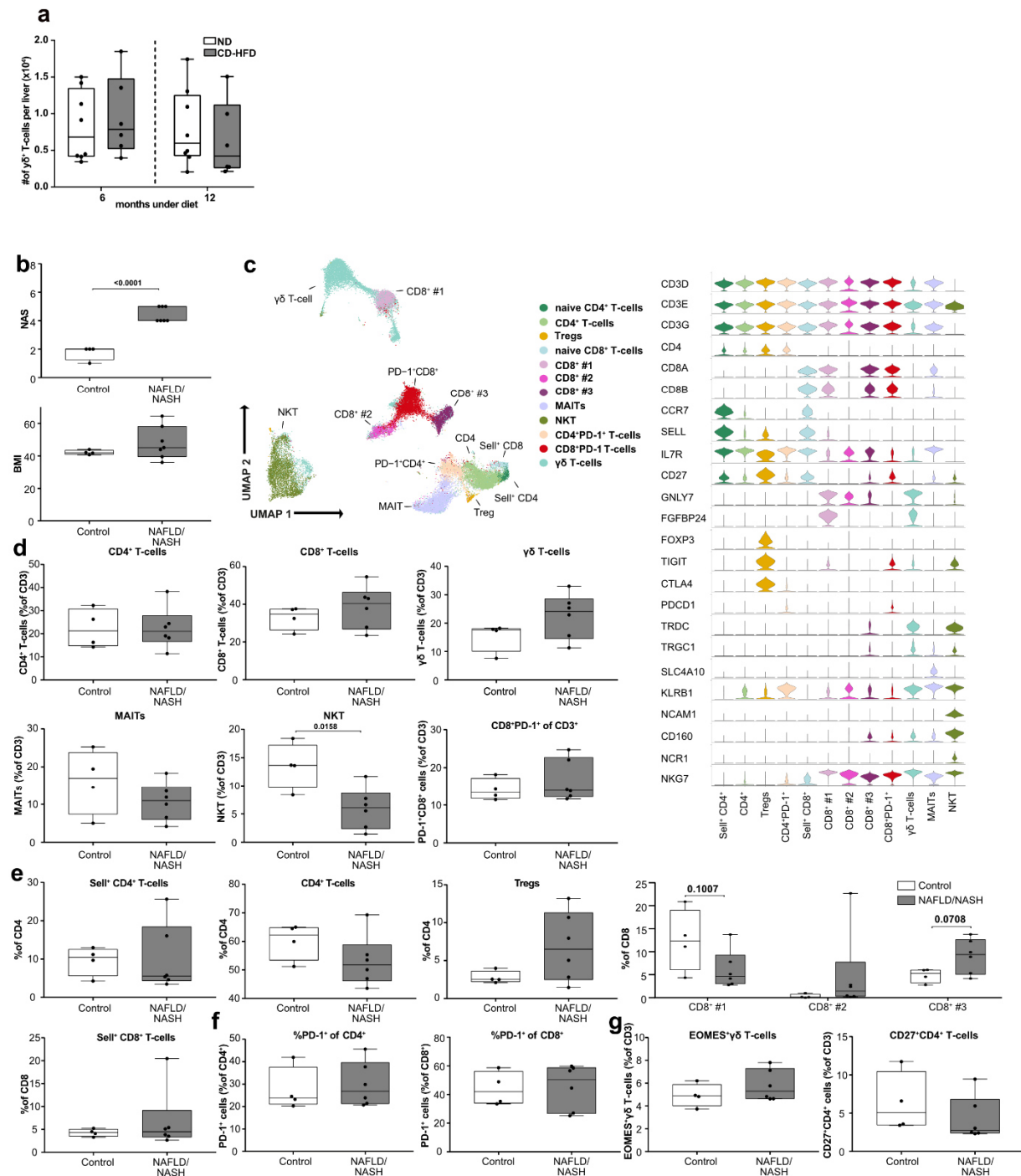


1699 As mentioned in point 8 we have performed a velocity analyses of the scRNA Seq data of  
1700 mouse CD4 T cells (see Rebuttal letter below). In mouse, no significant velocity flow was  
1701 detected in 12 months CD-HFD-fed mice, indicating, that CD4 cells are not transcriptionally  
1702 activated and driven by NASH-conditions or PD-1-targeted immunotherapy in NASH.  
1703 However, we again want to point out, that the velocity analysis of mouse CD4 T-cells need to  
1704 be taken with caution because we included 300-500 cells only per described subset. As a  
1705 consequence, we included the negative CD4 T-cell data not in the manuscript but in the  
1706 Rebuttal letter. Velocity analyses on human CD4 lead to comparable problems as seen in  
1707 mouse. As a consequence, we included the negative CD4 T-cell data not in the manuscript but  
1708 in the Rebuttal letter as **Rebuttal Figure 42**.

1709 We agree that  $\gamma\delta$  T-cells might be involved in underlying processes of NASH or NASH to HCC  
1710 transition – also in the context of PD1-related immunotherapy. In humans, our data is not  
1711 conclusive in all experiments, e.g. our data indicate for  $\gamma\delta$  T-cells, if we compare: bulk RNA-  
1712 seq indicates a reduced expression in severe NASH pathology of EOMES, TRDC, and TRGC1  
1713 (included in **Extended Data 28** and **Rebuttal Figure 41, 44, 45**), however, both flow cytometry  
1714 cohorts and the scRNA-seq cohort indicate no change of either  $\gamma\delta^+$  T-cells or  $\gamma\delta^+$  Eomes+ T-  
1715 cells comparing control vs NAFLD/NASH patients (included in **Extended Data 25, 27** and  
1716 **Rebuttal Figure 41, 44**).

1717 Corroborating the human flow cytometry data in our mouse model upon NASH establishment,  
1718 we detected no difference in hepatic abundance of  $\gamma\delta$ -T-cells between chow- or CD-HFD-fed  
1719 control mice. Furthermore, data presented in **Figures 1 and 4** and **Extended Data 3** argues  
1720 against the major contribution of gamma delta T-cells in the mouse model of NASH. Here, we  
1721 did not observe significant differences in the “other leukocytes” subset. In the revised  
1722 manuscript, we analyzed  $\gamma\delta$ -T-cells separately to strengthen the point, that these cells are not  
1723 significantly changed upon diet feeding (included in **Extended Data 3, 20-23** and **Rebuttal**  
1724 **Figure 28, 34, 44a**).

1725



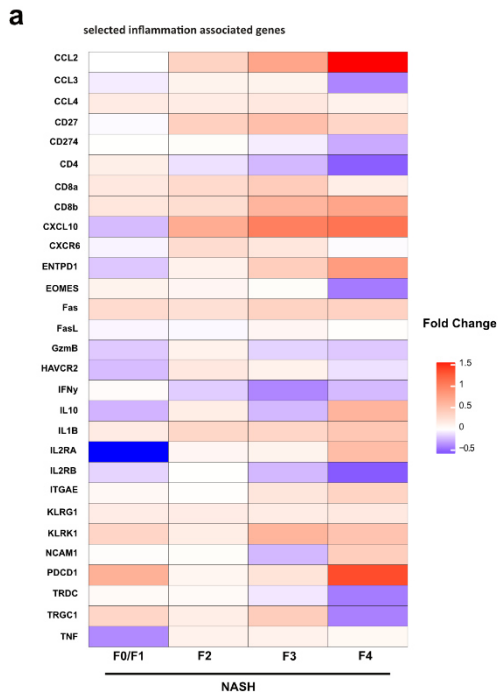
1726

1727

### Rebuttal Figure 44

1728 (a) Hepatic abundance of TCR $\gamma\delta$  T-cells of 6 or 12 months ND or CD-HFD fed mice (6 months  
 1729 ND n = 8 mice; CD-HFD n = 6 mice; 12 months ND n = 8 mice; CD-HFD n = 6 mice).

1730 (b) NAS and BMI of patients used for scRNA-seq analyses of patient-liver-derived T-cells of  
 1731 control and NAFLD/NASH patients (control n = 4 patients; NAFLD/NASH n = 7 patients). (c)  
 1732 UMAP representation, marker expression, (d) relative quantification and (e), (f), (g) polarization  
 1733 of defined T-cell subsets of defined T-cell subsets of patient-liver-derived T-cells by scRNA-  
 1734 seq of control and NAFLD/NASH patients (control n = 4 patients; NAFLD/NASH n = 7 patients).



1735

1736 **Rebuttal Figure 45**

1737 (a) RNA-sequencing data comparing NASH with varying fibrosis (F0 – F4 according to Brunt  
1738 classification) normalized to NAFLD from a total of n= 206 NAFLD/NASH patients corrected  
1739 for batch, gender and center

1740

1741 11. The patient data is not convincing, but also does not match their murine models. In Figure  
1742 5a, the authors show that CD8+GzmB+ cells are specifically lost in NASH samples which  
1743 seems to counteract the claim made by the authors that inflammatory CD8 T-cells cause liver  
1744 inflammation and associated carcinogenesis. The authors similarly show in S19a that IFNy,  
1745 Ccl3 and PD-L1 are in fact reduced in advanced NASH samples; does the loss of these  
1746 inflammatory genes not counteract the claims made in Figure 3g, S4d, S10, S11 and S13a?

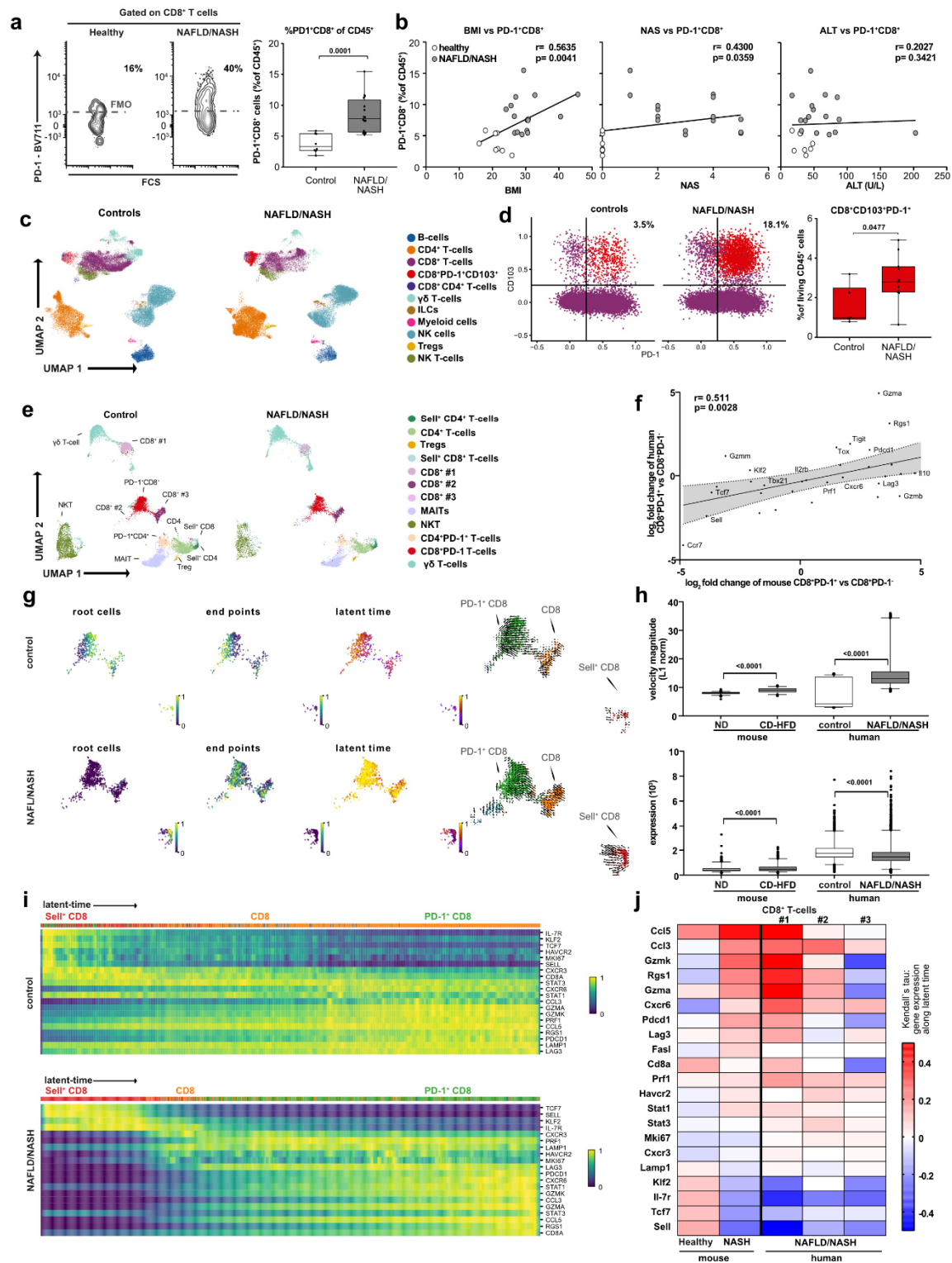
1747

1748 We thank Referee #2 for raising this important point and agree, that GzmB+CD8+ population  
1749 is decreased as well as GzmB expression in bulk RAN-seq (included in **Extended Data 28**  
1750 and **Rebuttal Figure 45**), other populations, on the other hand, are increased. GzmB is a  
1751 strong indication for inflammatory CD8+ T-cells. We would like to draw attention to the  
1752 improved cross-referencing to the co-submitted manuscript Dudek et al., in which Gzmb along  
1753 with other cytotoxic effector molecules (e.g. TNF) are key mediators of a hepatic inflammatory  
1754 environment, but not the executing molecules driving hepatocarcinogenesis. However, we  
1755 agree with Referee #2, that the data presented in **Figure 5** has limitations due to the small  
1756 sample size, although we could reproduce the cellular abundance between healthy vs

1757 NAFLD/NASH patients in a second cohort from a second center (included in **Figure 5** and  
1758 **Extended Data 25** and **Rebuttal Figure 41, 46**).

1759 We agree with Referee #2, that certain inflammatory genes (e.g. *Ifny*, *Ccl3*, *Cd274*) show  
1760 decreased expression along with NASH progression, however, how this translates into local  
1761 hepatic proteins-expression remains elusive (e.g. for human gene expression vs  
1762 immunohistochemical staining of *Pdcd1* in NASH F1-3 (included in **Figure 6** and **Rebuttal**  
1763 **Figure 47**); or F0-F4 for CD4, or CD274 (included in **Extended Data 28** and **Rebuttal Figure**  
1764 **47**)). As an example, human PD-L1 increases with NASH severity on IHC, which is  
1765 corroborated by the preclinical model (included in **Extended Data 3, 20, 22** and **Rebuttal**  
1766 **Figure 27, 33, 48**).

1767 To shed more light on the phenomena, we focused on our human scRNA-seq on the analyses  
1768 of CD8+ T-cells (included in **Figure 5**, **Extended Data 27** and **Rebuttal Figure 43f, 44, 46**)  
1769 and correlated these cells to the CD8+ T-cells analyzed from our preclinical model (included  
1770 in **Figure 5** and **Rebuttal Figure 46f**). These data match each other very well, strengthening  
1771 in our opinion hypotheses and conclusions drawn from the preclinical NASH-model. Therefore,  
1772 we do not think the results of the bulk RNA-seq counteracts the claims of previous figures from  
1773 the mouse model but allows an in-depth understanding of underlying inflammation in different  
1774 NASH stages (e.g. Referee #1: decrease activity of NASH with disease progression to HCC).



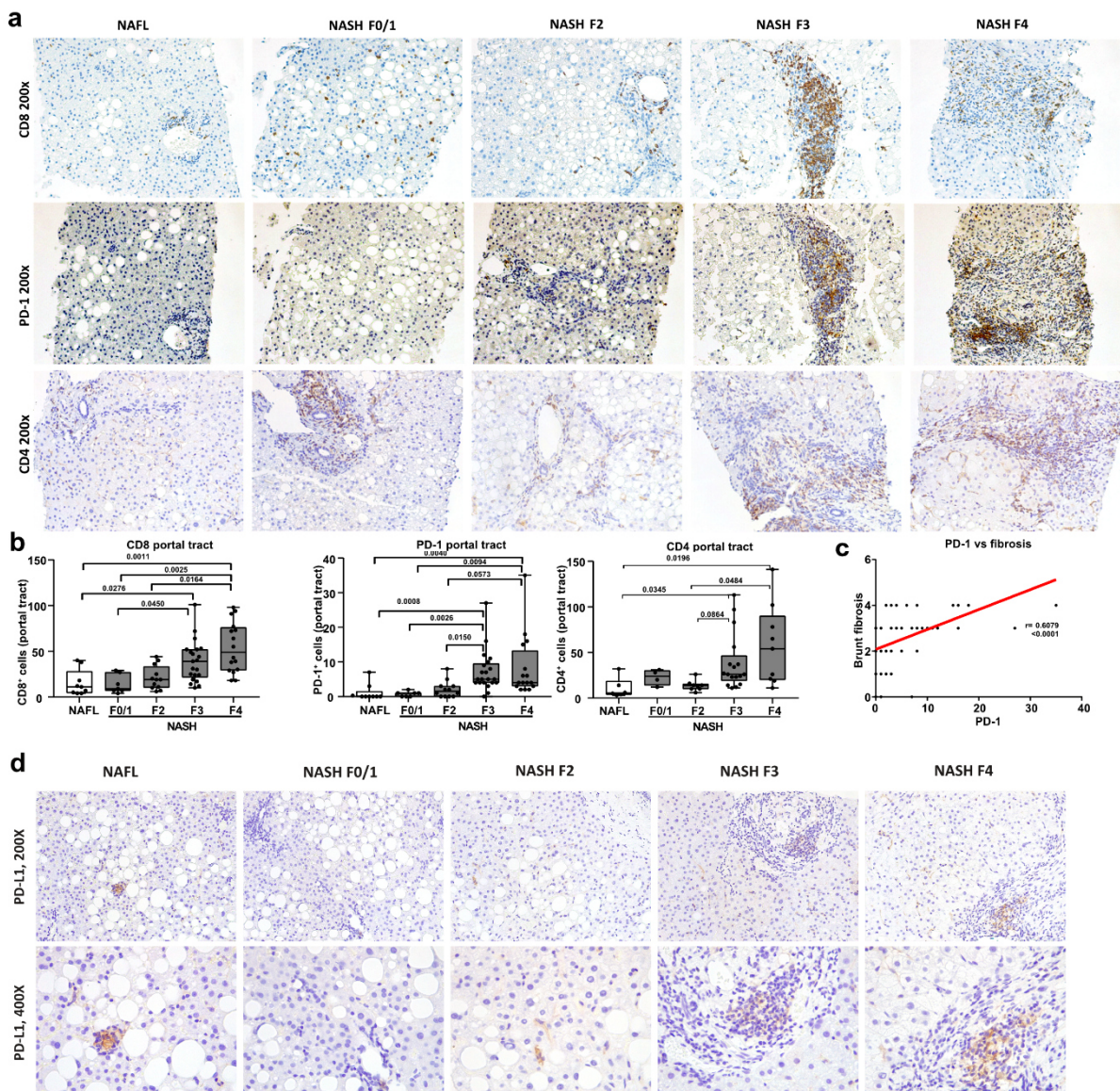
1775

1776

## Rebuttal Figure 46

1777 (a) Flow cytometry plots, quantification of patient-liver-derived PD-1+CD8+ T-cells, and (b)  
 1778 correlation of PD-1+CD8+ T-cells with BMI, NAS and ALT of healthy or NAFLD/NASH patients  
 1779 (Supplementary Table 1: healthy n= 8 patients; NAFLD/NASH n= 16 patients). (c) UMAP  
 1780 representation of randomly chosen CD45+ cells and (b) flow cytometry plots and quantification  
 1781 of CD8+PD-1+CD103+ derived from hepatic biopsies of control, or NAFLD/NASH patients

1782 (Supplementary Table 2: control n= 6 patients; NAFLD/NASH n= 11 patients) Populations:  
 1783 CD8+ (violet), CD8+PD-1+CD103+ (red). (e) UMAP representation of CD3+ cells and (f)  
 1784 analyses of differential gene expression by scRNA-seq of control, or NAFLD/NASH patients  
 1785 (control n= 4 patients; NAFLD/NASH n= 7 patients). (f) Correlation of significant differentially  
 1786 expressed genes in liver-derived CD8+PD-1+ compared to CD8+PD-1- T-cells subsets of 12  
 1787 months CD-HFD fed mice and NAFLD/NASH patients (mouse: n= 3 mice; human: n= 3  
 1788 patients). (g) Velocity analyses of scRNA-seq data showing (h) expression, transcriptional  
 1789 activity, (i) gene expression and (j) correlation of expression along the latent-time of selected  
 1790 genes along the latent-time of patient-liver-derived CD8+ T-cells of control, or NAFLD/NASH  
 1791 patients in comparison to mouse-liver-derived CD8+ T-cells (patients: NAFLD/NASH n= 3  
 1792 patients; mouse: n= 3 mice/group).

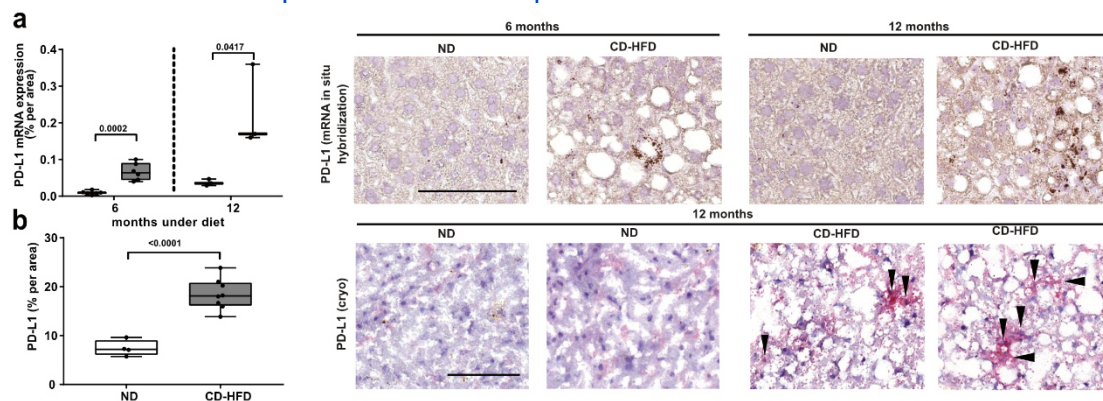


1793

1794 **Rebuttal Figure 47**

1795 (a) Immunohistochemical staining and (b) quantification of hepatic PD-1, CD8 and CD4  
 1796 expressing cells of NAFLD and NASH patients in Supplementary Table 3 with varying stages  
 1797 of fibrosis (NAFLD n= 9 patients; NASH F1/0 n= 7 patients; NASH F2 n= 12 patients; NASH  
 1798 F3 n= 21 patients; NASH F4 n= 16 patients; CD4: NAFL n= 6 patients; NASH F1/0 n= 4  
 1799 patients; NASH F2 n= 8 patients; NASH F3 n= 17 patients; NASH F4 n= 9 patients). (c)

1800 Correlation analysis of PD-1 against fibrosis scoring according to Brunt by  
1801 immunohistochemical staining by RNA-sequencing (NAFLD/NASH n= 65 patients). A total of  
1802 1656 patients were included in all three randomized trials, and 985 patients received a  
1803 checkpoint inhibitor (Supplementary Table 7). (d) Immunohistochemical staining of PD-L1 in  
1804 patient-derived liver samples. Scale bar: 50  $\mu$ m.



1805

1806 **Rebuttal Figure 48**

1807 (a) Quantification of hepatic PD-L1+ expression by RNA in situ hybridization of 6- or 12-months  
1808 ND or CD-HFD-fed mice (6 months: ND n= 13 mice; CD-HFD n= 11 mice; 12 months: ND n=  
1809 7 mice; CD-HFD n= 7 mice). Scale bar: 100  $\mu$ m. (b) Quantification of hepatic PD-L1+  
1810 expression by immunohistochemistry of 12 months ND or CD-HFD fed mice (6 months: ND n=  
1811 4 mice; CD-HFD n= 8 mice). Scale bar: 100  $\mu$ m.

1812

1813 12. Lastly, the majority of patient data are not significant and show weak effect sizes; is it fair  
1814 to draw strong conclusions on the basis of these data as the authors do?

1815

1816 We agree with Referee #2 and thus recruited additional patients to increase the number of  
1817 patients in our initial clinical cohort from 65 to 130 HCC patients under anti-PD(L)1-targeted  
1818 immunotherapy and validated our results in a second cohort of 118 HCC-patients under PD-  
1819 1-targeted immunotherapy (included in **Figure 6** and **Rebuttal Figure 49**).

1820 We agree with Referee #2, that the presented retrospective PD(L)1 targeted immunotherapy  
1821 treated NAFLD/NASH-associated HCC cohort - although unique for Europe and treatment not  
1822 officially licensed and thus reimbursement - is still small, although we would like to point out,  
1823 that prominent trends or effects can be seen in small retrospective cohorts as well. Thus, our  
1824 analyses of BCLC-C NAFLD/NASH-HCC vs other-etiological-HCC patients indicated, that  
1825 NAFLD/NASH-HCC have significantly reduced overall survival compared to other-etiological-  
1826 HCC in this small retrospective cohort. Of note, multivariate analyses identified NAFLD/NASH  
1827 as an independent factor for treatment response and thus identifying NAFLD/NASH as a  
1828 negative predictor for HCC immunotherapy (included in **Supplementary Table 8** and **Rebuttal**  
1829 **Figure 49**).

1830 We corroborated our hypothesis of non-viral (NASH-related) HCC being less responsive to  
1831 immunotherapy by a meta-analysis including 1656 patients of the three most important clinical  
1832 trials, identifying immunotherapy vs control for viral HCC as favorable treatment (HR(viral)=  
1833 0.64), in contrast, non-viral-HCC showed less benefit (HR(non-viral)= 0.92) for immunotherapy  
1834 (included in **Figure 6, Extended Data 30-32, Supplementary Table 9** and **Rebuttal Figure**  
1835 **50, 51**)).

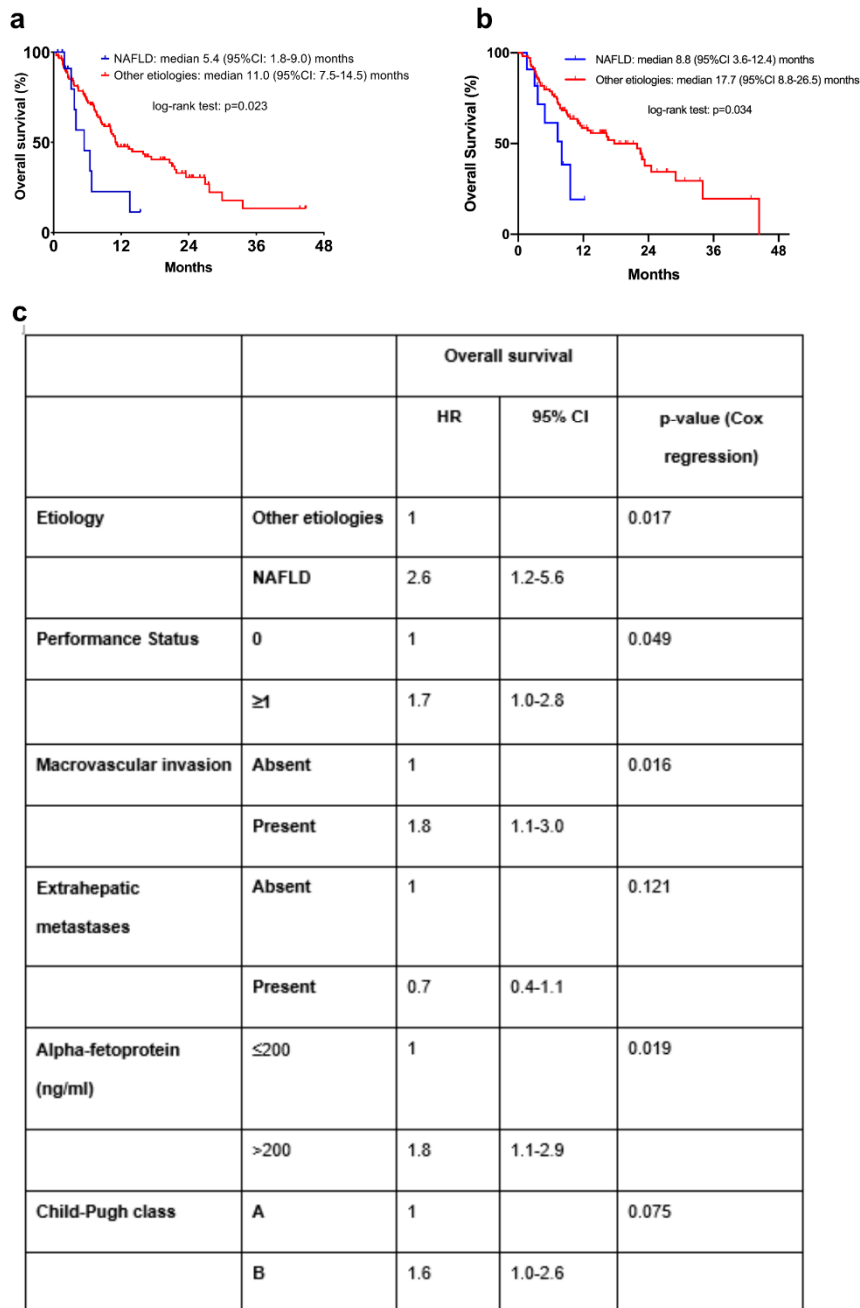
1836 Based on these data we want to point out that it is - as indicated by Referee#2 - of the highest  
1837 importance to us to specifically define/tone down appropriately the message of our manuscript:  
1838 Our manuscript does not indicate that immunotherapy is not beneficial for HCC patients at all.  
1839 Our manuscript rather demonstrates that HCC patients with viral etiologies do respond well  
1840 and achieve survival benefits - however, that patients with non-viral etiologies (e.g. NASH) do  
1841 not achieve a significant outcome benefit.

1842 We thus propose to stratify HCC patients who are very likely to profit from immunotherapy and  
1843 strengthen the argumentation to use immunotherapy in specific cohorts of HCC patients. We  
1844 agree with Referee#1 that this information needs to be articulated in the paper appropriately  
1845 not to deliver wrong messages but to be very specific.

1846 We truly believe that these are important clinical data, also providing the basis to test our  
1847 hypotheses in prospective studies on non-significantly beneficial effects in terms of OS for  
1848 immunotherapy in HCC patients with non-viral and NAFLD/NASH etiology, in particular.

1849 Moreover, we toned down the conclusions of our retrospective cohort in the manuscript and  
1850 would like to point out, that bigger cohorts and prospective clinical trials are of utmost  
1851 importance for the scientific community.





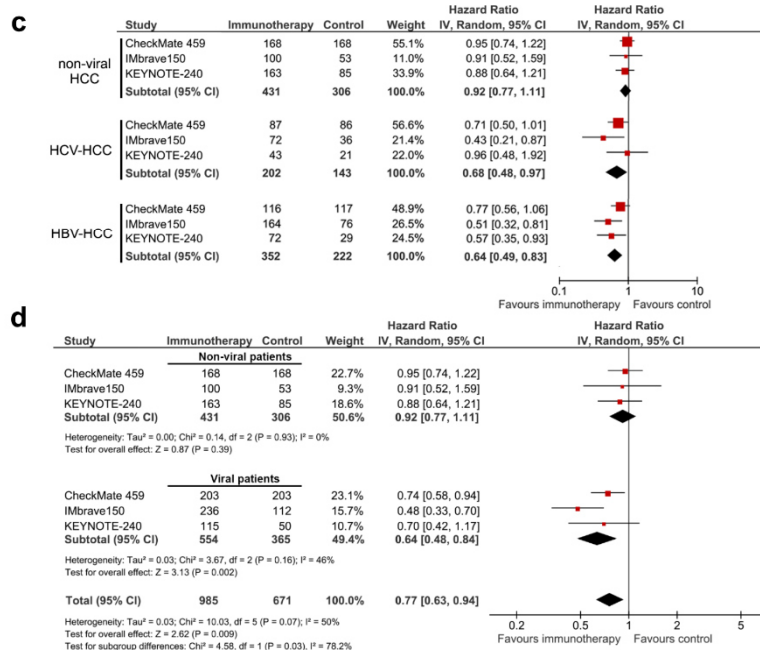
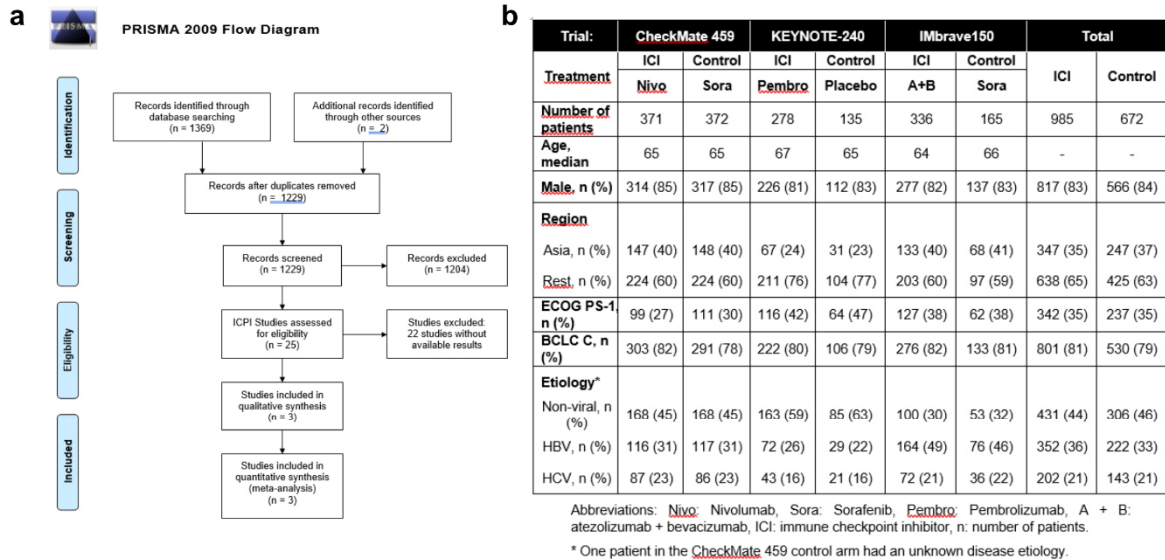
1852

1853

### Rebuttal Figure 49

1854 (a) Nonalcoholic fatty liver disease (NAFLD) is associated with a worse outcome in patients  
 1855 with hepatocellular carcinoma (HCC) treated with PD-(L)1-targeted immunotherapy. A total of  
 1856 130 patients with advanced HCC received PD-(L)1-targeted immunotherapy (Supplementary  
 1857 Table 8). Kaplan-Meier curve display overall survival of patients with NAFLD vs. those with  
 1858 any other etiology; all 130 patients were included in these survival analyses (NAFLD n=13, any  
 1859 other etiology n=117). (b) Validation cohort of patients with HCC treated with PD-(L)1-targeted  
 1860 immunotherapy. A total of 1180 patients with advanced HCC received PD-(L)1-targeted  
 1861 immunotherapy (Supplementary Table 10). Kaplan-Meier curve display overall survival of  
 1862 patients with NAFLD vs. those with any other etiology; all 118 patients were included in these

1863 survival analyses (NAFLD n=11, any other etiology n=107). (c) Multivariate analysis of  
 1864 prognostic factors in HCC patients treated with anti-PD-(L)1-based immunotherapy

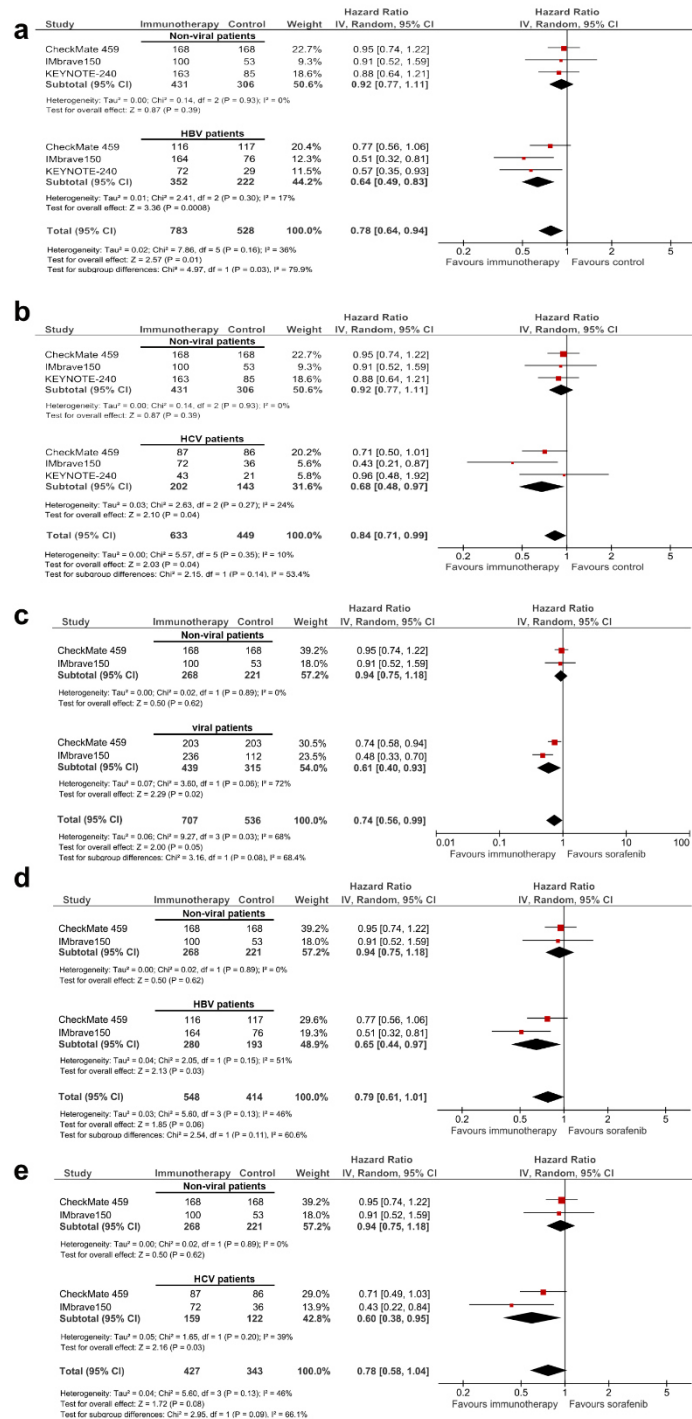


1865

1866 **Rebuttal Figure 50**

1867 (a) Selection of articles assessing the clinical outcome of immune checkpoint inhibitors in  
 1868 advanced HCC for inclusion in the systematic review and meta-analysis. ICPI: Immune  
 1869 checkpoint inhibitor. (b) Pooled baseline characteristics of the patients included in the meta-  
 1870 analysis (total n= 1656). (c) A total of 1656 patients were included in all three randomized trials,  
 1871 and 985 patients received a checkpoint inhibitor (Supplementary Table 7). (c) Separate meta-  
 1872 analyses were performed for each of the three etiologies: non-viral (including mostly NASH  
 1873 and alcohol intake), HCV and HBV. (d) HCV and HBV were pooled into a separate category,  
 1874 termed “viral”, and a subsequent meta-analysis comparing viral (n=919) and non-viral,  
 1875 including mostly NASH and alcohol intake (n=737) was performed. Hazard ratios for each trial

1876 are represented by squares, the size of the square represents the weight of the trial in the  
 1877 meta-analysis. The horizontal line crossing the square represents the 95% confidence interval  
 1878 (CI). The diamonds represent the estimated overall effect based on the meta-analysis random  
 1879 effect of all trials.



1880  
 1881 **Rebuttal Figure 51**

1882 A total of 1656 patients were included in all three randomized trials, and 985 patients received  
 1883 a checkpoint inhibitor. Subgroup analysis was performed to study the specific effects of  
 1884 immunotherapy comparing non-viral etiologies (n=737) with (a) HBV (n=574) or (b) HCV

1885 (n=345). Hazard ratios for each trial are represented by squares, the size of the square  
1886 represents the weight of the trial in the meta-analysis. The horizontal line crossing the square  
1887 represents the 95% confidence interval (CI). The diamonds represent the estimated overall  
1888 effect based on the meta-analysis random effect of all trials.

1889 A total of 1243 patients were included in two first-line trials comparing PD-1 or PD-L1 targeted  
1890 immunotherapy to sorafenib. 707 patients received an immune checkpoint inhibitor (either PD-  
1891 1 or anti-PD-1). (c) HCV and HBV were pooled into a separate category, termed “viral”, and a  
1892 subsequent meta-analysis comparing viral (n=754) and non-viral (n=489), mostly NASH and  
1893 alcohol intake, was performed. A subgroup analysis studying the specific effects of non-viral  
1894 etiologies (n=489) on the magnitude of effect of immunotherapy are presented, when  
1895 compared to (d) HBV (n=473) or (e) HCV (n=281). Hazard ratios for each trial are represented  
1896 by squares, the size of the square represents the weight of the trial in the meta-analysis. The  
1897 horizontal line crossing the square represents the 95% confidence interval (CI). The diamonds  
1898 represent the estimated overall effect based on the meta-analysis random effect of all trials.  
1899

1900 Minor points:

1901

1902 - Figure 1j lacks a color scale bar and proper description. How does one interpret the difference  
1903 between ND and CD-HFD in this plot?

1904

1905 We thank Referee #2 for highlighting the lack of a color bar in this panel, we have added a  
1906 color scale bar with a proper description. Figure 1j displays the median expression of selected  
1907 genes in the different T-cell populations observed in our scRNA-seq data set (included in  
1908 **Figure 1, Extended Data 5 and Rebuttal Figure 43**) and serves as a supplement to the 2-  
1909 dimensional tSNE plot. In this panel, we do not compare ND to CD-HFD rather simply allow  
1910 the readers to view the gene signatures characterizing the different populations. A comparison  
1911 of ND and CD-HFD is visualized using volcano plots in Figure 1. As this heatmap is rather a  
1912 technical information, but does not condense scientific explanation in great detail, we decided  
1913 to move this heatmap to **Extended Data 5**.

1914

1915 - Where is the ND + PD-1<sup>-/-</sup> in Figure 3b? Do these mice also get accelerated carcinogenesis?

1916

1917 We thank Referee #2 for highlighting this inconsistency. In line with the point raised by  
1918 Referee#2 we have improved this in a revised manuscript including PD-1<sup>-/-</sup> mice on ND.  
1919 Literature does not report accelerated hepatocarcinogenesis  
1920 (<http://www.informatics.jax.org/allele/allgenoviews/MGI:4397682>) and we did not observe any  
1921 hepatocarcinogenesis in PD1<sup>-/-</sup> under ND.

1922

1923 - There is no color scale bar in Figure 3e.

1924

1925 We thank Referee #2 for highlighting this inconsistency and improved our manuscript by  
1926 adding a scale bar.

1927

1928 - In Figure 5k, shouldn't progression-free survival and time to progression plots yield the exact  
1929 same data, but inversed? Why don't these curves match?

1930

1931 We thank Referee #2 for this question. TTP and PFS are different endpoints. TTP is defined  
1932 as the time from the date of treatment initiation until the date of first radiological tumor  
1933 progression. PFS is a composite endpoint. It is defined as the time from the date of treatment  
1934 initiation until radiological progression OR death, whatever comes first (Llovet et al., 2008). We  
1935 decided to leave out the non-significant data of TTP and PFS in our manuscript. Moreover,  
1936 upon recruiting the validation cohort of 118 HCC-patients under immunotherapy we decided  
1937 to not show TTP and PFS, but instead the multivariate analysis (included in **Supplemental**  
1938 **Table 9** and **Rebuttal Figure 49**).

1939

1940 - In Figure S1i, what is the parent population?

1941

1942 We thank Referee #2 for highlighting this inconsistency and improved our manuscript by  
1943 adding the description of the parent population. In the case of **Extended Data 1** the parental  
1944 populations are CD8+ (left) and respective CD4 or CD8 (right) T-cells.

1945

1946 - In Figure S4a, how does one distinguish ND from CD-HFD mice? The y-axis lacks a label.

1947

1948 We thank Referee #2 for highlighting this inconsistency and improved our manuscript by  
1949 adding the description of the y-axis.

1950

1951 - Figure 5c is plotted in a confusing manner (as the z-score scale is red independent of whether  
1952 it goes up or down), but it seems that the TNF signaling gene sets are actually decreasing in  
1953 expression.

1954

1955 We thank Referee #2 for highlighting this inconsistency. We decided after integration of the  
1956 new data, to leave that graph out as it communicates similar information already included in  
1957 **Extended Data 28**. Of note, if we change the labeling of z-score (similar to **Extended Data**  
1958 **28**), it clarifies, that TNF is indeed an increased pathway (similar to **Extended Data 28**).

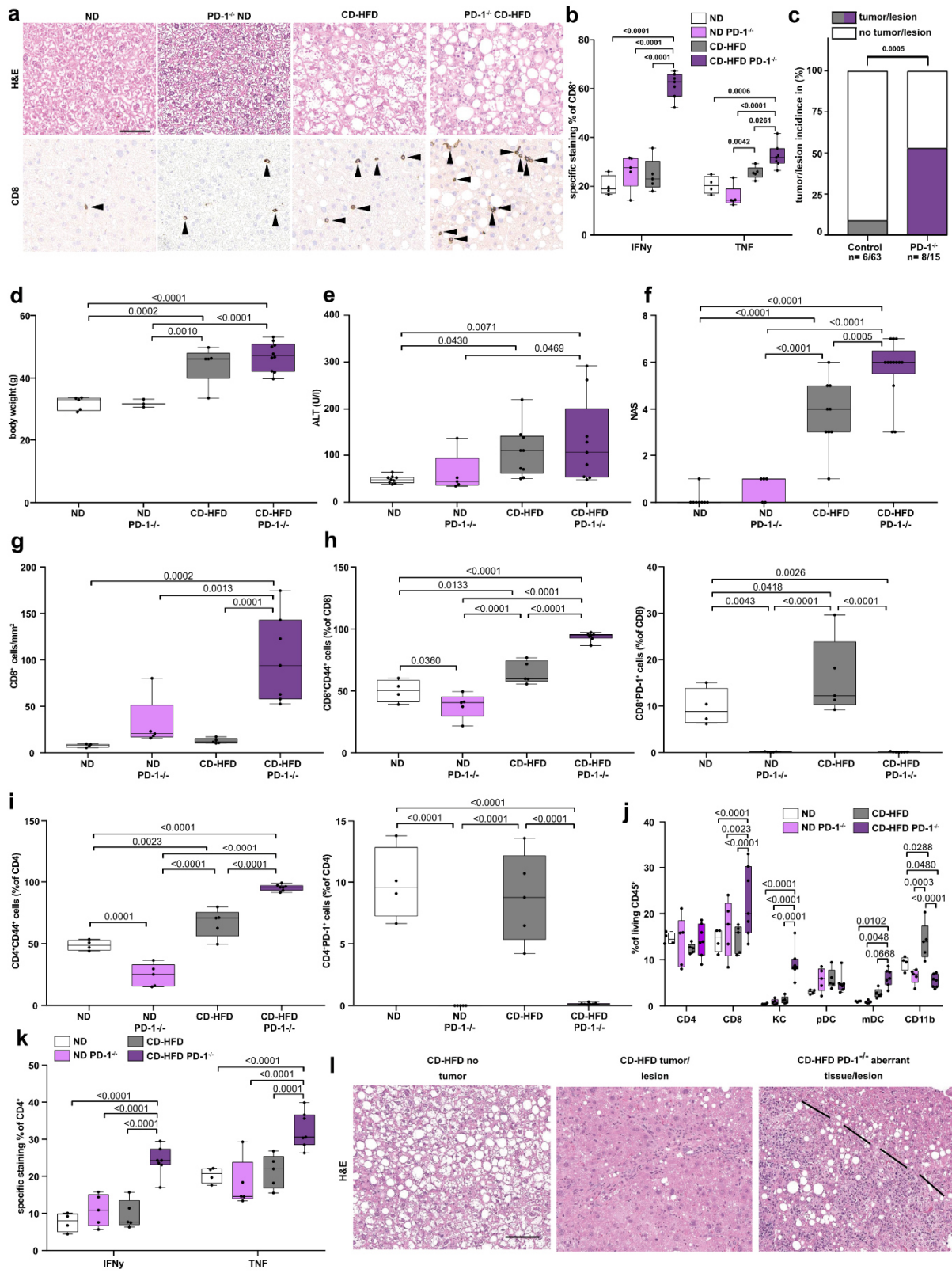
1959



1960 - Why do the PD-1<sup>-/-</sup> mice still express PD-1 (Fig. S12e)?

1961

1962 We thank Referee #2 for highlighting this inconsistency and improved our manuscript by re-  
1963 analyzing our flow cytometry data set (as gates have been set too loose – leading to a subset  
1964 of around 1% PD1 expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells). Analyses revealed that PD1<sup>-/-</sup> ND-fed  
1965 mice have no intrinsic higher immune cell abundance, or activation and hepatocarcinogenesis  
1966 compared to ND-fed wt control mice at 6 months under diet (included in **Figure 3** and  
1967 **Extended Data 14** and **Rebuttal Figure 52**). Moreover, as indicated no PD1-expression can  
1968 be observed.



1969

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1971

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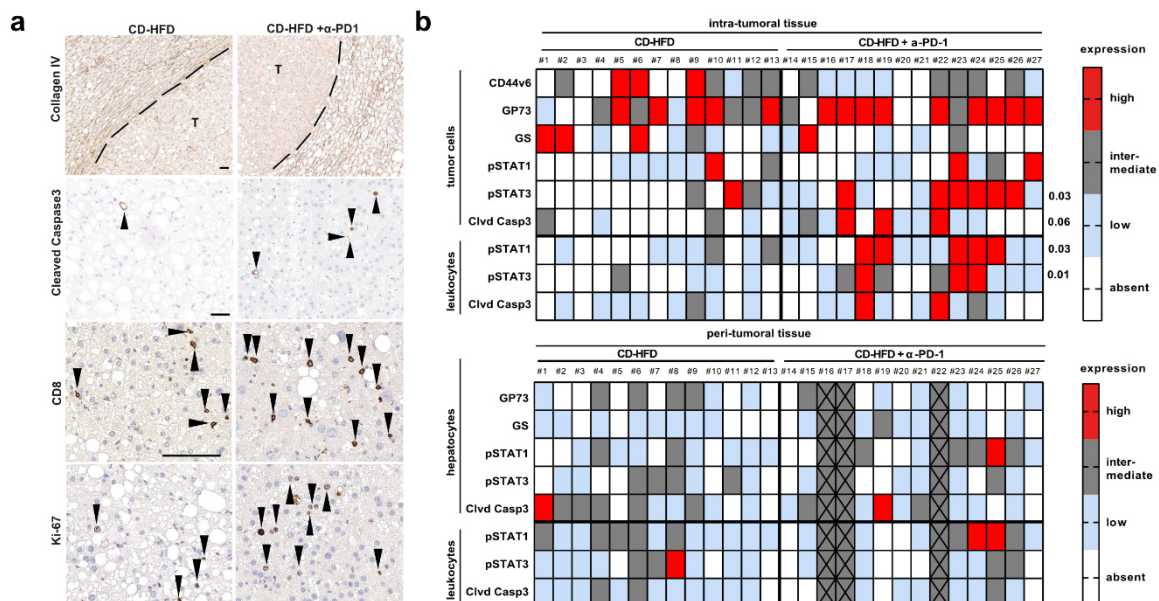
**Rebuttal Figure 52**

(a) Histological staining of hepatic tissue by H&E and CD8 of 6 months ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD fed mice (H&E: ND n= 8 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 9 mice; PD-1<sup>-/-</sup> CD-HFD n= 13 mice; CD8: ND n= 4 mice; CD-HFD n= 5 mice; PD-1<sup>-/-</sup> CD-HFD n= 7 mice).

1974 Arrowheads indicate CD8+ cells. Scale bar: 50  $\mu$ m. (b) Cytokine expression of hepatic CD8+  
 1975 T-cells of 6 months ND, PD-1-/- ND, CD-HFD or PD-1-/- CD-HFD fed mice (ND n= 4 mice; PD-  
 1976 1-/- ND n= 5 mice; CD-HFD n= 5 mice; PD-1-/- CD-HFD n= 6 mice). (c) Tumor/lesion incidence  
 1977 of 6 months CD-HFD or PD-1-/- CD-HFD fed mice (tumor incidence: CD-HFD n= 6  
 1978 tumors/lesions in 63 mice; PD-1-/- CD-HFD n= 6 tumors/lesions in 13 mice). (d) Body weight  
 1979 of 6 months ND, PD-1-/- ND, CD-HFD or PD-1-/- CD-HFD fed mice (ND n= 5 mice; PD-1-/-  
 1980 ND n= 3 mice; CD-HFD n= 5 mice; PD-1-/- CD-HFD n= 10 mice). (e) ALT levels of ND, PD-1-/  
 1981 -/- ND, CD-HFD or PD-1-/- CD-HFD (ND n= 9 mice; PD-1-/- ND n= 5 mice; CD-HFD n= 9 mice;  
 1982 PD-1-/- CD-HFD n= 10 mice). (c) NAS evaluation by H&E of ND, PD-1-/- ND, CD-HFD or PD-  
 1983 1-/- CD-HFD fed mice (ND n= 8 mice; PD-1-/- ND n= 5 mice; CD-HFD n= 9 mice; PD-1-/- CD-  
 1984 HFD n= 13 mice). (f) CD8 staining of hepatic tissue by immunohistochemistry of 6 months ND,  
 1985 PD-1-/- ND, CD-HFD or PD-1-/- CD-HFD fed mice (ND n= 4 mice; PD-1-/- ND n= 5 mice; CD-  
 1986 HFD n= 5 mice; PD-1-/- CD-HFD n= 7 mice). (g) – (j) Characterization of hepatic T-cells by  
 1987 flow cytometry of 6 months ND, PD-1-/- ND, CD-HFD or PD-1-/- CD-HFD fed mice (ND n= 4  
 1988 mice; PD-1-/- ND n= 5 mice; CD-HFD n= 5 mice; PD-1-/- CD-HFD n= 6 mice). (k) Relative  
 1989 quantification of hepatic leukocytes of 6 months CD-HFD or PD-1-/- CD-HFD fed mice (ND n=  
 1990 4 mice; PD-1-/- ND n= 5 mice; CD-HFD n= 5 mice; PD-1-/- CD-HFD n= 6 mice). (l) Histological  
 1991 staining of hepatic tissue by H&E of CD-HFD or PD-1-/- CD-HFD fed mice (ND n= 8 mice; CD-  
 1992 HFD n= 9 mice; PD-1-/- CD-HFD n= 13 mice). Dotted line indicates tumor/lesion border. Scale  
 1993 bar: 100  $\mu$ m.

1995 - In Figure S13k, the authors should present cleaved Caspase 3 and cleaved Caspase 8 if they  
 1996 want to conclude something about T-cell death, as total, uncleaved levels of these proteins do  
 1997 not indicate cell death.

1998  
 1999 We thank Referee #2 for highlighting this point. We have accordingly removed these plots and  
 2000 demonstrate cleaved caspase 3 by immunohistochemistry, which has the advantage that we  
 2001 not only see the Cleaved Caspase 3 directly but also which cells are undergoing apoptosis.  
 2002 These data are now included in **Extended Data 16** and **Rebuttal Figure 53**.



2003



2004 **Rebuttal Figure 53**

2005 (a) Histological staining of hepatic tumor tissue by Collagen IV, cleaved Caspase 3, CD8, Ki-  
2006 67 of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-1 (Collagen IV,  
2007 cleaved Caspase 3: CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD8, Ki-67: CD-HFD  
2008 n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 7 mice). Arrowheads indicate positive cells. Dotted line  
2009 indicates tumor/lesion rim. Tumor area is indicated by T. Scale bar: 100  $\mu$ m. (b) Scoring of  
2010 expression by immunohistochemistry staining of intra- and peri-tumoral hepatic tissue of 12  
2011 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-1 (CD-HFD n= 13 mice;  
2012 CD-HFD +  $\alpha$ -PD-1 n= 14 mice). Crossed out boxes indicate not sufficient tissue for analysis.  
2013

2014 - In Figure S16f, the FACS plot does not match the quantification on the left.

2015

2016 We thank Referee #2 for bringing this up and apologize for this inconsistency. We would like  
2017 to draw the attention, that in the flow cytometry plot the data is displayed as “%of CD8”, in  
2018 contrast in the box plot the data is displayed as “%of CD45” to give the reader a more  
2019 quantitative analysis.

2020

2021 - Regarding Figure S17b, the authors claim an increase in calcium levels in line 383 of their  
2022 manuscript, but this difference is not significant.

2023

2024 We agree with Referee #2. Thus, we have performed additional experiments – supporting our  
2025 initial finding that upon PD1-targeted immunotherapy calcium levels were increased on CD8+  
2026 but not CD4+ T-cells. This inconsistency was improved our manuscript accordingly.

2027

2028 - In Figure S18b, how does one interpret the difference between healthy, borderline NASH or  
2029 NASH patients? There is no explanation of the color scale bar. Also, what are “randomly  
2030 chosen CD45+ cells” as mentioned in the corresponding Figure Legend?

2031

2032 We thank Referee #2 for highlighting this inconsistency and improved our manuscript  
2033 accordingly by describing differences between patients and highlighting our analysis pipeline  
2034 for flow cytometric data according to (Brummelman et al., 2019). Moreover, we have added 2  
2035 more cohorts in the main Figure (**Figure 5**) and Extended Data and pooled borderline NASH  
2036 and NASH patient into one group of NAFLD/NASH patients after consultation with our  
2037 pathologists, who indicated that the difference between borderline NASH and NASH can be  
2038 regional – and thus is always is regarded as NASH (**Extended Figure 25** and **Rebuttal Figure**  
2039 **41, 44, 46**).

2040

2041 - Figure S19b is not legible.

2042

2043 We thank Referee #2 for this comment. In line, we have now changed the graph size and font  
2044 size.

2045

2046 - In lines 237-246 the authors describe that NK1.1-based depletion of immune populations did  
2047 not result in changed liver pathology, body weight, fibrosis ALT, hepatic cytokines and hepatic  
2048 chemokines. However, the animals who underwent this depletion also completely lacked liver  
2049 cancer development. How does this happen if the authors did not detect any changes? The  
2050 authors should perform NK1.1 depletion by itself to see if NK1.1+ cells, potentially depending  
2051 on CD8 cells, are in fact responsible for the authors' phenotype.

2052

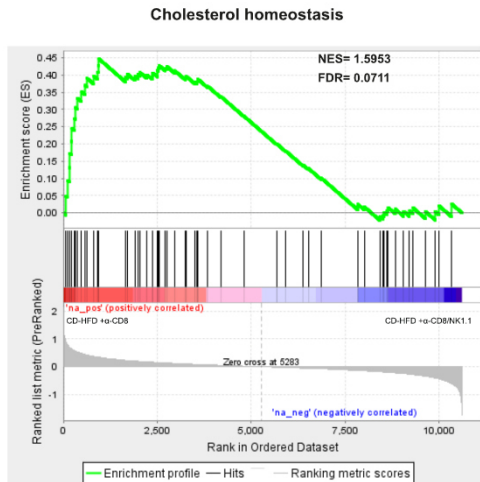
2053 We thank Referee #2 for highlighting this unprecise description of our data and improved our  
2054 manuscript by highlighting differences between CD8 depletion and CD8/NK1.1 co-depletion in  
2055 greater detail.

2056 We included additional GSEA analysis of RNA-seq data, which display changes in CD8/NK1.1  
2057 co-depleted in comparison to CD8 single depleted animals (CD8-single depleted animals  
2058 showed enrichment for "cholesterol homeostasis" (included in **Extended Data 9** and **Rebuttal  
2059 Figure 54**). Furthermore, we would like to draw attention to a previous study (Wolf et al., 2014),  
2060 in which NKT-cells were responsible for metabolic changes and CD8 T-cells driving hepatic  
2061 damage. We think, that the lack of liver cancer incidence is a result of CD8 depletion and a  
2062 reduction of a pro-tumorigenic environment - e.g. including pro-tumorigenic TNF signaling,  
2063 which is similarly enriched (TNF signaling via NFkB) in CD-HFD-fed control animals (NES(CD8  
2064 depletion vs control)= -1.6718) and NES(CD8/NK1.1 co-depletion vs control)= -1.6538)  
2065 (**Extended Data 8 and 9** and **Rebuttal Figure 31**, ). These data were also corroborated by  
2066 the analyses of the ICF signature which is strongly abrogated upon CD8 T cells depletion.

2067 Thus, we dissected the role of NK1.1 cells in greater detail by including the GSEA analysis of  
2068 RNA-seq data comparing CD8-depleted and CD8/NK1.1 co-depleted animals. Furthermore,  
2069 we improved cross-referencing to the co-submitted study Dudek et al. to highlight, that CD8 T-  
2070 cells are driving hepatocarcinogenesis.

2071 In line, together with Dudek et al. we generated new data using mouse strains with impaired  
2072 NKT cells - namely  $J\alpha 18^{-/-}$  and  $CD1d^{-/-}$  - under NASH-inducing diet. Both genetic knockout  
2073 mouse models develop NASH (including systemic obesity, fibrosis, ALT) and NASH-induced  
2074 hepatocarcinogenesis similar to WT control animals at 12-months diet-feeding. These data  
2075 argue against an essential role of NKT-cells to drive hepatocarcinogenesis at this time-point.

a



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### Rebuttal Figure 54

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(a) Gene set enrichment analysis of RNA sequencing data of hepatic tissue comparing or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed mice with CD-HFD + co-depletion of  $\alpha$ -CD8/NK1.1 of 12 months ND, CD-HFD, CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 fed or CD-HFD + co-depletion of  $\alpha$ -CD8/NK1.1 (n= 5 mice/group).

2083

- Sentence 289-292 is unclear.

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2089

We thank Referee #2 for highlighting the imprecise description and have now improved this in the main text of the revised manuscript. The sentence now reads as follows: "Next, we investigated the mechanisms underlying the increased occurrence of liver cancer incidence/liver tumor formation associated with anti-PD-1 treatment in the context of NASH."

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- When discussing GSEA, the authors frequently use the wording 'reduced enrichment (e.g. line 241)' when talking about enrichment in the opposite phenotype. This is incorrect, as the absolute amount of enrichment is often similar just, as mentioned, in the opposite direction.

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2099

We thank Referee #2 for highlighting this imprecise description. We altered this in the revised manuscript. The changes read now as follows e.g.: "Gene set enrichment analysis (GSEA) of RNA sequencing data from whole liver tissue of CD8<sup>+</sup> depleted mice revealed enrichment for DNA repair, oxidative phosphorylation, complement, and TNF signaling compared to CD-HFD-fed control)".

**2100 Referee #3 (Remarks to the Author):**

2101 This full article manuscript is novel, and the experimentation to support the conclusions is  
2102 exhaustive and solid for the most part. In essence, the findings indicate that, in NASH livers,  
2103 there is an accumulation/expansion of a pathogenic CD8 T-cell population that expresses PD-  
2104 1 and exacerbates NASH pathology and fosters hepatocellular carcinogenesis and  
2105 progression. The inflammatory and tissue-damaging functions of this pathogenic CD8 T-cells  
2106 are repressed by PD-1 blockade that is common clinical practice for second-line treatment of  
2107 advanced HCC and is under clinical trials for earlier stages of the disease. In fact, PD-L1  
2108 blockade plus anti-VEGF will soon become the standard of treatment for advanced HCC in  
2109 first line. According to the findings in this paper upon PD-1 blockade, authors document an  
2110 exacerbation of carcinogenesis and liver damage that questions the indication of PD-1  
2111 blockade in NASH-associated liver cancer. A balanced presentation of preclinical and  
2112 supportive clinical results in patient specimens very much enhances the significance of this  
2113 study.

2114

2115 [We thank Referee #3 for the positive feedback and the statement that our study is “novel, and](#)  
2116 [the experimentation to support the conclusions is exhaustive and solid for the most part” . We](#)  
2117 [would like to address his/her concerns in the following section point-by-point by presenting](#)  
2118 [new experimental data sets experiments, rephrasing, and re-analysis of the underlying data-](#)  
2119 [sets.](#)

2120

2121 Questions and comments:

2122

2123 1. TNF seems to be an actionable therapeutic target for the observed harmful effects of this  
2124 CD8 T-cell population. It would be interesting to know if TNF could be blocked preserving anti-  
2125 cancer immunity (especially under checkpoint inhibition therapy) but preventing tissue damage  
2126 and carcinogenesis promotion.

2127

2128 [We thank Referee #3 for raising this important concern and thus have performed anti-TNF](#)  
2129 [with/without anti-PD-1-related immunotherapy in the context of NASH/HCC. Anti-TNF](#)  
2130 [treatment alone - without PD1-targeted immunotherapy - leads to liver cancer formation](#)  
2131 [comparable to control-treated CD-HFD-fed mice.](#)

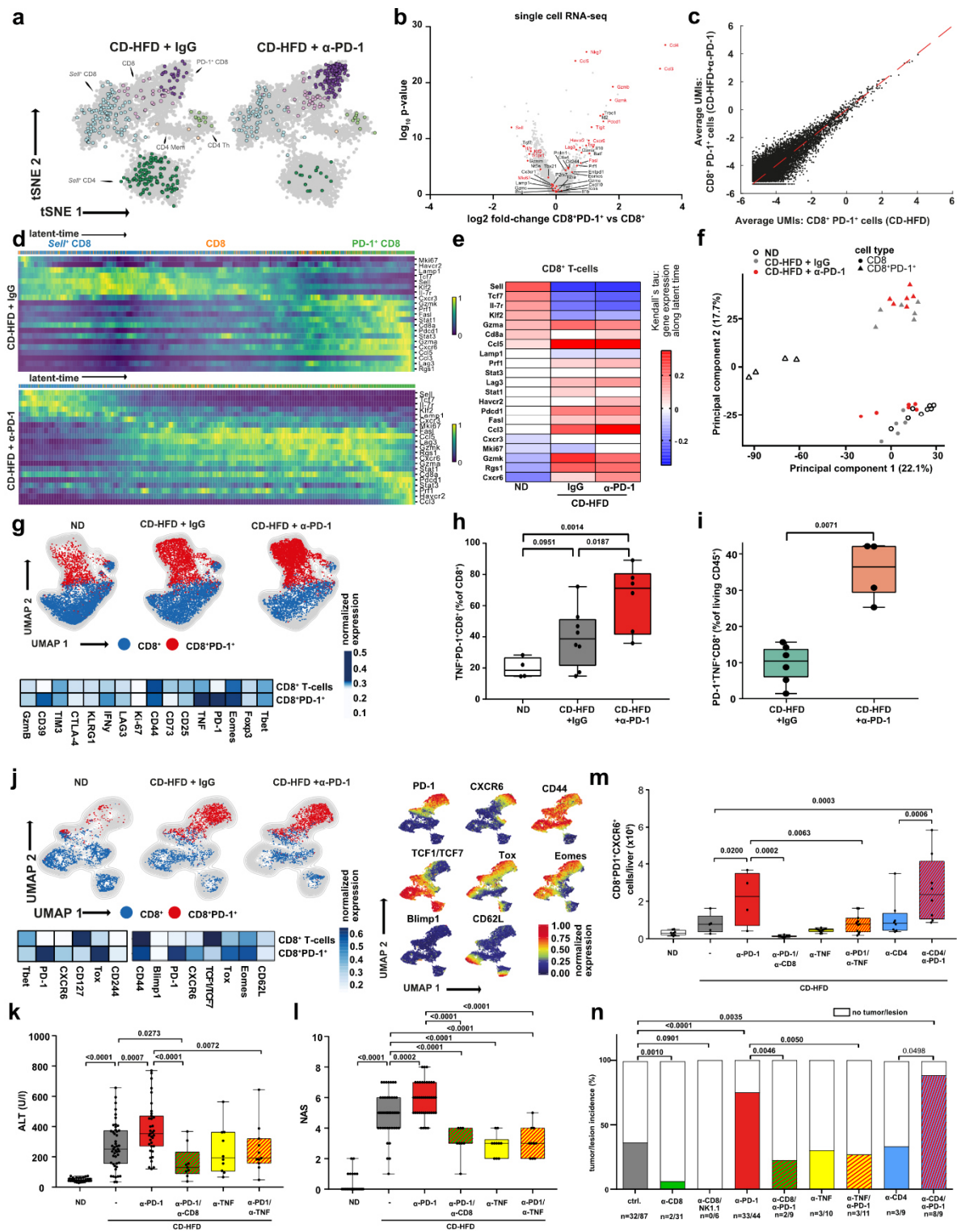
2132 [However, anti-TNF treatment in the context of PD1-targeted immunotherapy leads to a](#)  
2133 [significant reduction of tumor incidence \(tumor incidence\(anti-PD-1\)= 75% vs tumor](#)  
2134 [incidence\(anti-TNF/anti-PD-1\)= 25%, p= 0.0024\), liver damage \(ALT\(anti-PD-1\)= 381.6 U/L vs](#)

2135 ALT(anti-TNF/anti-PD-1)= 250 U/L,  $p= 0.0072$ ) and NAFLD-activity score (NAS(anti-PD-1)=  
2136 5.875 vs NAS (anti-TNF/anti-PD-1)= 3.1,  $p= <0.0001$ ), when compared to anti-PD1 treated  
2137 CD-HFD-fed mice alone. This indicates that TNF exerts key functions of the observed adverse  
2138 effects of PD1-targeted immunotherapy, namely contributing to increased  
2139 hepatocarcinogenesis (included in **Figure 4, Extended Data 20 and 21** and **Rebuttal Figure**  
2140 **55-57**).

2141 Moreover, the combination of anti-PD1 therapy with CD8-T cell depleting antibodies fully  
2142 eliminated the adverse, NAS increasing and pro-carcinogenic effects of CD8+ T-cells. These  
2143 data emphasize that CD8+ T-cells are a major cell population mediating increased  
2144 hepatocarcinogenesis through a TNF-dependent mechanism upon PD1-targeted  
2145 immunotherapy (included in **Figure 4, Extended Data 20 and 21** and **Rebuttal Figure 55-57**).  
2146 On one hand, the mechanisms could be executed by CD8 T-cell derived TNF itself or by  
2147 mechanisms that depend on TNF-signaling on other cells (e.g. myeloid cells). For example,  
2148 we see a drastic reduction of myeloid attracting chemokines (MCP-1, CCL3, CCL4, MIP-2) but  
2149 also cytokines of liver inflammation (e.g. IL-17A, IL-10, IL-13, IL-33), all cytokines/molecules  
2150 which might fuel liver inflammation and thus hepatocarcinogenesis in PD-1-targeted  
2151 immunotherapy in NASH mice.

2152 Importantly, comparing mouse-human of CD8+ T-cells isolated from liver tissue of NASH mice  
2153 or patients through classical flow cytometry, CYTOF, and on scRNA-seq level we identified  
2154 similar populations and transcriptional activation of CD8+ PD1+ in a total of three independent  
2155 center patient cohorts (included in **Figure 5, Extended Data 25-27** and **Rebuttal Figure 58-**  
2156 **61**). These data indicate that results obtained and hypotheses built from the preclinical NASH  
2157 model are relevant for human disease and are in line with published results, where TNF  
2158 blockade uncouples mediated toxicity in dual CTLA-4 and PD-1 immunotherapy (Perez-Ruiz  
2159 et al., 2019).

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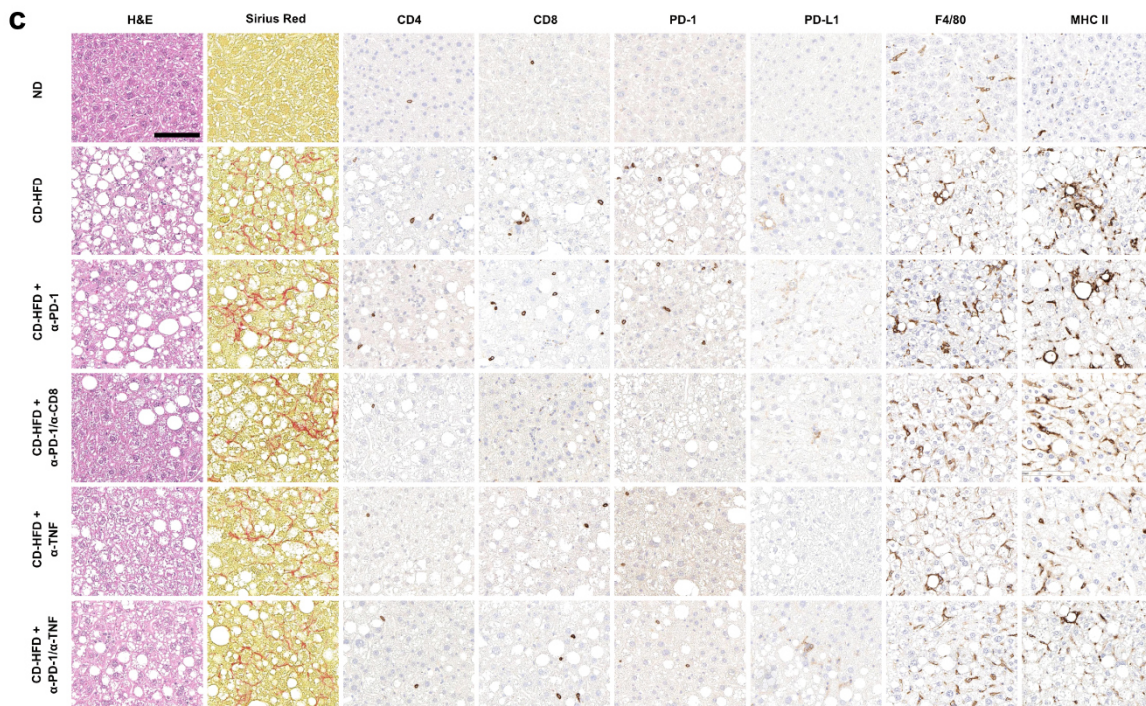
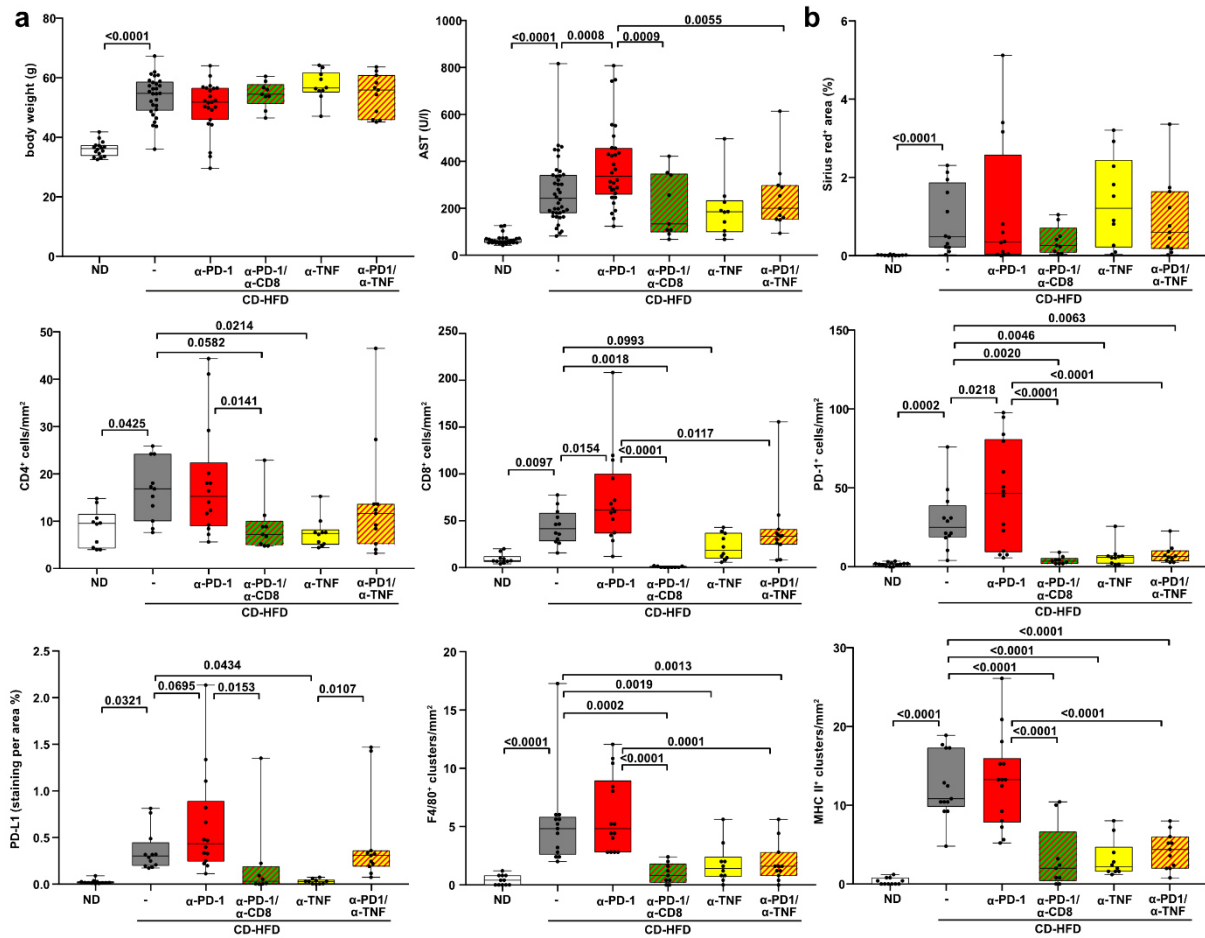
**Rebuttal Figure 55**2163 (a) ScRNA-seq analysis of hepatic TCRβ<sup>+</sup> cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 or α-CD8 antibodies (n = 3 mice/group).2164 (b) Selected marker expression in hepatic CD8<sup>+</sup> T-cells by scRNA-seq comparing CD8<sup>+</sup> with CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by α-PD-12165 antibodies (n = 3 mice/group). (c) Average UMI comparison of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (n =

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2168

2169 3 mice/group). (d) RNA velocity analyses of scRNA-seq data showing expression and (e)  
2170 correlation of expression along the latent-time of selected genes along the latent-time (n= 3  
2171 mice/group). Root cells: yellow cells indicate root cells, blue cells indicate cells farthest away  
2172 from root by RNA velocity. End points: yellow cells indicate end point cells, blue cells indicate  
2173 cells farthest away from defined end point cells by RNA velocity. Latent time: pseudo-time by  
2174 RNA velocity, dark color indicate start of RNA velocity, yellow color indicate end point of latent  
2175 time. RNA velocity flow: Blue cluster defined as start point, orange cluster as intermediate,  
2176 green cluster as end point. Arrows indicate trajectory of cells. (f) PCA plot of hepatic CD8+ or  
2177 CD8+PD-1+ T-cells sorted TCR $\beta$ + cells by mass spectrometry of 12 months ND, CD-HFD or  
2178 CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD8+: ND n= 6 mice, CD-HFD  
2179 + IgG n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice; CD8+PD-1+: ND n= 4 mice, CD-HFD + IgG n=  
2180 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (g) UMAP representation showing the FlowSOM-guided  
2181 clustering, heatmap showing the median marker expression, and (h) quantification of hepatic  
2182 CD8+ T-cells of 12 months ND, CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  
2183  $\alpha$ -PD-1 antibodies (ND n= 4 mice; CD-HFD + IgG n= 8 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (i)  
2184 Quantification of CellCNN analyzed flow cytometry data of hepatic CD8+ T-cells of 12 months  
2185 CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD +  
2186 IgG n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice). (j) UMAP representation showing the FlowSOM-  
2187 guided clustering, the expression intensity of the indicated marker and heatmap showing the  
2188 median marker expression of flow cytometry data of hepatic CD8+PD-1+ T-cells of 12 months  
2189 ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 6 mice;  
2190 CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (k) ALT and (l) NAS evaluation of 12 months  
2191 ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF, or  $\alpha$ -  
2192 PD-1/ $\alpha$ -TNF antibodies (ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice;  
2193 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF  
2194 n= 11 mice). (m) Quantification of hepatic CD8+PD-1+CXCR6+ T-cells ND, CD-HFD, CD-  
2195 HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4,  
2196 or  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35  
2197 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-  
2198 1/ $\alpha$ -TNF n= 11 mice); CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice). (n)  
2199 Quantification of tumor incidence of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks  
2200 treatment by  $\alpha$ -CD8,  $\alpha$ -CD8/NK1.1,  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or  
2201  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (tumor incidence: CD-HFD n= 32 tumors/lesions in 87 mice; CD-  
2202 HFD +  $\alpha$ -CD8 n= 2 tumors/lesions in 31 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 0 tumors/lesions in  
2203 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 33 tumors/lesions in 44 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 2  
2204 tumors/lesions in 9 mice; CD-HFD +  $\alpha$ -TNF n= 3 tumors/lesions in 10 mice; CD-HFD +  $\alpha$ -PD-  
2205 1/ $\alpha$ -TNF n= 3 tumors/lesions in 11 mice); CD-HFD +  $\alpha$ -CD4 n= 3 tumors/lesions in 9 mice; CD-  
2206 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 tumors/lesions in 9 mice).

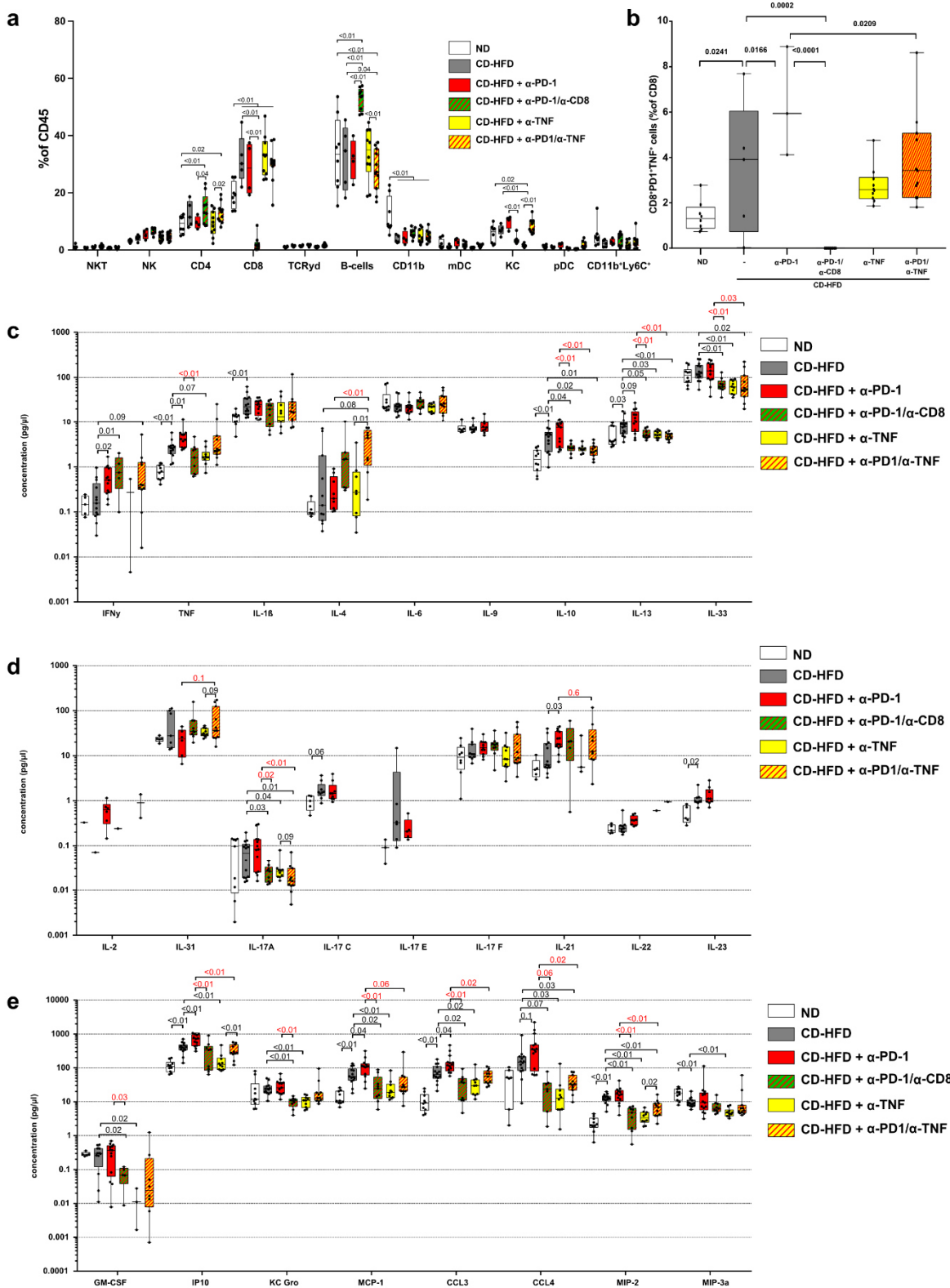


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**2210 Rebuttal Figure 56**

2211 (a) Body weight, AST, and histological evaluation by (b) Sirius red, CD4, CD8, PD-1, PD-L1,  
2212 F4/80, MHC-II and (c) staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  
2213  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (body weight: ND n= 16 mice; CD-  
2214 HFD n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-  
2215 HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; AST: body weight: ND n= 30  
2216 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
2217 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; Sirius red: ND n=  
2218 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
2219 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD4: ND n= 10  
2220 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
2221 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8: ND n= 10  
2222 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-  
2223 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n=  
2224 11 mice; PD-1: ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD  
2225 +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 8 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 10  
2226 mice; PD-L1: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  
2227  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice;  
2228 F4/80: ND n= 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-  
2229 1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  
2230  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1  
2231 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  
2232  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice).



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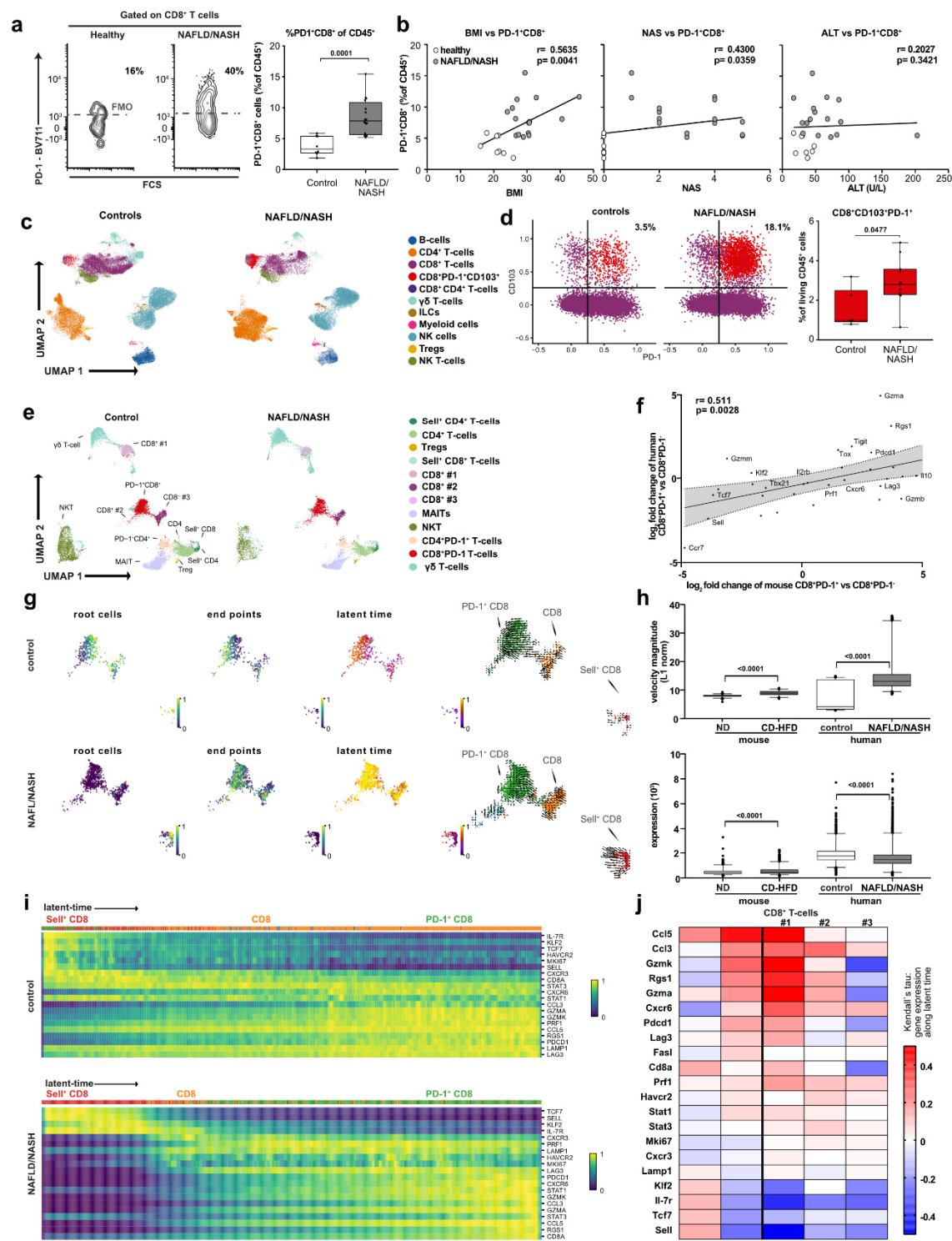
2236

**Rebuttal Figure 57**

(a) Quantification of hepatic immune cell composition and (b) CD8<sup>+</sup>PD-1<sup>+</sup>TNF<sup>+</sup> T-cells by flow cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by α-PD-1,



2237  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (Hepatic immune cell composition: ND n= 8  
2238 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice;  
2239 CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8+PD-1+TNF+: ND  
2240 n= 8 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 3 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
2241 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). (c) and (d)  
2242 multiplex ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12  
2243 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -  
2244 TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n=  
2245 13 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-  
2246 1/ $\alpha$ -TNF n= 11 mice).



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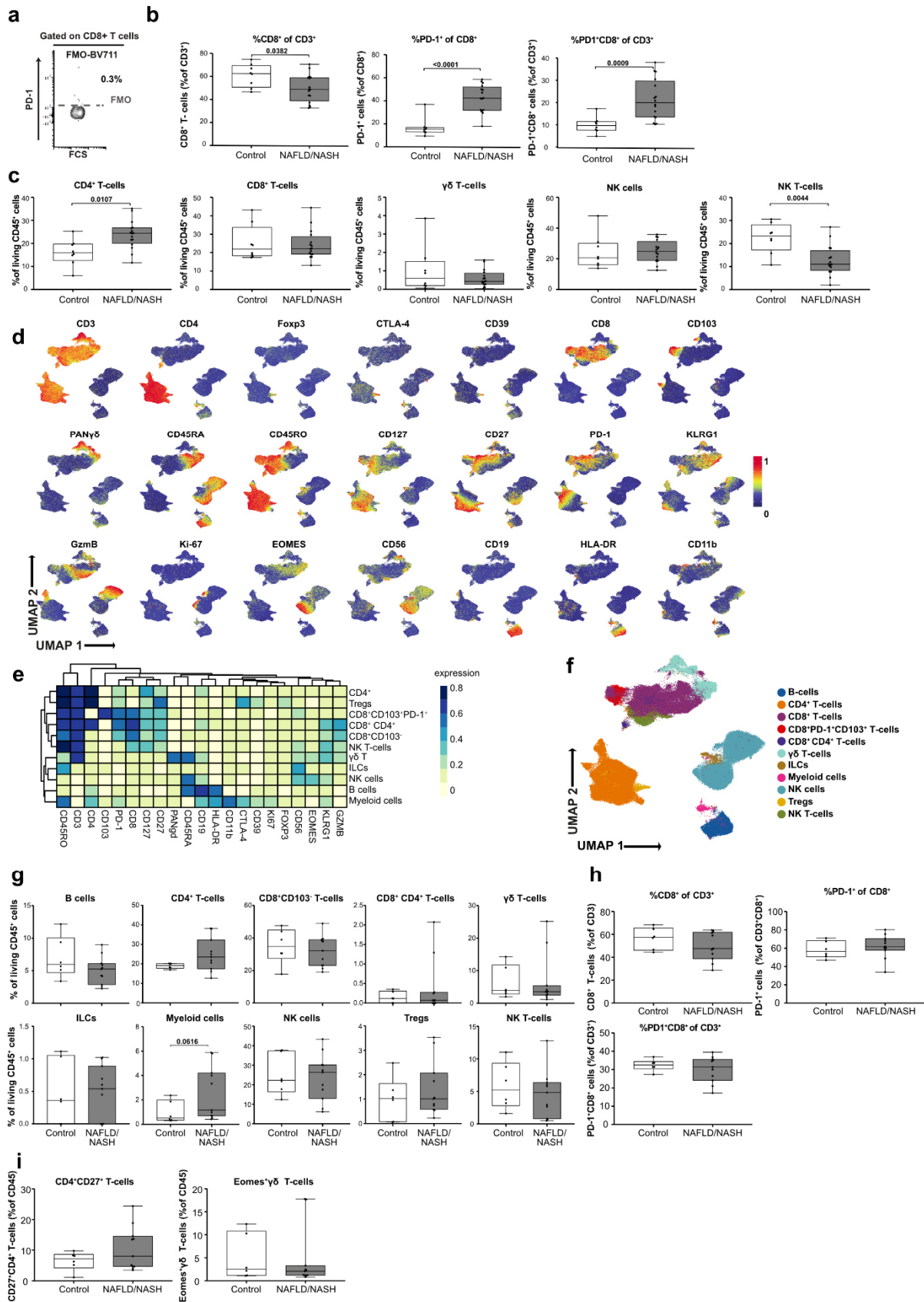
**Rebuttal Figure 58**

2249 (a) Flow cytometry plots, quantification of patient-liver-derived PD-1+CD8+ T-cells, and (b)  
 2250 correlation of PD-1+CD8+ T-cells with BMI, NAS and ALT of healthy or NAFLD/NASH patients  
 2251 (Supplementary Table 1: healthy n= 8 patients; NAFLD/NASH n= 16 patients). Fluorescence-  
 2252 minus-one (FMO) defined in Extended Data 25. (c) UMAP representation showing the  
 2253 FlowSOM-guided clustering of CD45<sup>+</sup> cells and (d) flow cytometry plots and quantification of



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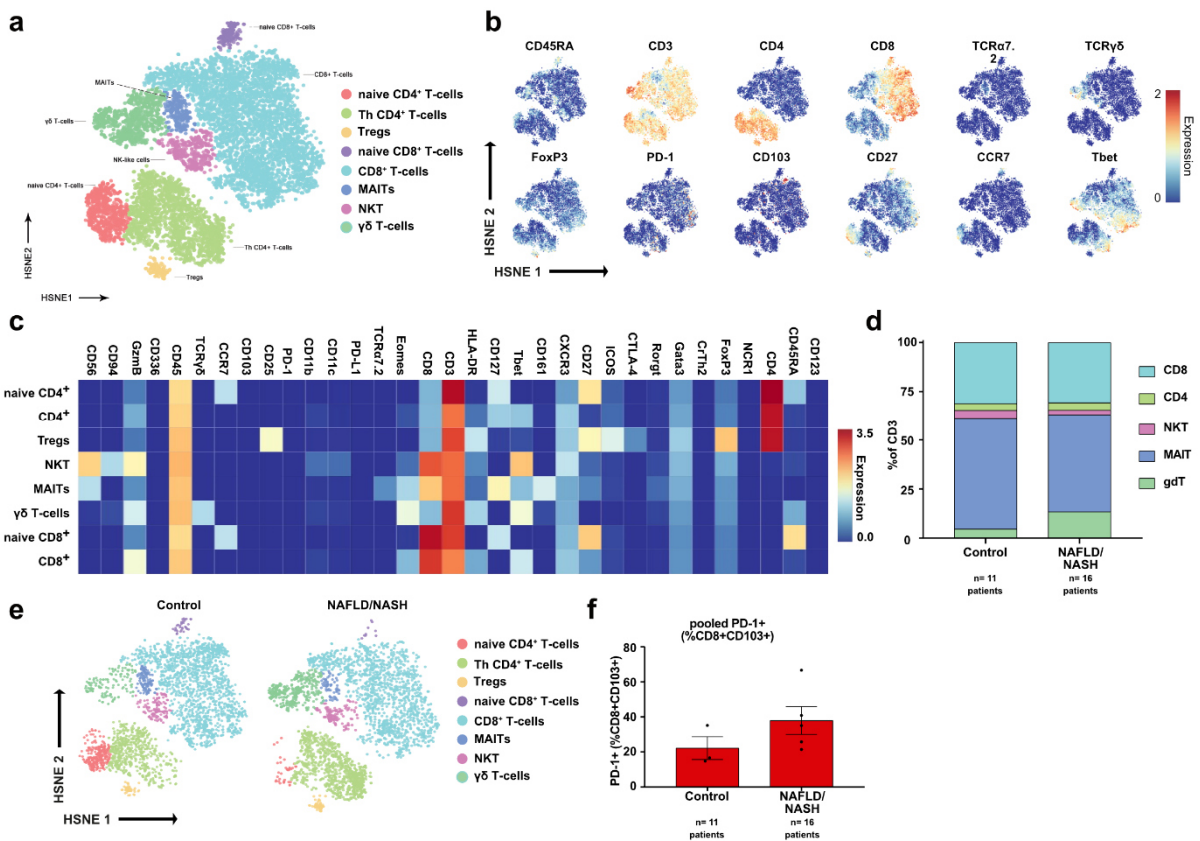
2254 CD8+PD-1+CD103+ derived from hepatic biopsies of control, or NAFLD/NASH patients  
2255 (Supplementary Table 2: control n= 6 patients; NAFLD/NASH n= 11 patients) Populations:  
2256 CD8+ (violet), CD8+PD-1+CD103+ (red). (e) UMAP representation of CD3+ cells and analyses  
2257 of differential gene expression by scRNA-seq of control, or NAFLD/NASH patients (control n=  
2258 4 patients; NAFLD/NASH n= 7 patients). (f) Correlation of significant differentially expressed  
2259 genes in liver-derived CD8+PD-1+ compared to CD8+PD-1- T-cells subsets of 12 months CD-  
2260 HFD-fed mice and NAFLD/NASH patients (mouse: n= 3 mice; human: n= 3 patients). (g)  
2261 Velocity analyses of scRNA-seq data showing (h) expression, transcriptional activity, (i) gene  
2262 expression and (j) correlation of expression along the latent-time of selected genes along the  
2263 latent-time of patient-liver-derived CD8+ T-cells of control, or NAFLD/NASH patients in  
2264 comparison to mouse-liver-derived CD8+ T-cells (patients: NAFLD/NASH n= 3 patients;  
2265 mouse: n= 3 mice/group). Root cells: yellow cells indicate root cells, blue cells indicate cells  
2266 farthest away from the root by RNA velocity. End points: yellow cells indicate end point cells,  
2267 blue cells indicate cells farthest away from defined end point cells by RNA velocity. Latent time:  
2268 pseudo-time by RNA velocity, dark color indicate start of RNA velocity, yellow color indicate  
2269 end point of latent time. RNA velocity flow: Blue cluster defined as start point, orange cluster  
2270 as intermediate, green cluster as end point. Arrows indicate the trajectory of cells.



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2273 **Rebuttal Figure 59**

2274 (a) Flow cytometry plot of FMO control, (b) quantification of patient-liver-derived PD-1+CD8+  
 2275 T-cells, and (c) quantification of CD4, CD8,  $\gamma\delta$ , NK and NKT cells healthy or NAFLD/NASH  
 2276 patients (Supplementary Table 1: healthy n= 8 patients; NAFLD/NASH n= 16 patients). (d)  
 2277 Analysis of randomly chosen CD45+ cells and (e) average marker expression of defined  
 2278 CD45+ subsets by flow cytometry derived from hepatic biopsies of control and NAFLD/NASH  
 2279 patients to define distinct marker expression (Supplementary Table 2: control n= 6 patients;  
 2280 NAFLD/NASH n= 11 patients). (f) Definition of cellular subsets, (g) relative quantification of  
 2281 defined cellular subsets of randomly chosen CD45+ cells, (h) polarization of CD8+ T-cells and  
 2282 (i) quantification of CD4+CD27+, or  $\gamma\delta$  TCR+Eomes+, T-cells by flow cytometry derived from  
 2283 hepatic biopsies of healthy and NAFLD/NASH patients (Supplementary Table 2: control n= 6  
 2284 patients; NAFLD/NASH n= 11 patients).

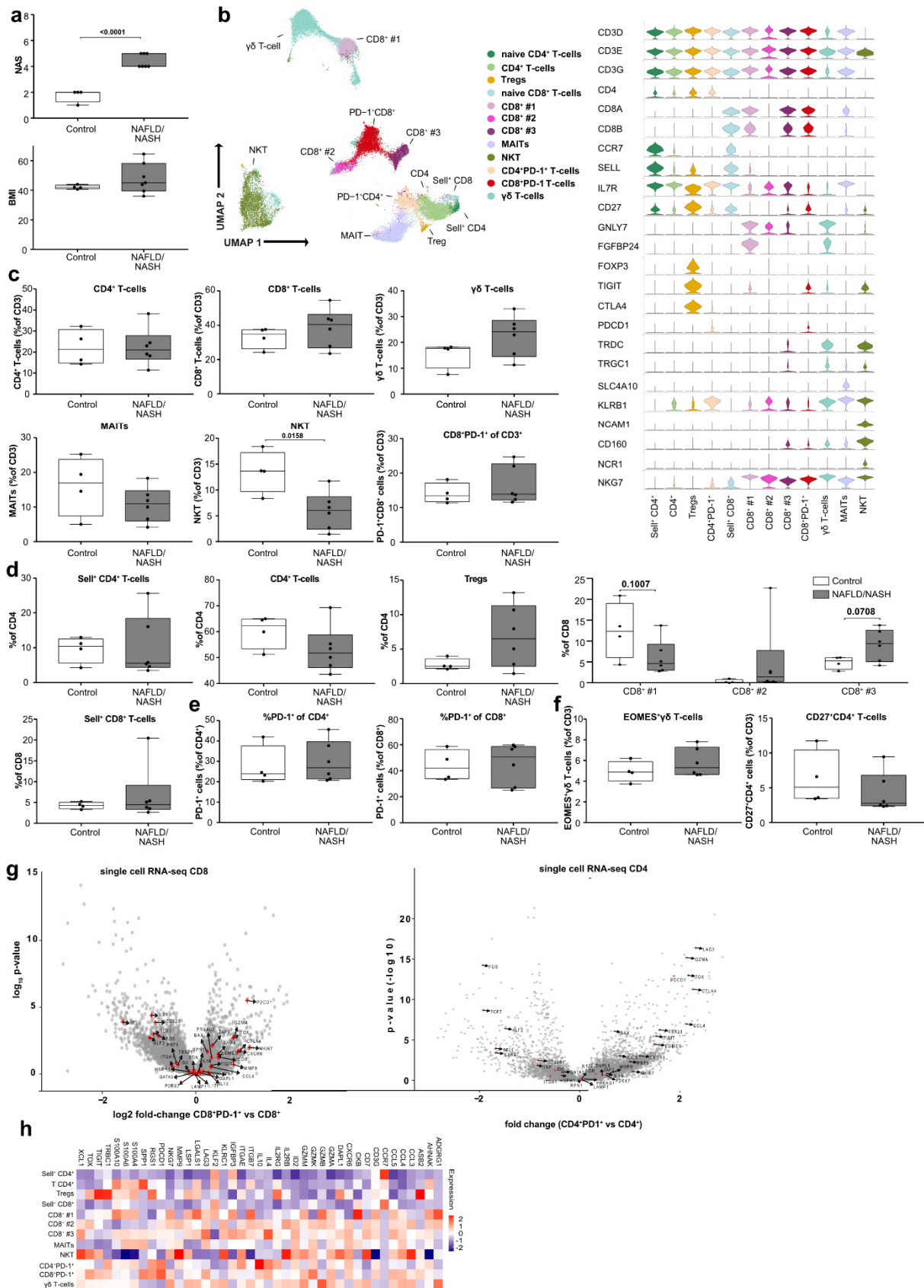


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 2286 **Rebuttal Figure 60**

2287 (a) tSNE representation, (b) marker expression, (c) average marker expression of defined T-  
 2288 cell subsets of patient-liver-derived T-cells analyzed by CyTOF of control and NAFLD/NASH  
 2289 patients (control n= 11 patients pooled in 3 analyses; NAFLD/NASH n= 16 patients pooled in  
 2290 5 analyses). (d) Composition, (e) HSNE representation of defined T-cell subsets and (f)  
 2291 quantification of CD8+CD103+PD-1+ cells of of patient-liver-derived T-cells analyzed by  
 2292 CyTOF of control and NAFLD/NASH patients (control n= 11 patients pooled in 3 analyses;  
 2293 NAFLD/NASH n= 16 patients pooled in 5 analyses).

2294





2297 **Rebuttal Figure 61**

2298 (a) NAS and BMI of patients used for scRNA-seq analyses of patient-liver-derived T-cells of  
2299 control and NAFLD/NASH patients (control n= 4 patients; NAFLD/NASH n= 7 patients). (b)  
2300 UMAP representation, marker expression, (c) relative quantification and (d), (e), (f) polarization  
2301 of defined T-cell subsets of defined T-cell subsets of patient-liver-derived T-cells by scRNA-  
2302 seq of control and NAFLD/NASH patients (control n= 4 patients; NAFLD/NASH n= 7 patients).  
2303 (g) Differential gene expression of CD4+PD-1+ vs CD4+ T-cells and (h) selected average  
2304 marker expression in CD4+ and CD8+ T-cell subsets of by scRNA-seq of control and  
2305 NAFL/NA2SH patients (control n= 4 patients; NAFLD/NASH n= 7 patients).  
2306

2307 2. Would PD-L1 blockade enhance liver cancer and tissue damage as well? Which cells are  
2308 expressing PD-L1 in the system? This becomes important given the recent approval of  
2309 atezolizumab + bevacizumab.

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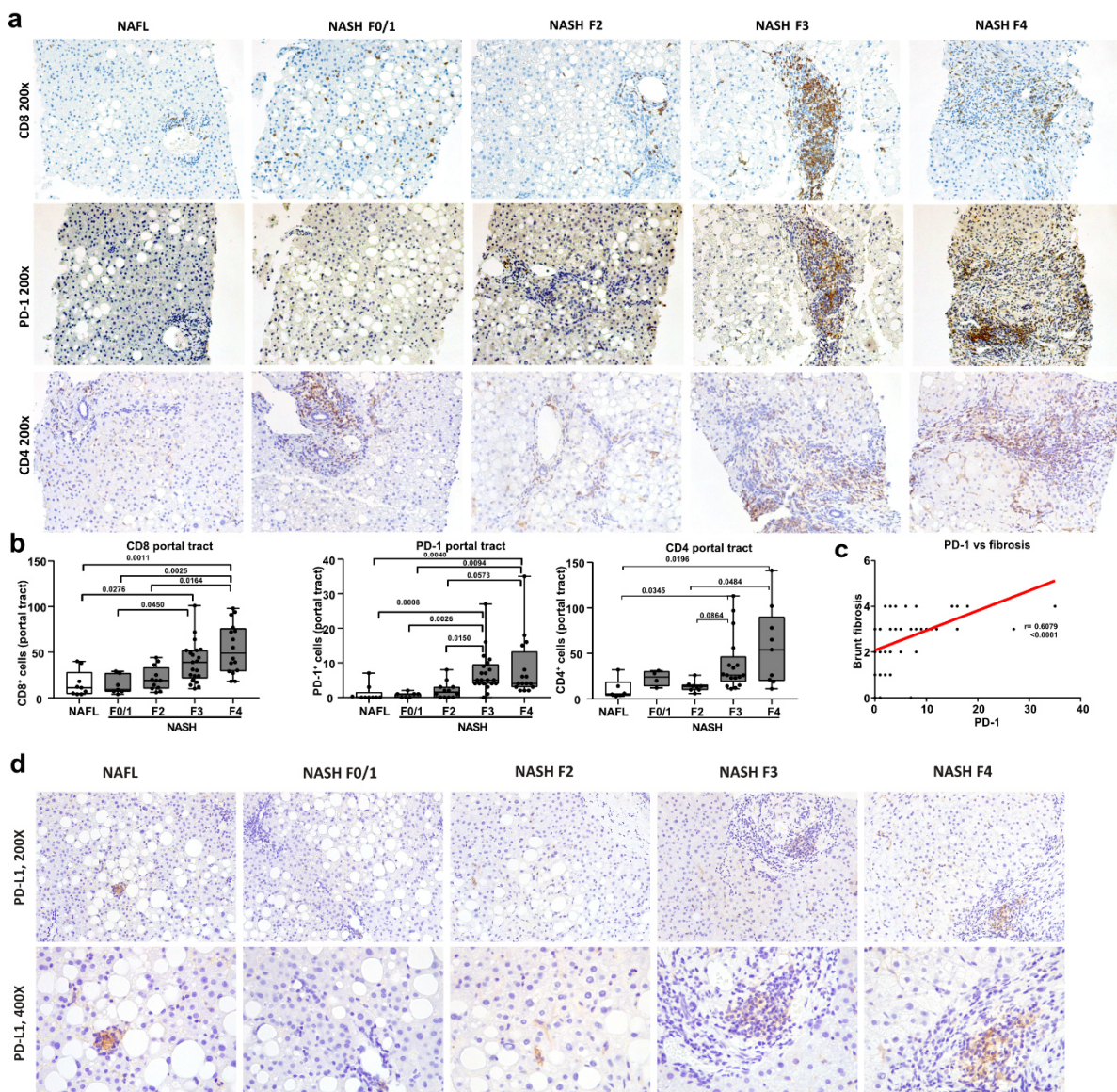
2311 We agree with Referee #3 for raising the point that dissection of anti-PD-L1-targeted  
2312 immunotherapy is of major concern, especially in the light of the recent results of the  
2313 IMBrave150 study. Data we have received from RNA in situ hybridization and  
2314 immunohistochemistry indicate that PD-L1 is expressed with increased level over time – with  
2315 progression of NASH disease (in mice and men). In summary, PDL1 staining in the preclinical  
2316 model is mainly associated with inflammatory cells, positive cells can be observed in the  
2317 sinusoidal space as well (included in **Extended Data 3, 20, 22** and **Rebuttal Figure 56, 62-  
2318 64**). In humans, PDL1 positivity was observed in aggregates of inflammatory cells in the  
2319 parenchyma and the portal tract area. Focally, positivity was also seen in sinusoidal lining cells  
2320 (included in **Extended Data 28** and **Rebuttal Figure 62**).

2321

2322 The cells expressing PD-L1 in NASH-affected mice are mainly lymphocytes but also some  
2323 parenchymal cells (see **Extended Data 3+7, 20+22** and **Rebuttal Figure 63**).

2324 In line with the comment of Referee #3, we have also performed anti-PD-L1 targeted  
2325 immunotherapy in mice with and without established liver cancer (included in **Extended Data  
2326 7** and **Rebuttal Figure 63**). Results from these experiments indicate that similar to anti-PD1 -  
2327 anti-PDL1-treatment does not induce an anti-cancer effect for NASH-induced HCC but induces  
2328 - similar to anti-PD1 treatment - a pro-inflammatory and pro-carcinogenic effect (e.g. increased  
2329 NAS, strong trend in increased hepatic CD8 abundance by IHC (p= 0.0546), cytokines like IL-  
2330 21 and CCL3) (included **Extended Data 7+13** and **Rebuttal Figure 63, 65**). These data  
2331 indicate, that in the preclinical NASH model both PD1 or PDL1-targeted immunotherapy  
2332 induces adverse effects. This is corroborated by our increased, retrospective cohort HCC-  
2333 patients of different etiologies under PD(L)1-targeted immunotherapy, in which multivariate  
2334 analysis results in NAFLD/NASH being an independent negative factor for overall survival and

2335 validated these results in a second cohort of 118 HCC-patients (included in **Figure 6** and  
 2336 **Rebuttal Figure 66**). Furthermore, we corroborated our hypothesis of non-viral (NASH-  
 2337 related) HCC being less responsive to immunotherapy by a meta-analysis including 1656  
 2338 patients of the three most important clinical trials, identifying immunotherapy vs control for viral  
 2339 HCC as favorable treatment (HR(viral)= 0.64), in contrast, non-viral-HCC showed less benefit  
 2340 (HR(non-viral)= 0.92) for immunotherapy (included in **Figure 6, Extended Data 30-32,**  
 2341 **Supplementary Table 9 and Rebuttal Figure 67, 68**)).



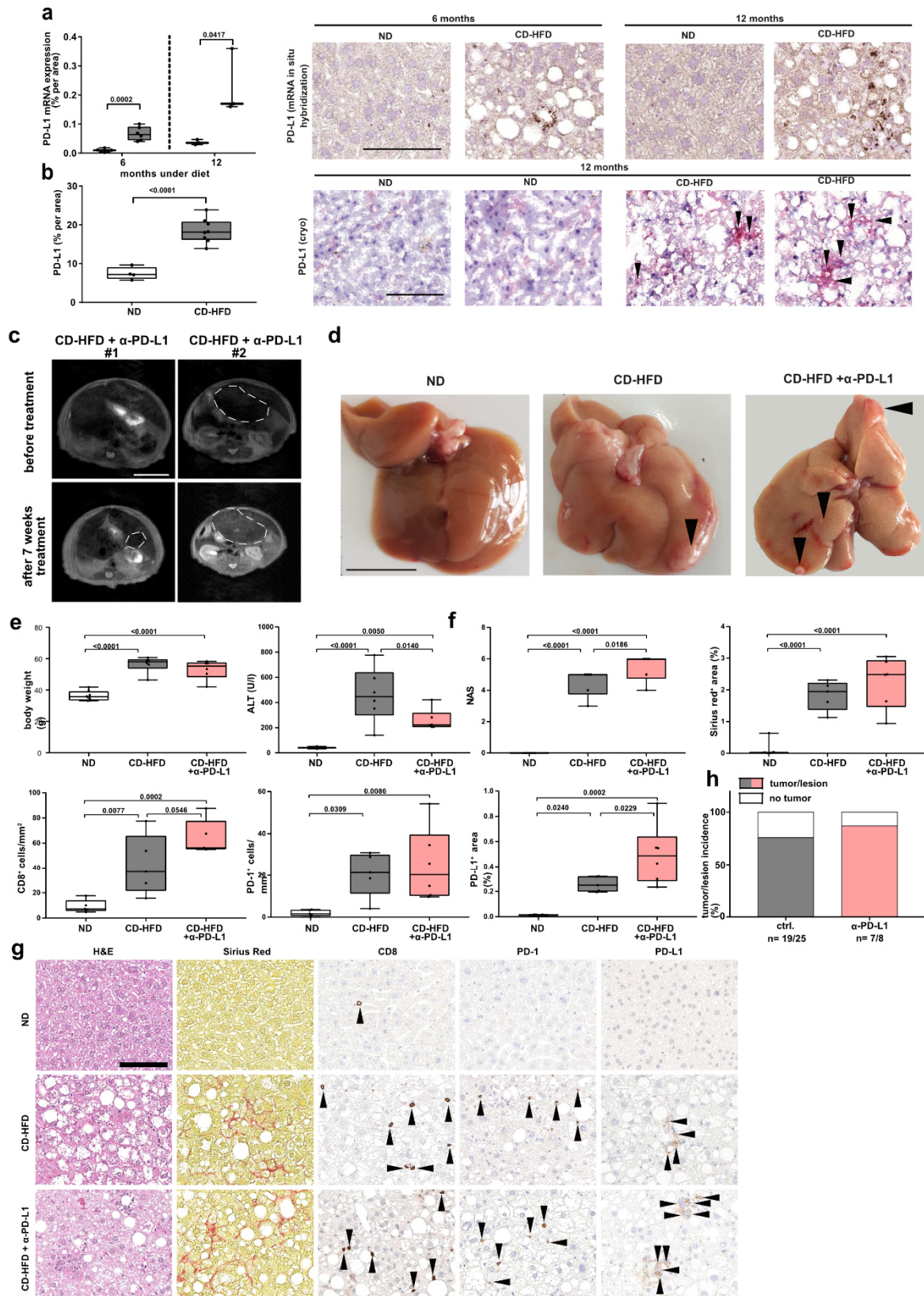
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2343 **Rebuttal Figure 62**

2344 (a) Immunohistochemical staining and (b) quantification of hepatic PD-1, CD8 and CD4  
 2345 expressing cells of NAFLD and NASH patients in Supplementary Table 3 with varying stages  
 2346 of fibrosis (NAFLD n= 9 patients; NASH F1/0 n= 7 patients; NASH F2 n= 12 patients; NASH  
 2347 F3 n= 21 patients; NASH F4 n= 16 patients; CD4: NAFL n= 6 patients; NASH F1/0 n= 4  
 2348 patients; NASH F2 n= 8 patients; NASH F3 n= 17 patients; NASH F4 n= 9 patients).



2349 Correlation analysis of PD-1 against fibrosis scoring according to Brunt by  
2350 immunohistochemical staining by RNA-sequencing (NAFLD/NASH n= 65 patients). A total of  
2351 1656 patients were included in all three randomized trials, and 985 patients received a  
2352 checkpoint inhibitor (Supplementary Table 7). (d) Immunohistochemical staining of PD-L1 in  
2353 patient-derived liver samples. Scale bar: 50  $\mu$ m.  
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**Rebuttal Figure 63**

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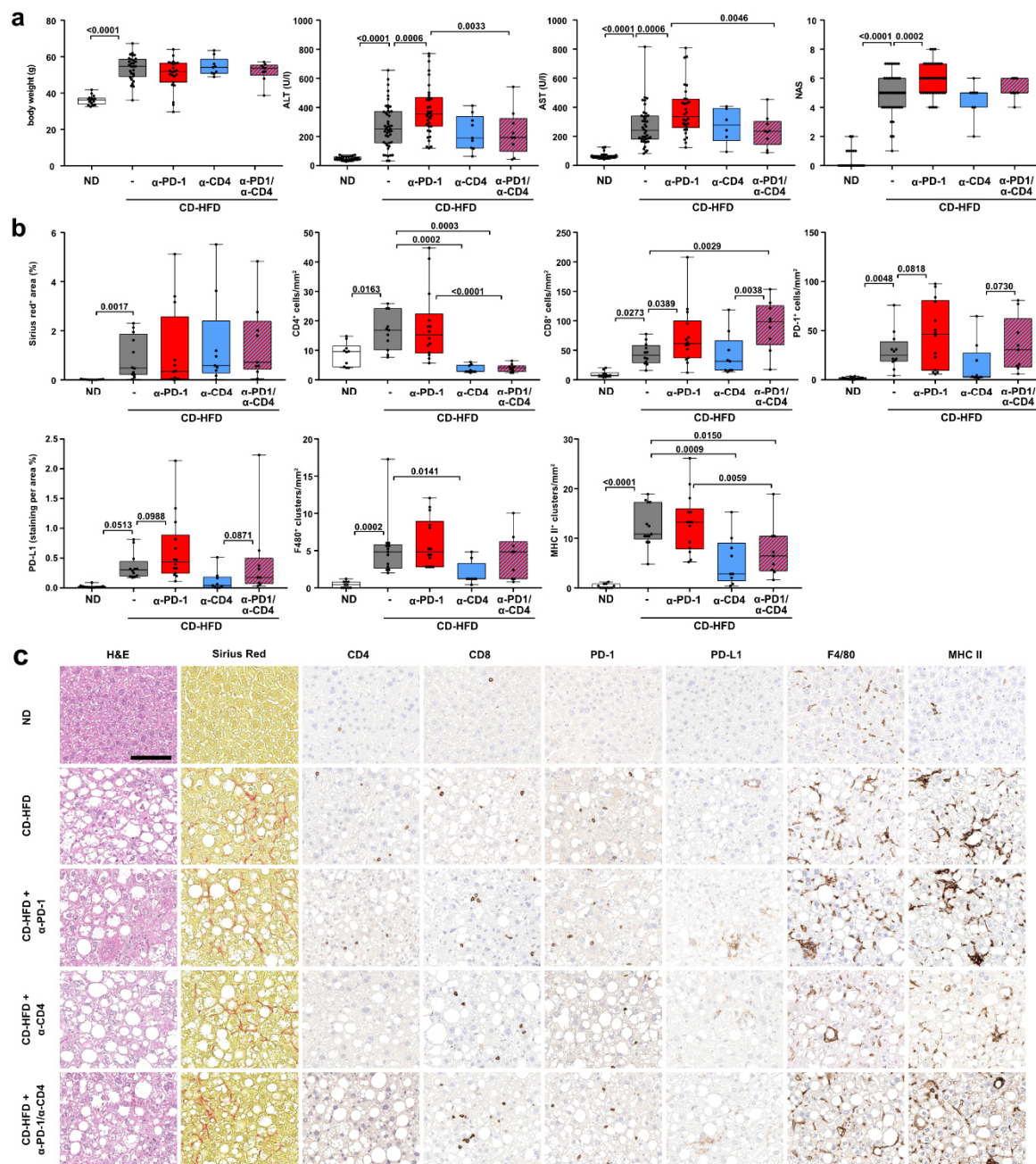
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(a) Quantification of hepatic PD-L1+ expression by RNA in situ hybridization of 6- or 12-months ND or CD-HFD-fed mice (6 months: ND n= 13 mice; CD-HFD n= 11 mice; 12 months: ND n=



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2359 7 mice; CD-HFD n= 7 mice). Scale bar: 100  $\mu$ m. (b) Quantification of hepatic PD-L1+  
2360 expression by immunohistochemistry of 12 months ND or CD-HFD fed mice (6 months: ND n=  
2361 4 mice; CD-HFD n= 8 mice). Scale bar: 100  $\mu$ m. (c) MRI pictures of liver of mice after 10  
2362 months CD-HFD and 7 weeks later after assignment to CD-HFD or CD-HFD-fed mice + 7  
2363 weeks treatment of  $\alpha$ -PD-L1 (CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-L1 n= 8 mice). Lines  
2364 indicate tumor nodule. Scale bar: 10 mm. (d) Macroscopy of liver of 12 months ND, CD-HFD  
2365 or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-L1. Arrowheads indicate tumor/lesions.  
2366 Scale bar: 10 mm. (e) Body weight, ALT levels of 12 months ND, CD-HFD or CD-HFD-fed  
2367 mice + 8 weeks treatment of  $\alpha$ -PD-L1 (Body weight, ALT, : ND n= 8 mice; CD-HFD n= 6 mice;  
2368 CD-HFD +  $\alpha$ -PD-L1 n= 6 mice) (f) and (g) NAS evaluation by H&E, Fibrosis evaluation of Sirius  
2369 Red staining, quantification of CD8, PD-1 and PD-L1 staining of hepatic tissue by  
2370 immunohistochemistry of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment  
2371 of  $\alpha$ -PD-L1 (NAS: ND n= 7 mice; CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; Sirius  
2372 Red: ND n= 7 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice ; CD8, : ND n= 5 mice;  
2373 CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 5 mice; PD-1, PD-L1: ND n= 5 mice; CD-HFD n=  
2374 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice). Scale bar: 100  $\mu$ m. (h) Tumor/Lesion incidence in CD-  
2375 HFD or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-L1 (CD-HFD n= 19 tumors/lesions in  
2376 25 mice; CD-HFD +  $\alpha$ -PD-L1 n= 7 tumors/lesions in 8 mice)

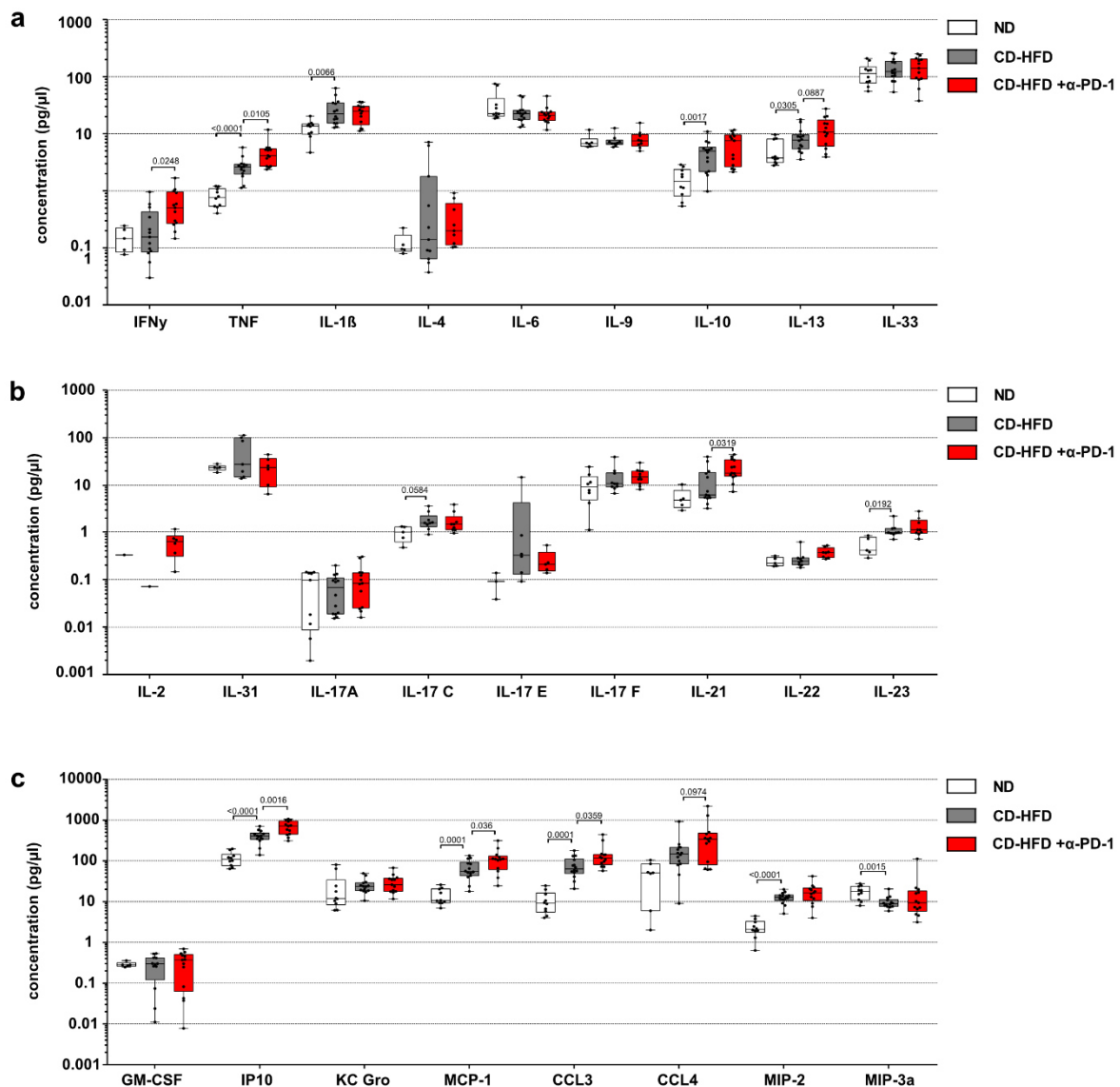


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### Rebuttal Figure 64

(a) Body weight, ALT, AST, NAS, and histological evaluation by (b) Sirius Red, CD4, CD8, PD-1, PD-L1, F4/80, MHC-II and (c) staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (body weight: ND n= 16 mice; CD-HFD n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; ALT ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; AST: ND n= 30 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; NAS: ND n= 31 mice; CD-HFD n= 46 mice; CD-HFD +  $\alpha$ -PD-1 n= 40 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice; Sirius red: ND n= 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; CD4: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-

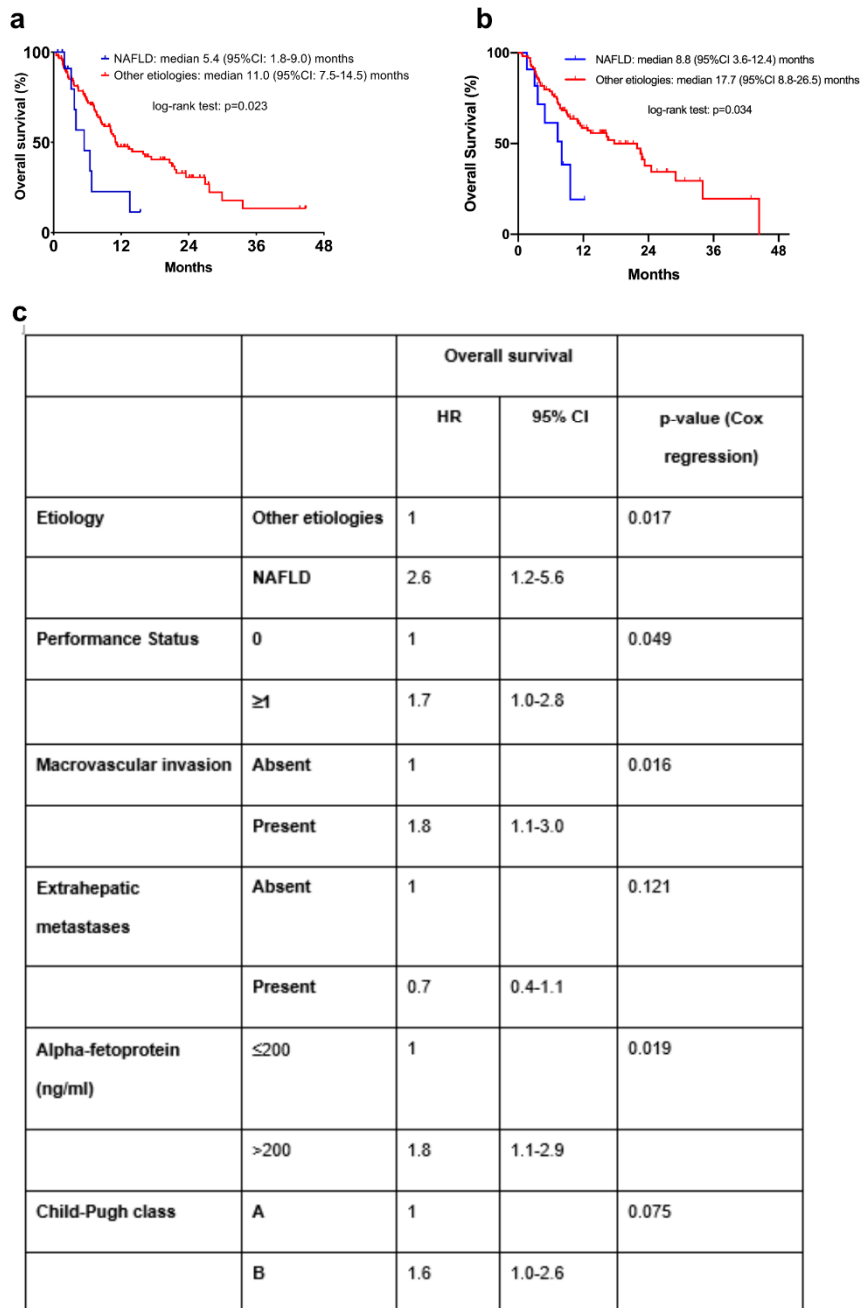
2390 1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 11 mice; CD8: ND  
 2391 n= 10 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice;  
 2392 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-1: ND n= 13 mice; CD-HFD n= 12 mice; CD-HFD +  
 2393  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-L1:  
 2394 ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9  
 2395 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; F4/80: ND n= 11 mice; CD-HFD n= 13 mice; CD-  
 2396 HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice;  
 2397 MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -  
 2398 PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice). Scale  
 2399 bar: 100  $\mu$ m.



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### Rebuttal Figure 65

(a) and (b) multiplex ELISA concentrations of hepatic inflammation-associated cytokines and (c) chemokines of 12 months ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-1 or CD-HFD (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 13 mice).

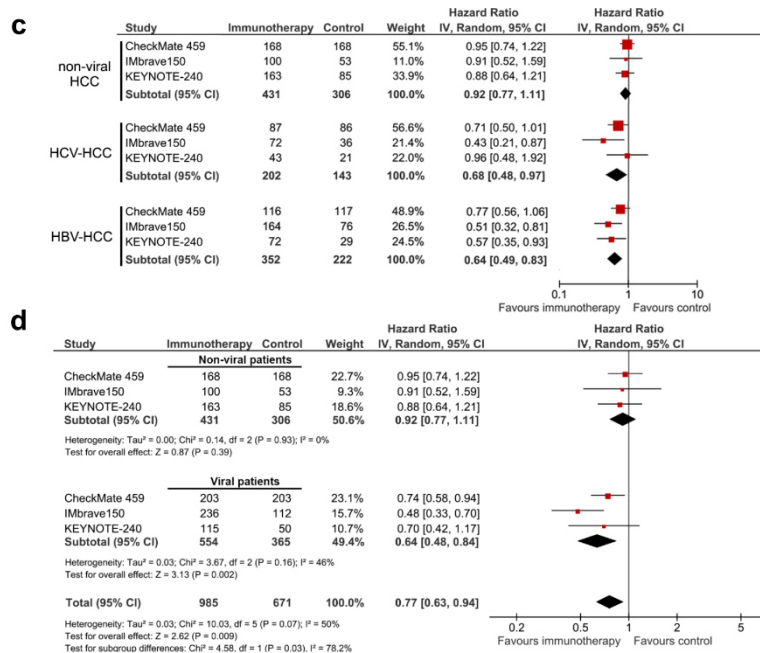
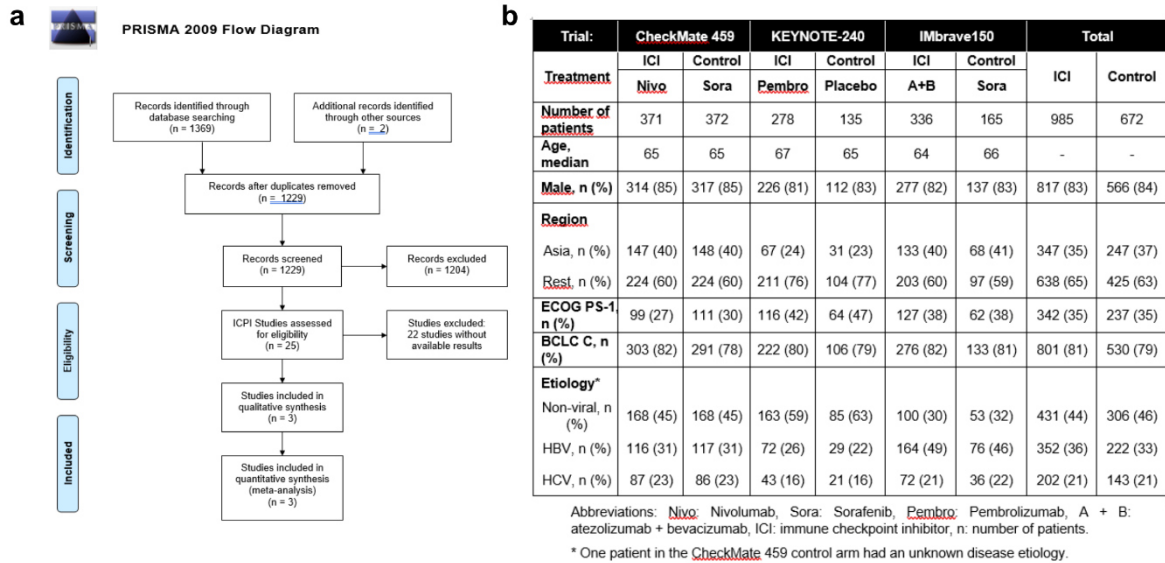


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### Rebuttal Figure 66

(a) Nonalcoholic fatty liver disease (NAFLD) is associated with a worse outcome in patients with hepatocellular carcinoma (HCC) treated with PD-(L)1-targeted immunotherapy. A total of 130 patients with advanced HCC received PD-(L)1-targeted immunotherapy (Supplementary Table 8). Kaplan-Meier curve display overall survival of patients with NAFLD vs. those with any other etiology; all 130 patients were included in these survival analyses (NAFLD n=13, any other etiology n=117). (b) Validation cohort of patients with HCC treated with PD-(L)1-targeted immunotherapy. A total of 1180 patients with advanced HCC received PD-(L)1-targeted immunotherapy (Supplementary Table 10). Kaplan-Meier curve display overall survival of patients with NAFLD vs. those with any other etiology; all 118 patients were included in these survival analyses (NAFLD n=11, any other etiology n=107). (c) Multivariate analysis of prognostic factors in HCC patients treated with anti-PD-(L)1-based immunotherapy





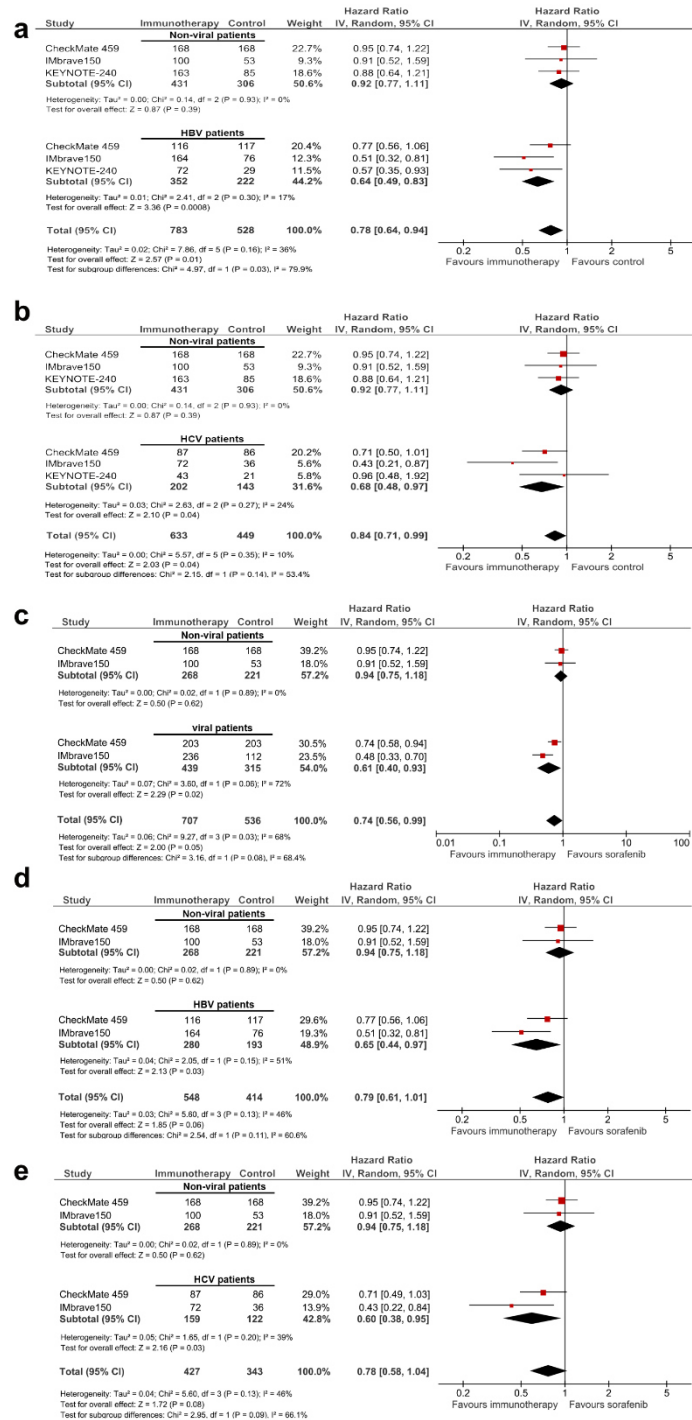
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**Rebuttal Figure 67**

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(a) Selection of articles assessing the clinical outcome of immune checkpoint inhibitors in advanced HCC for inclusion in the systematic review and meta-analysis. ICPI: Immune checkpoint inhibitor. (b) Pooled baseline characteristics of the patients included in the meta-analysis (total n= 1656). (c) A total of 1656 patients were included in all three randomized trials, and 985 patients received a checkpoint inhibitor (Supplementary Table 7). (c) Separate meta-analyses were performed for each of the three etiologies: non-viral (including mostly NASH and alcohol intake), HCV and HBV. (d) HCV and HBV were pooled into a separate category, termed “viral”, and a subsequent meta-analysis comparing viral (n=919) and non-viral, including mostly NASH and alcohol intake (n=737) was performed. Hazard ratios for each trial are represented by squares, the size of the square represents the weight of the trial in the meta-analysis. The horizontal line crossing the square represents the 95% confidence interval

2431 (CI). The diamonds represent the estimated overall effect based on the meta-analysis random  
 2432 effect of all trials.



2433

2434 **Rebuttal Figure 68**

2435 A total of 1656 patients were included in all three randomized trials, and 985 patients received  
 2436 a checkpoint inhibitor. Subgroup analysis was performed to study the specific effects of  
 2437 immunotherapy comparing non-viral etiologies (n=737) with (a) HBV (n=574) or (b) HCV  
 2438 (n=345). Hazard ratios for each trial are represented by squares, the size of the square  
 2439 represents the weight of the trial in the meta-analysis. The horizontal line crossing the square

2440 represents the 95% confidence interval (CI). The diamonds represent the estimated overall  
2441 effect based on the meta-analysis random effect of all trials.

2442 A total of 1243 patients were included in two first-line trials comparing PD-1 or PD-L1 targeted  
2443 immunotherapy to sorafenib. 707 patients received an immune checkpoint inhibitor (either PD-  
2444 1 or anti-PD-1). (c) HCV and HBV were pooled into a separate category, termed “viral”, and a  
2445 subsequent meta-analysis comparing viral (n=754) and non-viral (n=489), mostly NASH and  
2446 alcohol intake, was performed. A subgroup analysis studying the specific effects of non-viral  
2447 etiologies (n=489) on the magnitude of effect of immunotherapy are presented, when  
2448 compared to (d) HBV (n=473) or (e) HCV (n=281). Hazard ratios for each trial are represented  
2449 by squares, the size of the square represents the weight of the trial in the meta-analysis. The  
2450 horizontal line crossing the square represents the 95% confidence interval (CI). The diamonds  
2451 represent the estimated overall effect based on the meta-analysis random effect of all trials.  
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2453 3. Results on NASH in human samples are compelling and supportive of the relevance of the  
2454 findings. It would be interesting to know in such livers which cells express PD-L1.

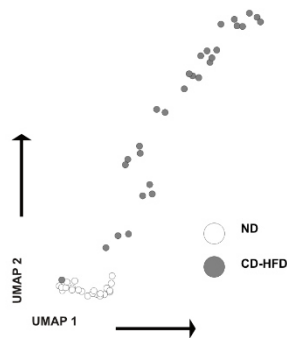
2455  
2456 We thank Referee #3 for highlighting this important aspect of our data – and have consequently  
2457 performed PD-L1 expression analyses by immunohistochemistry in human specimens  
2458 described in the previous point raised by Referee #3. Although analysis by bulk RNA-seq of  
2459 liver tissue indicates a decrease of PDL1/CD274 expression with the severity of NASH  
2460 pathology, immunohistochemistry indicates an increase of PDL1 positivity with the severity of  
2461 NASH pathology. PDL1 positivity was observed in aggregates of inflammatory cells in the  
2462 parenchyma and the portal tract area. Focally, positivity was also seen in sinusoidal lining cells  
2463 (included in **Extended Data 28** and **Rebuttal Figure 62d**).

2464  
2465 4. What do you think is the fibrogenic factor/s promoted by pathogenic CD8 cells? Any  
2466 candidates from the extensive transcriptomic analyses?

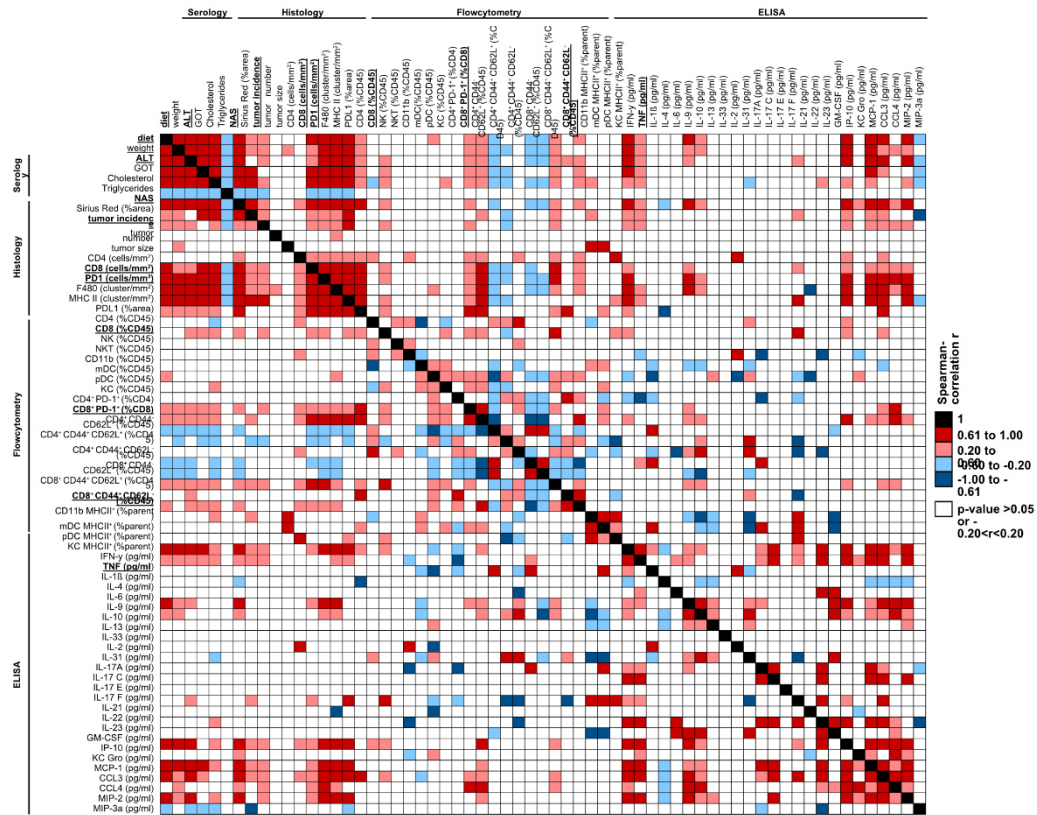
2467  
2468 We thank Referee #3 for pointing out, that the fibrogenic factor is of major concern to prevent  
2469 HCC in subgroups of NASH patients. Our transcriptomic data-set has so far not pointed  
2470 towards specific fibrogenic factors, indicating that the chronic inflammatory environment  
2471 correlating with pathogenic CD8 cells drives fibrosis in our mice. To strengthen this hypothesis  
2472 AI-based analyses of a broad range of parameters of our 12 months CDHFD-fed mice  
2473 revealed, that Sirius red staining correlates negatively within CD8 depleted animals, indicating  
2474 that CD8-associated inflammation or CD8-dependent mechanisms might be functionally linked  
2475 with fibrosis (included in **Figure 1**, **Extended Data 4 and 24** and **Rebuttal Figure 69, 70**).  
2476 Moreover, in 12 months CDHFD-fed mice fibrosis correlated positively with CD8 T-cells  
2477 abundance, CD8+PD-1+ (%CD8), pDC+MHCII+ polarization, and hepatic TNF concentration.  
2478 Therefore, we cannot point out one specific factor driving fibrosis on pathogenic CD8 cells.

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**a**



**b**



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**Rebuttal Figure 69**

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(a) UMAP representation of 63 parameters (serology, flow cytometry, histology) indicating

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NASH pathology severity measured of 12 months ND or CD-HFD fed mice (ND n= 22 mice;

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CD-HFD n= 31 mice). (b) Data gathered from hepatic tissue analyses was binary correlated

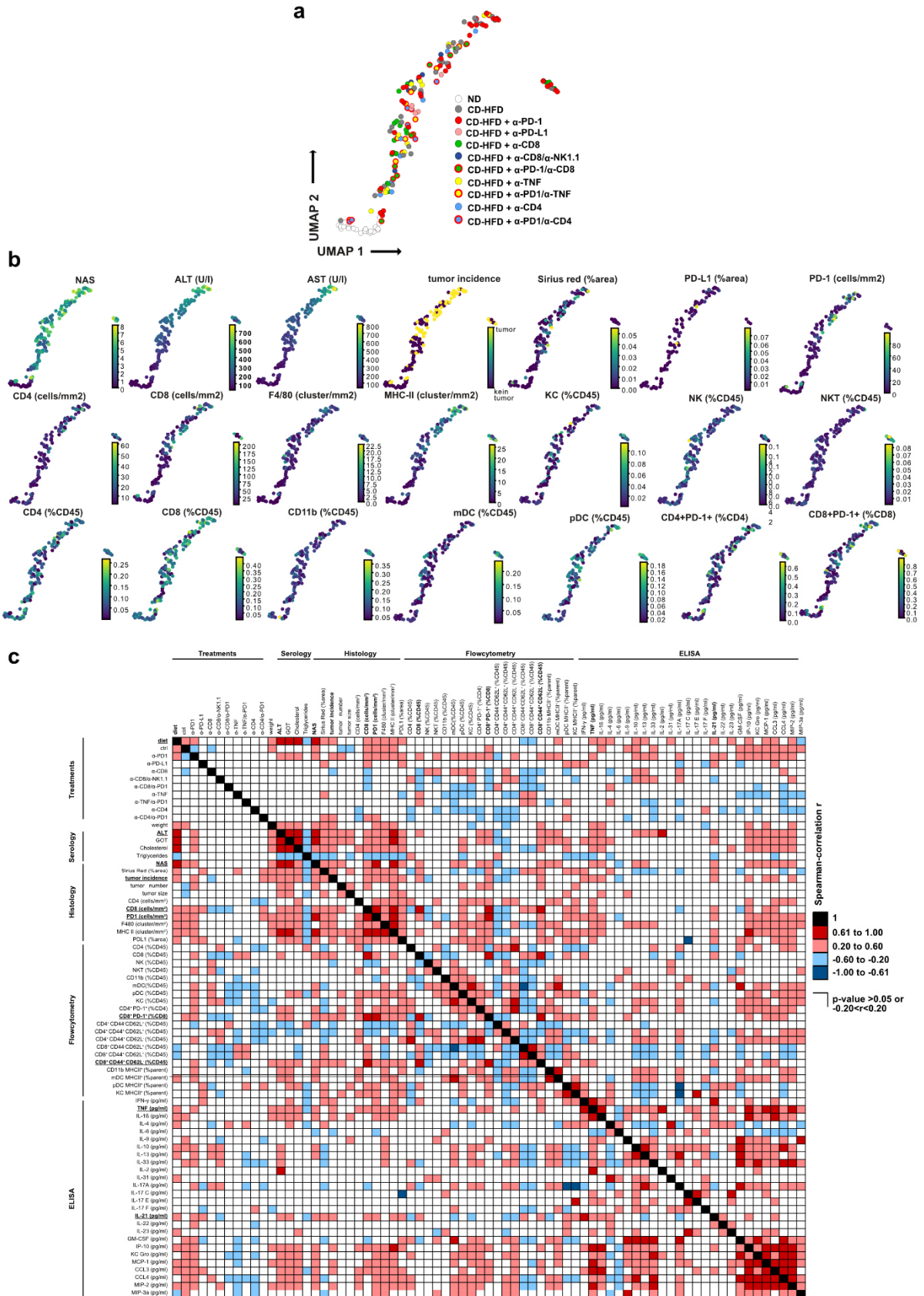
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with each other of 6- or 12-months ND or CD-HFD fed mice (ND n= 47 mice; CD-HFD n= 72

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mic).

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2488 **Rebuttal Figure 70**

2489 (a) UMAP representation of 63 parameters (serology, flow cytometry, histology) and (b)  
2490 selected display of analyzed parameters indicating NASH pathology severity measured of 12  
2491 months ND or CD-HFD fed mice (ND n= 22 mice; CD-HFD n= 31 mice; CD-HFD +  $\alpha$ -PD-1 n=  
2492 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; CD-HFD +  $\alpha$ -CD8 n= 24 mice; CD-HFD +  $\alpha$ -  
2493 CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice;  
2494 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4  
2495 n= 9 mice). (c) Data gathered from hepatic tissue analyses was binary correlated with each  
2496 other of 6- or 12-months ND, CD-HFD or CD-HFD + 8 weeks treatment of  $\alpha$ -CD8,  $\alpha$ -CD8/ $\alpha$ -  
2497 NK1.1;  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or  $\alpha$ -PD-1/ $\alpha$ -CD4 fed mice (ND  
2498 n= 47 mice; CD-HFD n= 72 mice; CD-HFD +  $\alpha$ -PD-1 n= 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6  
2499 mice; CD-HFD +  $\alpha$ -CD8 n= 29 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -  
2500 CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD  
2501 +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).

2503 5. Are Kupffer cells involved in the CD8-dependent pathogenesis mechanisms?

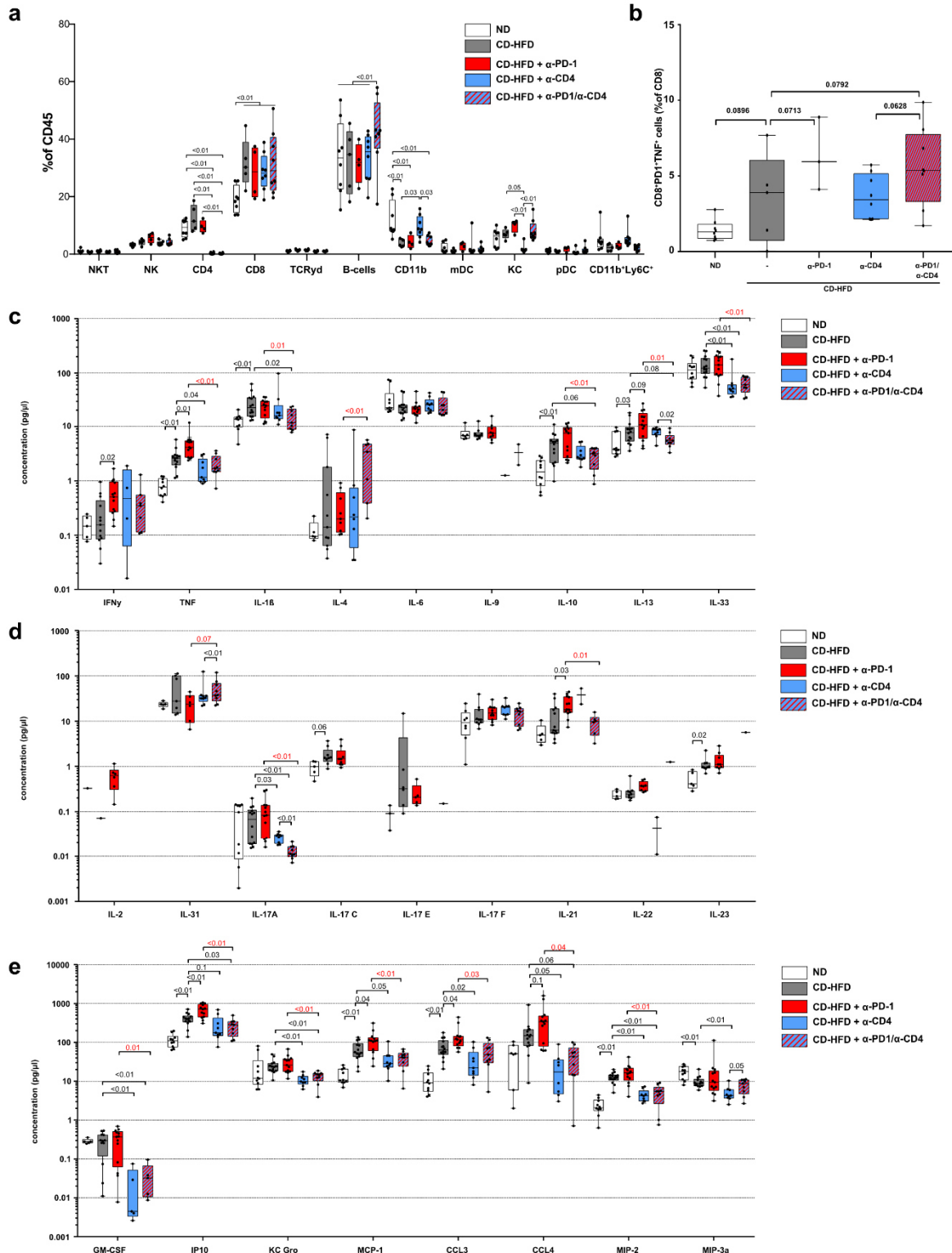
2504

2505 We thank Referee #3 for asking the important question about Kupffer cells (KC). A study  
2506 (Malehmir et al., 2019) reports, that KCs have a crucial role in the pathogenesis of NASH, but  
2507 activation of monocytes and myeloid-derived macrophages correlates with disease  
2508 progression. Data presented in **Extended Data 8 and 11** cannot exclude KC-dependent  
2509 mechanisms, however, they seem to have a minor role, especially concerning the co-submitted  
2510 manuscript Dudek et al. in which CD8+ cells drive pathogenesis in KC-independent ways.

2511 We have further performed analyses on how KC correlate with varying degrees of inflammation  
2512 induced by our antibody treatments (anti-CD8, anti-CD8/anti-NK1.1, anti-CD8/anti-PD1, anti-  
2513 PD1, anti-PDL1, anti-TNF, anti-TNF/anti-PD1, and as control experiment anti-CD4 and anti-  
2514 CD4/anti-PD1) by our AI-based analysis approach (included in **Figure 1, Extended Data 4,**  
2515 **20-24** and **Rebuttal Figure 56, 57, 64, 69, 70**). Under baseline conditions (12 months CD-  
2516 HFD-fed animals receiving no treatments) KC abundance does not correlate with any  
2517 serological or histological marker, but KC activation (measured by MHCII+ polarization)  
2518 correlates strongly with tumor size and IL-21 (included in **Extended Data 4** and **Rebuttal**  
2519 **Figure 69**). However, when applying treatments (e.g. PD-1-targeted immunotherapy) KC  
2520 correlates with treatments as well as activation of hepatic KC (measured by MHCII+) correlate  
2521 positively with CD8+PD-1+ (%CD8), Sirius Red staining, tumor incidence, tumor number,  
2522 tumor size, and IL-21 (included in **Extended Figure 24** and **Rebuttal Figure 70**).

2523 In summary, we believe in line with our own study (Malehmir et al., 2019) and recent literature  
2524 (Remmerie et al., 2020) that Kupffer cells are an important cell type on whose basis not  
2525 inflammatory pathologies are initiated and maintained, but also in end-stage disease fresh  
2526 KC/KC-like cells (attracted by cytokines e.g. MCP-1, CCL3, MIP-2 (included in **Extended 2,**

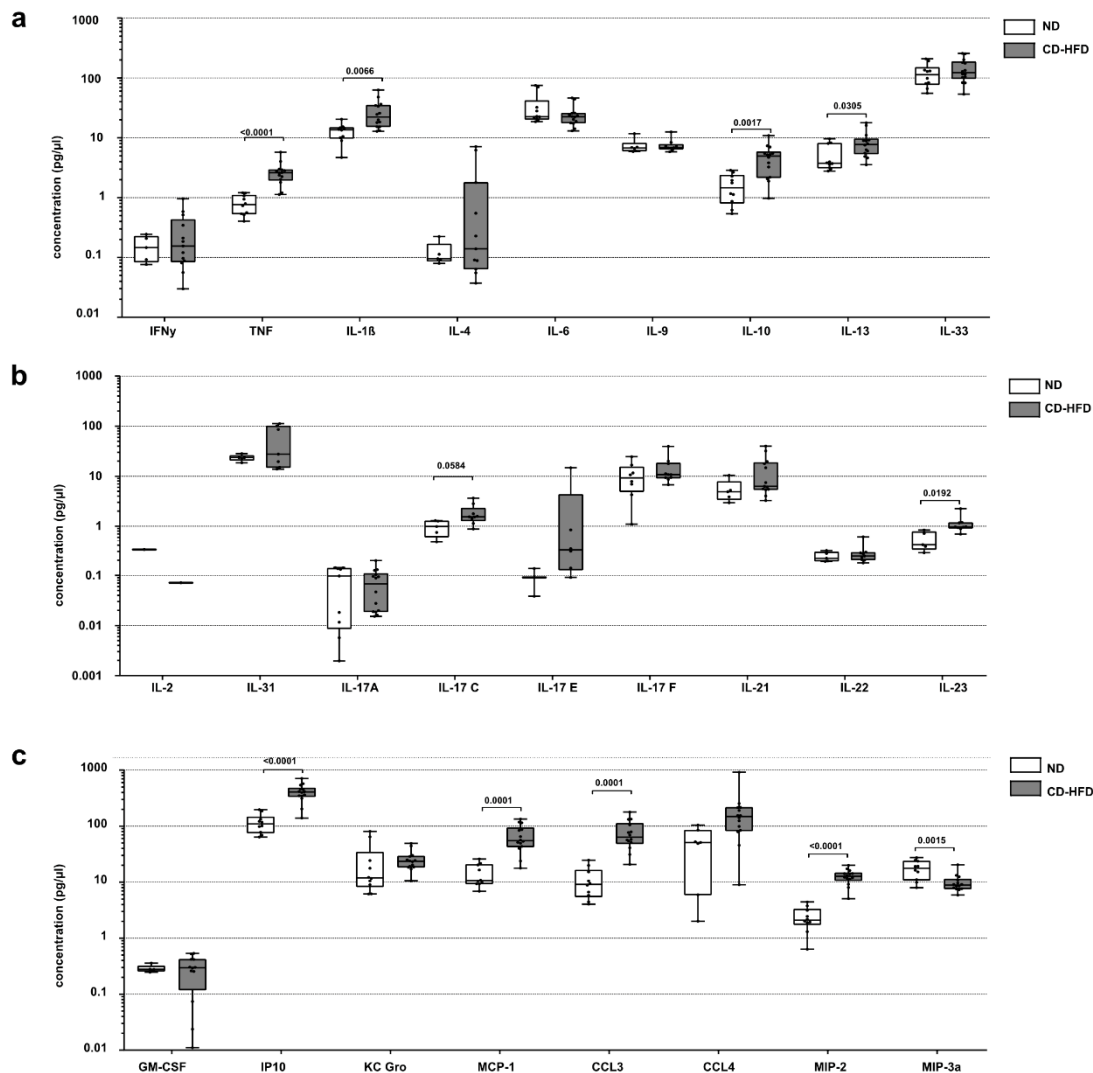
2527 **13, 21 and 23 and Rebuttal Figure 57, 65, 71, 72)** activation might be detrimental as indicated  
 2528 by our correlation analysis. – laying the ground for adaptive immune cell reactions.



2529  
 2530  
 2531

2532 **Rebuttal Figure 71**

2533 (a) Quantification of hepatic immune cell composition and (b) CD8+PD-1+TNF+ T-cells by flow  
 2534 cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  
 2535  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (Hepatic immune cell composition: ND n= 8 mice; CD-HFD  
 2536 n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -  
 2537 CD4 n= 8 mice; CD8+PD-1+TNF+: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n=  
 2538 3 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice). (c) and (d) multiplex  
 2539 ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12 months ND,  
 2540 CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4  
 2541 antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 13 mice; CD-HFD +  
 2542  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).  
 2543


 2544 **Rebuttal Figure 72**  
 2545

2546 (a) and (b) multiplex ELISA of hepatic inflammation associated cytokines and (c) chemokines  
 2547 of 12 months ND or CD-HFD-fed mice (ND n= 10 mice; CD-HFD n= 14 mice). All data are  
 2548 shown as mean  $\pm$  SEM. All data were analyzed by two-tailed Student t test.  
 2549



2550 6. Obesity and response to PD-1 associations have been reported (PMID: 30420753 and  
2551 PMID: 30813970). According to these studies, obesity relates to T-cell dysfunction that PD-1  
2552 blockade derepresses and results in better responsiveness. The models of NASH should suffer  
2553 overweight as well as perhaps the patients in the reported series. This point should be  
2554 addressed if possible and at least discussed. Authors may gain insight with their comparisons  
2555 of the models with and without choline in the diet. As a potential consequence, would it be the  
2556 case that in HCC patients, obese patients respond worse to treatment contrary to other  
2557 indications? Of clinical note, advanced HCC patients frequently experience cachexia but  
2558 perhaps less frequently so those with presumed or documented NASH etiology.

2559

2560 We thank Referee #3 for highlighting these important studies of checkpoint inhibition in the  
2561 frame of obese cancer patients. (Wang et al., 2018) shows - similar to our study - convincingly  
2562 that increased PD-1 expression is a hallmark of diet-induced obesity, thus we cite the study in  
2563 our introduction and improved cross-referencing in our discussion. Potential differences in the  
2564 outcome of PD-1-targeted immunotherapy might be a consequence of the use of obesity-but,  
2565 not NASH-inducing high-fat diet, which we show is crucial to induce hallmarks of NASH by  
2566 comparing HFD with CD-HFD in **Extended Data 1**. Moreover, we would like to draw attention  
2567 to the different cancer entities, which potentially affect immunotherapy-responsiveness. Wang  
2568 et al. use subcutaneous tumor models of lung carcinoma (3LL) and melanoma (B16-F0), but  
2569 not spontaneous developed liver cancer in a chronic inflammatory metabolically challenged  
2570 hepatic microenvironment. Notably, obese animals have bigger tumor-volumes and anti-PD-1  
2571 reactive animals do not control tumor-volume to a smaller absolute tumor-volume compared  
2572 to non-obese controls (Figures 2 and 4 in (Wang et al., 2018)).

2573 The second study of (Cortellini et al., 2019) corroborates the preclinical data of (Wang et al.,  
2574 2018) nicely in lung-, renal-carcinoma, or melanoma patients, but not liver cancer. No grading  
2575 of obese patients was performed (e.g. we report in Supplementary Table 1: healthy/control  
2576 liver, NAFLD/NASH), which we show in **Figure 5** is crucial for hepatic CD8 and PD-1  
2577 abundance. Supporting our manuscript, (Cortellini et al., 2019) report significantly more  
2578 likelihood of obese patients experiencing immune-related-Adverse-Effects (irAEs) “compared  
2579 to non-overweight patients (55.6% vs. 25.2%,  $p < 0.0001$ )”. Unfortunately, no subgroup  
2580 analyses about differences of hepatic irAEs between obese/non-obese patients are shown.

2581 We included the study of (Cortellini et al., 2019) in our introduction and discussion.

2582 Our NAFLD/NASH cohort without immunotherapy treatment indicate a correlation of BMI with  
2583 CD8+PD-1+ T-cells (included in **Figure 5** and **Rebuttal Figure 58**). In our conducted meta-  
2584 analysis, no BMIs were reported, thus statements about treatment response remain

2585 hypothetical. Furthermore, our retrospective HCC-patient cohort under PD(L)1 immunotherapy  
2586 was too small for subgroup analysis, however, there was no significant difference in BMI  
2587 between NAFLD/NASH-HCC and other etiologies-HCC patients, indicative of obesity (included  
2588 in **Supplementary Table 7**).

2589

2590 7. The retrospective series of patients with advanced HCC treated cannot be considered  
2591 conclusive at this point and only hypothesis-generating. The wording there needs to be  
2592 carefully down-toned.

2593

2594 We agree with Referee #3, that the presented retrospective PD-(L)1 targeted immunotherapy  
2595 treated NAFLD/NASH-associated HCC cohort – although unique for Europe and treatment not  
2596 officially licensed and thus reimbursement - is still small, although we would like to point out,  
2597 that prominent trends or effects can be seen in small retrospective cohorts as well.

2598 Thus, our analyses of BCLC-C NAFLD/NASH-HCC vs other-etiological-HCC patients  
2599 indicated, that NAFLD/NASH-HCC has significantly reduced overall survival compared to  
2600 other-etiological-HCC in this small retrospective cohort, which we validated in a second cohort  
2601 of 118 HCC patients under immunotherapy (included in **Figure 6** and **Rebuttal Figure 66**). Of  
2602 note, multivariate analyses identified NAFLD/NASH as an independent factor for treatment  
2603 response and thus identifying NAFLD/NASH as a negative predictor for HCC immunotherapy  
2604 (included in **Supplementary Table 9** and **Rebuttal Figure 66**).

2605 We corroborated our hypothesis of non-viral (NASH-related) HCC being less responsive to  
2606 immunotherapy by a meta-analysis including 1656 patients of the three most important clinical  
2607 trials (IMbrave 150; Checkmate 459; Keynote 240), identifying immunotherapy vs control for  
2608 viral HCC as favorable treatment ( $HR(viral) = 0.64$ ), in contrast, non-viral-HCC showed less  
2609 benefit ( $HR(non-viral) = 0.92$ ) for immunotherapy (included in **Figure 6**, **Extended Data 30-32**,  
2610 **Supplementary Table 7** and **Rebuttal Figure 67-68**).

2611 Based on these data we want to point out that it is - as indicated by Referee#3 - of the highest  
2612 importance to us to specifically define/tone down appropriately the message of our manuscript:  
2613 Our manuscript does not indicate that immunotherapy is not beneficial for HCC patients at all.  
2614 Our manuscript rather demonstrates that HCC patients with viral etiologies do respond well  
2615 and achieve survival benefits - however, that patients with non-viral etiologies (e.g. NASH) do  
2616 not achieve a significant outcome benefit.

2617 We thus propose to stratify HCC patients who are very likely to profit from immunotherapy and  
2618 strengthen the argumentation to use immunotherapy in specific cohorts of HCC patients. We

2619 agree with Referee#1 that this information needs to be articulated in the paper appropriately  
 2620 not to deliver wrong messages but to be very specific.

2621 We truly believe that these are important clinical data, also providing the basis to test our  
 2622 hypotheses in prospective studies on non-significantly beneficial effects in terms of OS for  
 2623 immunotherapy in HCC patients with non-viral and NAFLD/NASH etiology, in particular.

2624 Moreover, we toned down the conclusions of our retrospective cohort in the manuscript and  
 2625 would like to point out, that bigger cohorts and prospective clinical trials are of utmost  
 2626 importance for the scientific community.

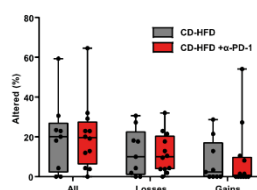
2627

2628 8. An important message of this paper is that progression following PD-(L)1 treatment in NASH  
 2629 patients could be the development of a second primary malignancy rather than from the same  
 2630 one. Can this point be addressed in the models? Is multifocal cancer more common in those  
 2631 cases? The more CD8 pathogenic T-cells in the infiltrate, the more multifocal the tumors?  
 2632

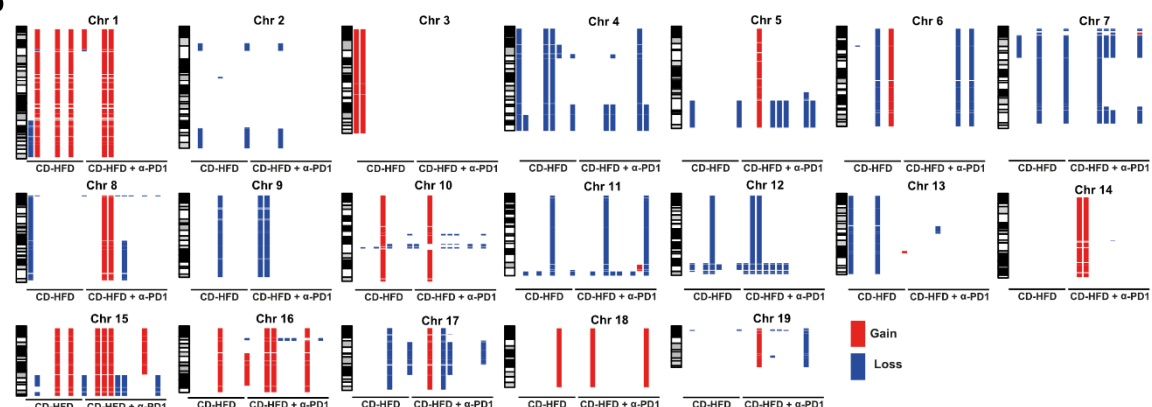
2633 We thank Referee #3 for asking this important question. In our opinion dissection of  
 2634 primary/second primary malignancy is overstepping the limitation of the preclinical model,  
 2635 indicated by the variability of immunohistochemical staining and by the similarity of genomic  
 2636 aberrations (included in **Extended Data 16** and **Rebuttal Figure 73**).

2637 We further have performed correlation analyses (e.g. CD8, PD-1, PD-L1, NAS, fibrosis, liver  
 2638 damage, tumor size, and tumor load) to allow readers a more detailed description of the  
 2639 presented data (included in **Figure 1**, **Extended Data 4+24** and **Rebuttal Figure 69, 70**).

**a**



**b**



2640



2641 **Rebuttal Figure 73**

2642 (a) Quantification of genomic aberrations (b) by array comparative genomic hybridization  
2643 (aCGH) of tumor tissues of mice after 12 months on CD-HFD (n= 9) or 12 months on CD-HFD-  
2644 fed mice + 8 weeks treatment with  $\alpha$ -PD-1 (n= 12).

2645

2646 9. The companion back to back paper shows more data on the physiology of the pathogenic  
2647 CD8 T-cells that I would otherwise ask to this article. Therefore, proper cross-reference of  
2648 those findings is needed at least in discussion.

2649

2650 We thank Referee #3 for highlighting the importance of the co-submitted paper Dudek et al.

2651 and therefore, we improved cross-referencing in the discussion.

2652 **Referee #4 (Remarks to the Author):**

2653 This is an interesting and quite original study of the role of immunity in promoting liver cancer.  
2654 There are data from the mouse models presented which show that CD8+ T-cells can contribute  
2655 to the pathology of NASH and the risk of cancers. The implication is that checkpoint blockade  
2656 which can accentuate the function of CD8 populations can worsen disease. There are also  
2657 some human data which are fairly consistent with this idea. It is perhaps not surprising that  
2658 checkpoint inhibition might worsen an inflammatory condition, although inducing a cancer risk  
2659 is very interesting.

2660 Overall the authors do a very good job in describing the cellular responses and the impact of  
2661 depletion/blockade. There seemed to be a bit of a gap around defining the mechanisms in  
2662 terms of how the CD8+ T-cell population induced cancer. Also it was somewhat unclear what  
2663 the specificity of these T-cells was and what was triggering their initial responsiveness in  
2664 NASH. So although a strong case is made for the pro-tumor role the actual pathways to  
2665 disease were less concrete.

2666

2667 [We thank Referee #4 for appreciating our study's originality in shedding new light on the role](#)  
2668 [of immunity promoting liver cancer, with fairly consistent human data correlating with the](#)  
2669 [findings in the preclinical model.](#)

2670 [We thank Referee #4 for pointing out the limitations of our study which has helped us to](#)  
2671 [increase the quality of our manuscript and address the respective points. We would like to](#)  
2672 [address the concerns of Referee #4 in the following section point-by-point by newly performed](#)  
2673 [experiments \(addressing all questions raised in full\), re-phrasing, re-analysis of the underlying](#)  
2674 [data-sets and would like to draw attention to the improved cross-referencing to the co-](#)  
2675 [submitted manuscript Dudek et al., which dissect the molecular and cellular mechanism of](#)  
2676 [CD8+ T-cell dependent pathogenesis in NASH.](#)

2677

2678 Figure 1: There do not appear to be any iNKT-cells in the UMAP or tise plots – these are  
2679 discussed latter in the text. That seems a little surprising as they are quite dominant in the  
2680 mouse liver and have a clear transcriptional profile. Could the authors clarify where these cells  
2681 lie. It would be also useful to know whether other unconventional cell subsets including GD T-  
2682 cells and MAIT-cells are incorporated in this, although they are likely much rarer. The latter  
2683 may be relevant even if rare as they have been linked to liver fibrosis. The same questions  
2684 would also apply to the scRNAseq of the human samples.

2685



Research for a Life without Cancer

2686 We thank Referee #4 for raising this important point. We have now dissected mouse NK1.1+  
2687 cells in the revised version of our manuscript into NK1.1+TCRb+ as NKT and NK1.1+TCRb-  
2688 as NK cells (included in **Figure 1** and **Rebuttal Figure 74**). Similarly, we highlighted NKT-  
2689 cells, MAITs, and  $\gamma\delta$  T-cells in our patient-derived hepatic lymphocytes analysis by flow  
2690 cytometry, newly performed scRNA-seq, and CYTOF analysis (included in **Figure 5**,  
2691 **Extended Data 25-27** and **Rebuttal Figure 74**).

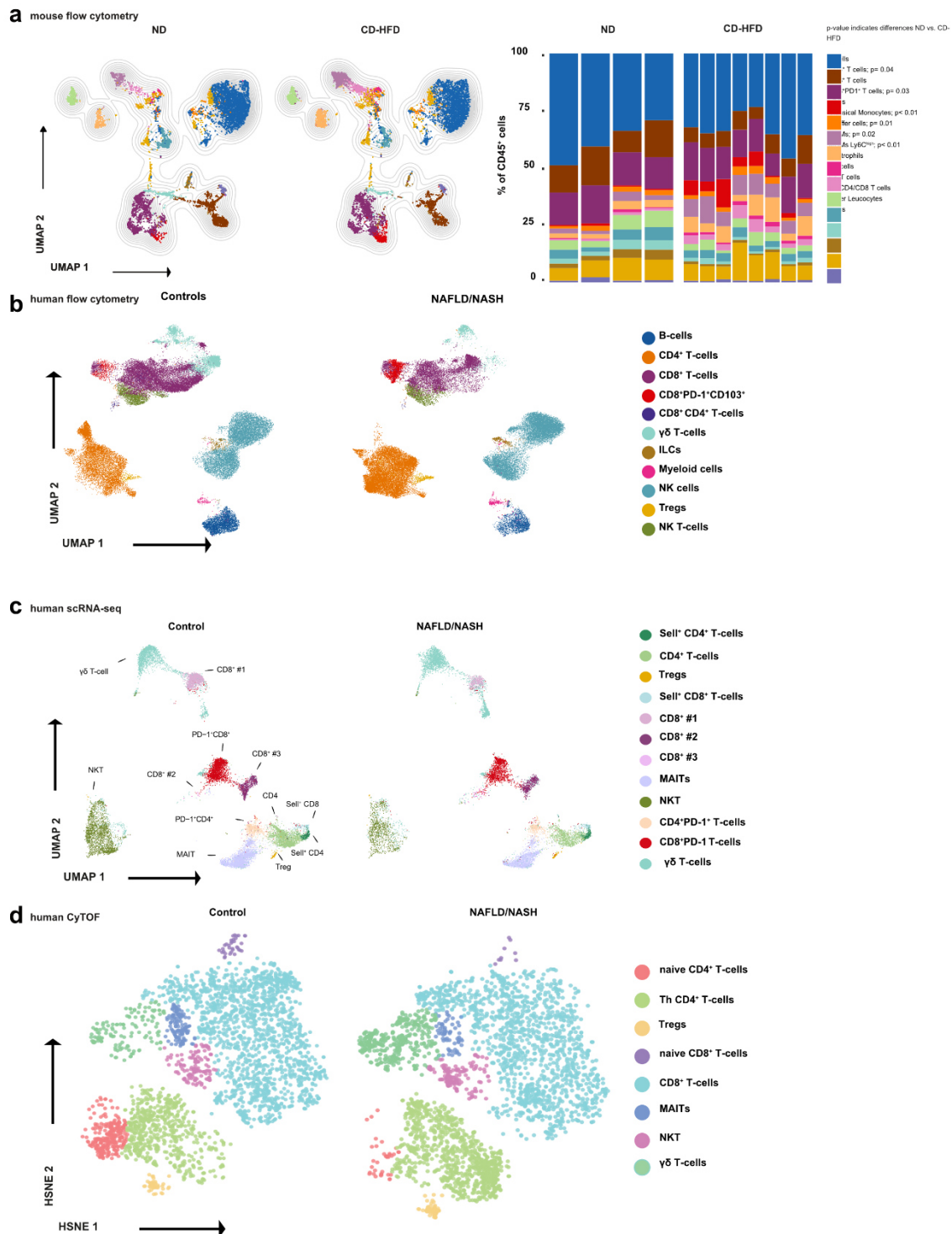
2692 We agree with Referee #4, that MAITs might be important and thus included quantification of  
2693 MAITs in our newly performed scRNA-seq and CYTOF analyses of patient-derived hepatic  
2694 lymphocytes. In these analyses, no change of relative abundance of MAITs was observed  
2695 when comparing control vs. NAFLD/NASH patients. Moreover, we would like to draw attention  
2696 to the co-submitted manuscript Dudek et al., which analyzed - together with us - CD-HFD-fed  
2697 Ja18<sup>-/-</sup> and CD1d<sup>-/-</sup> mice. The latter did not display significant changes in pathology compared  
2698 to CD-HFD-fed control mice at time points of established NASH.

2699 We agree with Referee #4, that  $\gamma\delta$  T-cells may be important, however in our mouse model  
2700 upon NASH establishment, we detected no difference in hepatic abundance of  $\gamma\delta$  T-cells  
2701 between chow or CD-HFD-fed control mice (included in **Extended Data 3**). Furthermore, data  
2702 presented in **Figures 1 and 4** and **Extended Data 3** argue against a major direct contribution  
2703 of  $\gamma\delta$  T-cells in the preclinical model at time points of 6 or 12 months of diet-feeding.

2704 We agree that  $\gamma\delta$  T-cells might be important in the pathogenesis of NASH and NASH to HCC  
2705 transition, however, e.g. rather in collaboration with CD8<sup>+</sup> T cells, also in the context of PD1-  
2706 related immunotherapy.

2707 In humans, our data is not conclusive in all experiments, e.g. our data indicate for  $\gamma\delta$  T-cells, if  
2708 we compare: bulk RNA-seq indicates a reduced expression in severe NASH pathology of  
2709 EOMES, TRDC, and TRGC1 (included in **Extended Data 28** and **Rebuttal Figure 75, 76, 77**),  
2710 however, both flow cytometry cohorts and the scRNA-seq cohort indicate no change of either  
2711  $\gamma\delta$ <sup>+</sup> T-cells or  $\gamma\delta$ <sup>+</sup> Eomes<sup>+</sup> T-cells comparing control vs NAFLD/NASH patients (included in  
2712 **Extended Data 25, 27** and **Rebuttal Figure 75, 76**).

2713 Corroborating the human flow cytometry data in our mouse model upon NASH establishment,  
2714 we detected no difference in hepatic abundance of  $\gamma\delta$  T-cells between chow- or CD-HFD-fed  
2715 control mice. Furthermore, data presented in **Figures 1** and **Extended Data 3** argues against  
2716 the major contribution of  $\gamma\delta$ T-cells in the mouse model of NASH. Here, we did not observe  
2717 significant differences in the “other leukocytes” subset. In the revised manuscript, we analyzed  
2718  $\gamma\delta$ -T-cells separately to strengthen the point, that these cells are not significantly changed upon  
2719 diet feeding (included in **Extended Data 3, 20-23** and **Rebuttal Figure 76a, 78, 79**).



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2721

**Rebuttal Figure 74**

2722

(a) UMAP representation of 5000 randomly chosen CD45+ cells and quantification of hepatic immune cell composition by flow cytometry of 12 months ND or CD-HFD-fed mice (ND n= 4

2723

mice; CD-HFD n= 8 mice). (b) UMAP representation of randomly chosen CD45+ cells and

2724

(b) flow cytometry plots and quantification of CD8+PD-1+CD103+ derived from hepatic

2725

biopsies of control, or NAFLD/NASH patients (Supplementary Table 2: control n= 6 patients;

2726

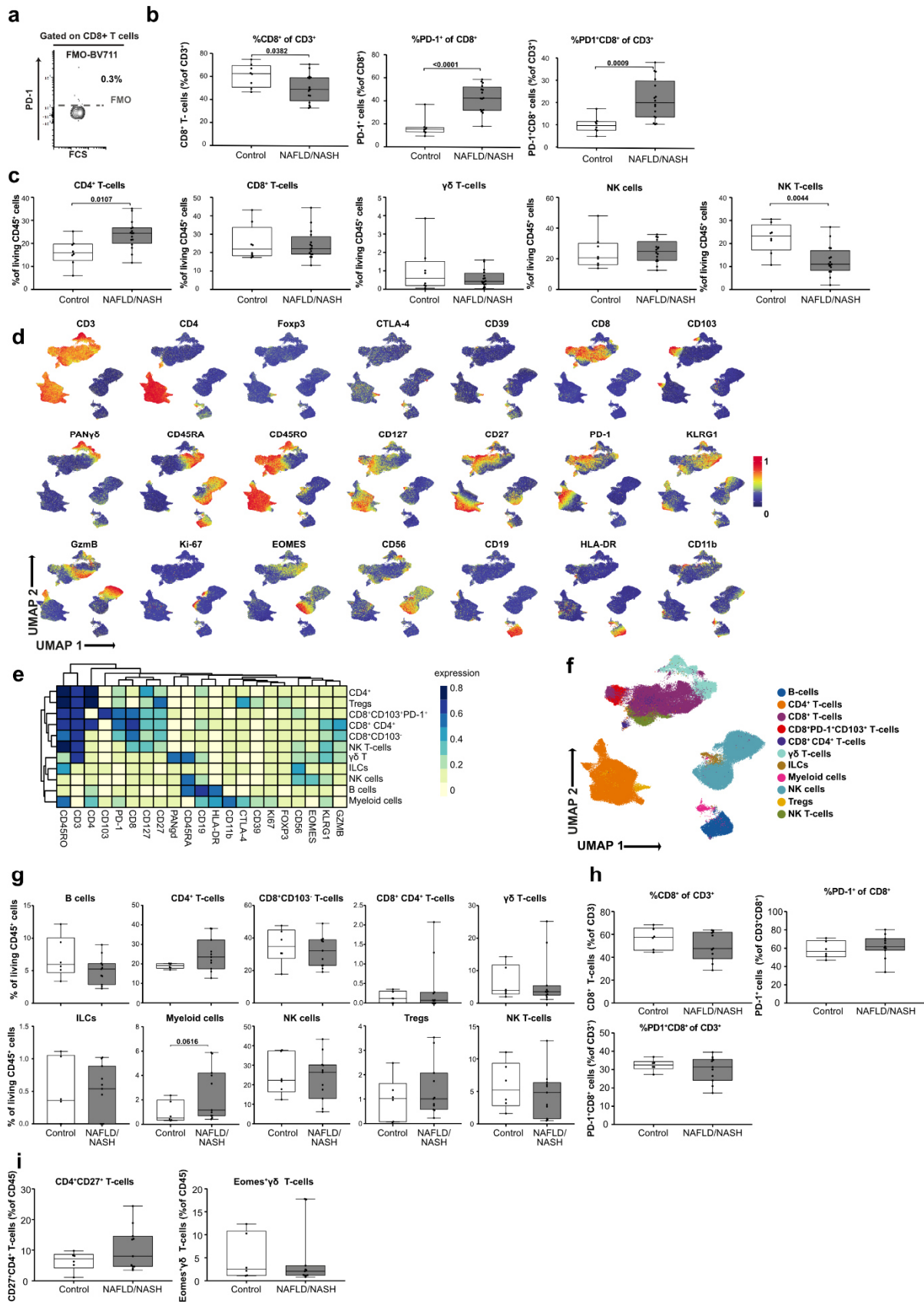
NAFLD/NASH n= 11 patients). (c) UMAP representation of CD3+ cells by scRNA-seq of

2727



2728 control, or NAFLD/NASH patients (control n= 4 patients; NAFLD/NASH n= 7 patients). (d)  
2729 HSNE representation of defined T-cell subsets of patient-liver-derived T-cells analyzed by  
2730 CyTOF of control and NAFLD/NASH patients (control n= 11 patients pooled in 3 analyses;  
2731 NAFLD/NASH n= 16 patients pooled in 5 analyses).  
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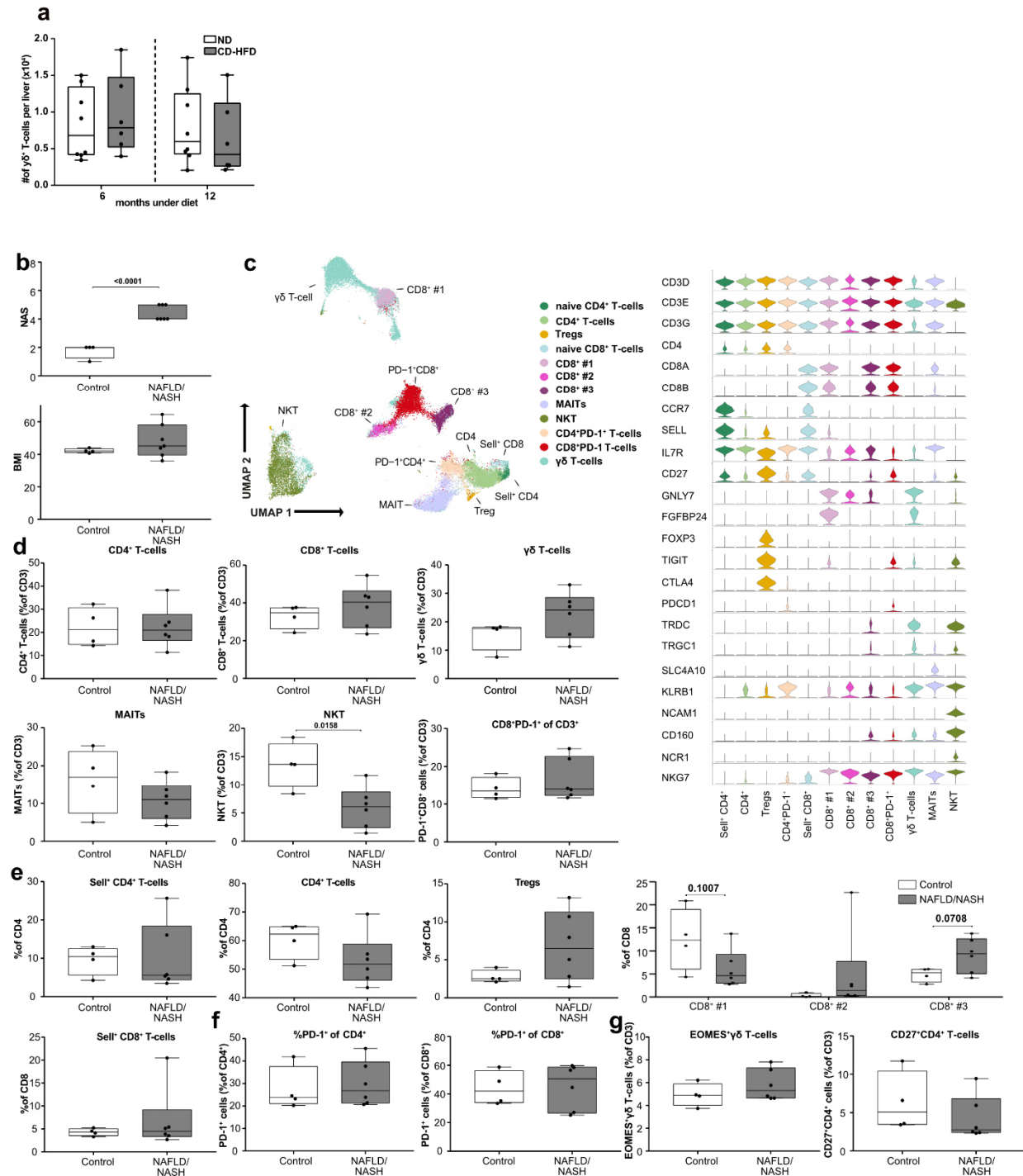




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**2736 Rebuttal Figure 75**

2737 (a) Flow cytometry plot of FMO control, (b) quantification of patient-liver-derived PD-1+CD8+  
2738 T-cells, and (c) quantification of CD4, CD8,  $\gamma\delta$ , NK and NKT cells healthy or NAFLD/NASH  
2739 patients (Supplementary Table 1: healthy n= 8 patients; NAFLD/NASH n= 16 patients). (d)  
2740 Analysis of randomly chosen CD45+ cells and (e) average marker expression of defined  
2741 CD45+ subsets by flow cytometry derived from hepatic biopsies of control and NAFLD/NASH  
2742 patients to define distinct marker expression (Supplementary Table 2: control n= 6 patients;  
2743 NAFLD/NASH n= 11 patients). (f) Definition of cellular subsets, (g) relative quantification of  
2744 defined cellular subsets of randomly chosen CD45+ cells, (h) polarization of CD8+ T-cells and  
2745 (i) quantification of CD4+CD27+, or  $\gamma\delta$  TCR+Eomes+, T-cells by flow cytometry derived from  
2746 hepatic biopsies of healthy and NAFLD/NASH patients (Supplementary Table 2: control n= 6  
2747 patients; NAFLD/NASH n= 11 patients).



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2749

**Rebuttal Figure 76**

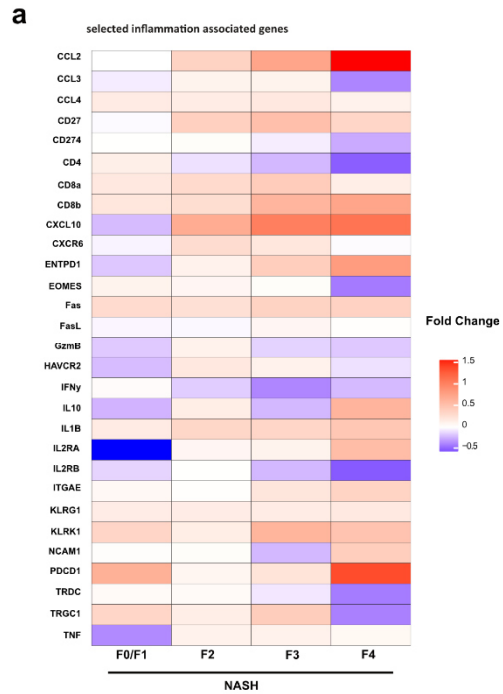
2750 (a) Hepatic abundance of TCR $\gamma\delta$  T-cells of 6 or 12 months ND or CD-HFD fed mice (6 months  
 2751 ND n = 8 mice; CD-HFD n = 6 mice; 12 months ND n = 8 mice; CD-HFD n = 6 mice).

2752 (b) NAS and BMI of patients used for scRNA-seq analyses of patient-liver-derived T-cells of  
 2753 control and NAFLD/NASH patients (control n = 4 patients; NAFLD/NASH n = 7 patients).

2754 (c) UMAP representation, marker expression, (d) relative quantification and (e), (f), (g) polarization  
 2755 of defined T-cell subsets of defined T-cell subsets of patient-liver-derived T-cells by scRNA-  
 2756 seq of control and NAFLD/NASH patients (control n = 4 patients; NAFLD/NASH n = 7 patients).



2757



2758

### Rebuttal Figure 77

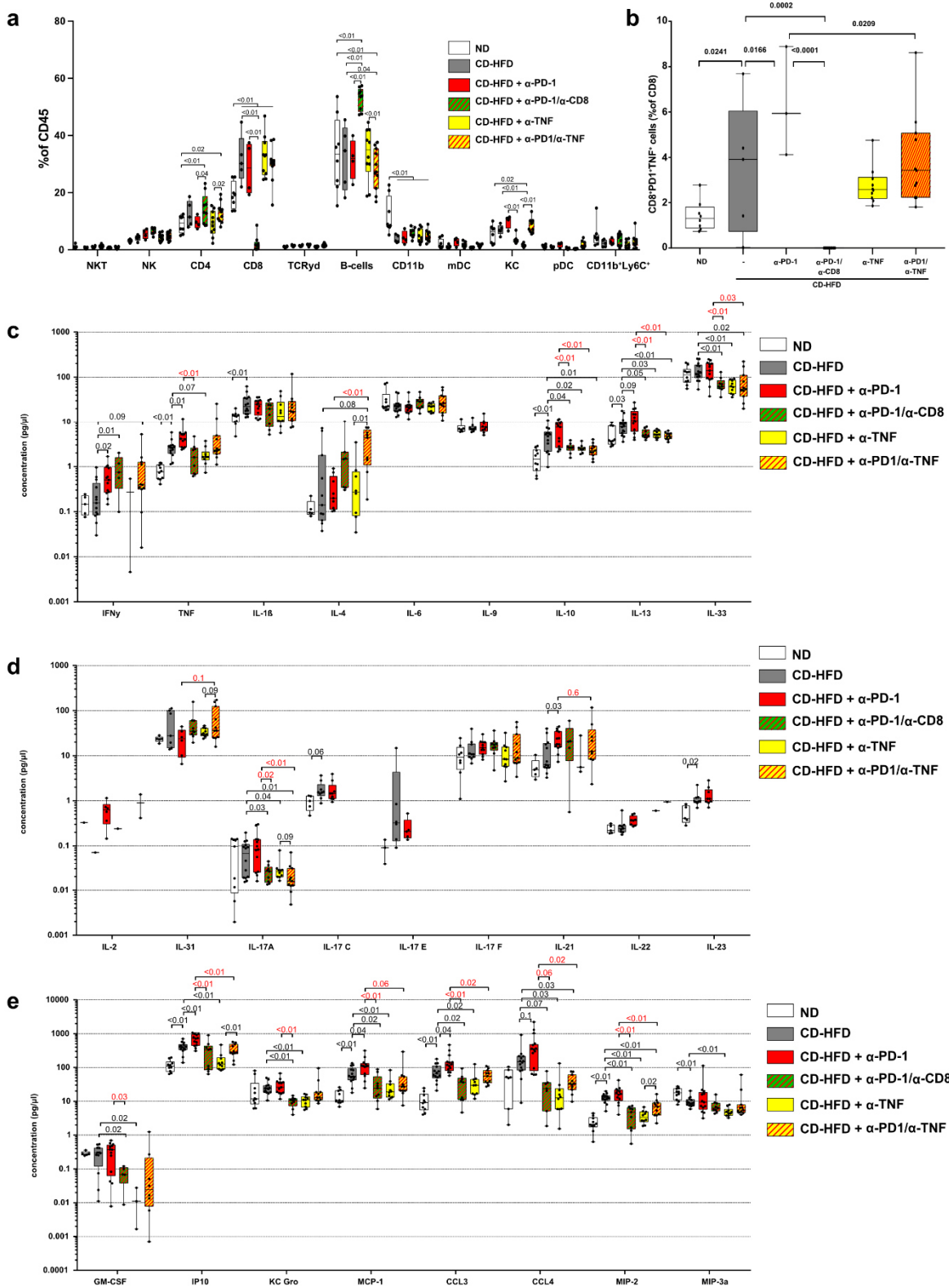
2759

(a) RNA-sequencing data comparing NASH with varying fibrosis (F0 – F4 according to Brunt classification) normalized to NAFLD from a total of n= 206 NAFLD/NASH patients corrected

2760

for batch, gender and center

2761

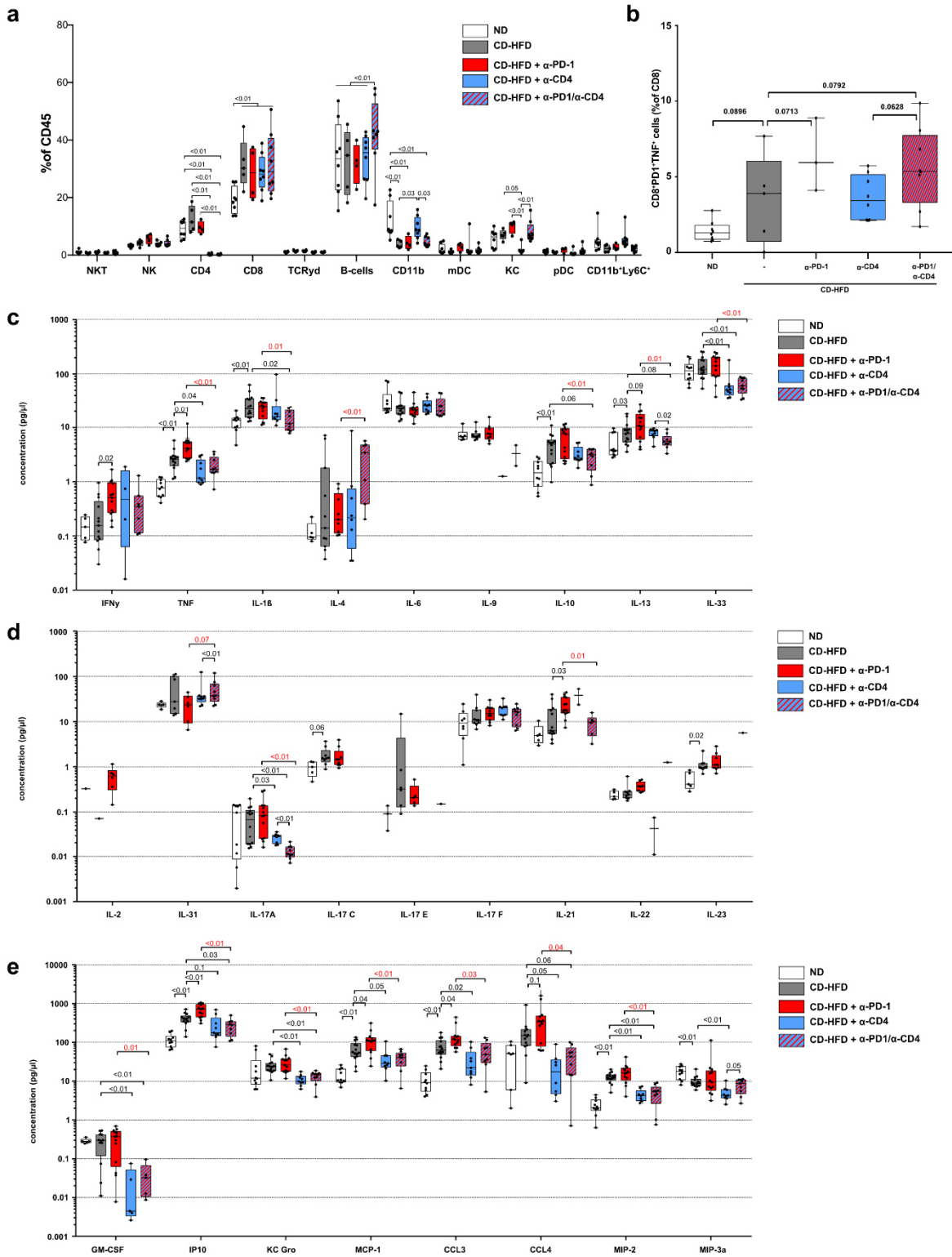


2762 **Rebuttal Figure 78**

2763 (a) Quantification of hepatic immune cell composition and (b) CD8+PD-1+TNF+ T-cells by flow  
 2764 cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  
 2765  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (Hepatic immune cell composition: ND n= 8



2766 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice;  
2767 CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8+PD-1+TNF+: ND  
2768 n= 8 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 3 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
2769 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). (c) and (d)  
2770 multiplex ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12  
2771 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -  
2772 TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n=  
2773 13 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-  
2774 1/ $\alpha$ -TNF n= 11 mice).



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**Rebuttal Figure 79**

(a) Quantification of hepatic immune cell composition and (b) CD8+PD-1+TNF+ T-cells by flow cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-CD4, α-PD-1/α-CD4 antibodies (Hepatic immune cell composition: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD + α-PD-1 n= 4 mice; CD-HFD + α-CD4 n= 8 mice; CD-HFD + α-PD-1/α-CD4 n= 8 mice; CD8+PD-1+TNF+: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD + α-PD-1 n=

2782 3 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice). (c) and (d) multiplex  
2783 ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12 months ND,  
2784 CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4  
2785 antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 13 mice; CD-HFD +  
2786  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).

2787  
2788 Figure 1e: What are the p values on the right referencing? The difference in the PD1+  
2789 population does not appear to be significant. How valid is the PD1+ subset as a subcluster and  
2790 also what are the critical significant differences apart from elevated PD1 expression – some  
2791 justification for this early on would be helpful. Often PD1 expression is more of a gradient (even  
2792 within PD1+ cells) so a binary distinction needs a bit more justification. Does this group of cells  
2793 have distinct TCRs from the non-PD1 (or lower PD1) subset or are they the same population  
2794 with distinct expression? Some data on this would address the question about specificity –  
2795 although this would be better addressed by defining actual TCR-specific (or independent)  
2796 functionality.

2797

2798 We thank Referee #4 for raising important points about **Figure 1**. We have now improved our  
2799 manuscript by clarifying, that the p-values on the right-side reference to abundance in CD-  
2800 HFD-fed mice compared to chow-fed control mice.

2801 We agree with Referee 4, that the CD8+PD-1+ subpopulation was (initially) not significantly  
2802 changed ( $p= 0.09$ ). Upon adding novel data, and re-analysis according to the comment of  
2803 Referee #4, by highlighting NKT cells, CD8+PD1+ ( $p= 0.03$ ) are significantly changed.  
2804 Furthermore, by using AI-based analysis of various parameters displaying our used CD-HFD-  
2805 fed cohorts as a total, we observed that pathology severity correlated with the hepatic  
2806 abundance of CD8+ T-cells and PD1 polarization of these cells (included in **Figure 1 and 4**,  
2807 **Extended Data 4 and 24** and **Rebuttal Figure 80-83**). These analyses indicate, that besides  
2808 changes e.g. in myeloid subsets, CD8+PD1+ cells are a key subset in NASH-diseased mice  
2809 as well as in human patients (see also **Figure 5** and **Rebuttal Figure 84**). To underline the  
2810 importance of a CD8+PD-1+ subset -expressing effector/exhaustion markers correlating with  
2811 disease progression- we have connected the data of **Figure 1** more closely to single-cell RNA-  
2812 seq data presented in **Figure 1** (e.g. the unique transcriptional activity in NASH-derived CD8+  
2813 T-cells (included in **Figure 1** and **Rebuttal Figure 80**) and improved cross-referencing to the  
2814 data co-submitted manuscript Dudek et al. in the discussion.

2815 Furthermore, we have included in the revised manuscript, that we did not observe for CD8+ T-  
2816 cells a sufficient/non-binary gradient of PD-1 expression, allowing dissection into PD-  
2817  $1^{\text{negative}}$ /PD-1 $^{\text{intermediate}}$ /PD-1 $^{\text{high}}$  subsets upon 12 months CD-HFD-feeding, (included in



2818 **Extended Data 3**). Moreover, we functionally show that CD8<sup>+</sup> T-cell are indeed the drivers of  
2819 anti-PD1-related therapy induced liver cancer.

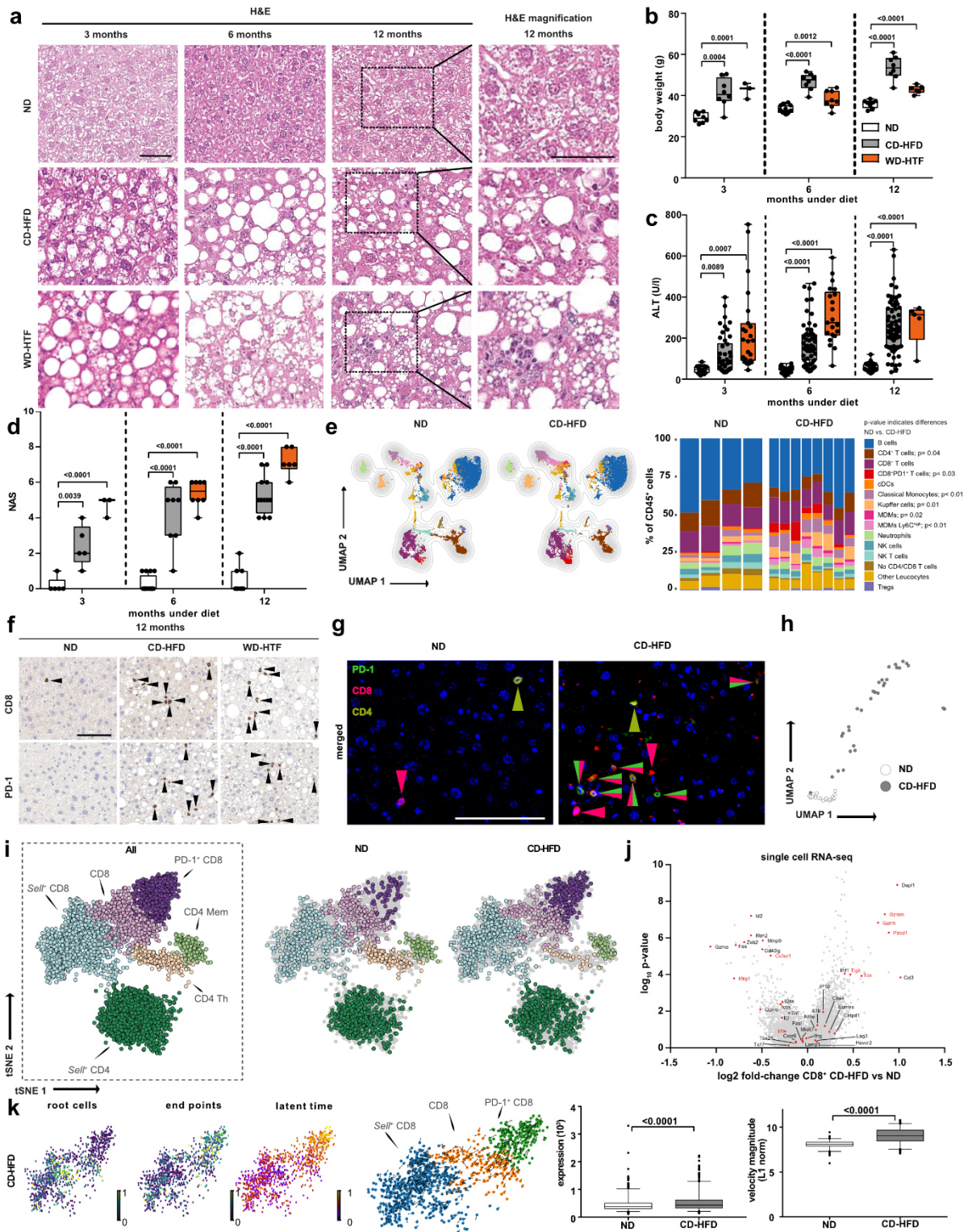
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2821 We thank Referee #4 for pointing out the question about TCR dependency and thus would like  
2822 to draw the attention to the co-submitted manuscript Dudek et al., which describes TCR-  
2823 independent mechanisms on a cellular and molecular level driving CD8<sup>+</sup> T cell-mediated  
2824 hepatocyte cell death. NASH-diet feeding experiments using mice with impaired TCR-  
2825 dependent effector function have been performed in collaboration with Dudek et al.

2826 12-months CD-HFD-fed perforin<sup>-/-</sup> mice developed NASH (including systemic obesity, fibrosis,  
2827 ALT) and NASH-induced hepatocarcinogenesis similar to WT control animals. We have now  
2828 addressed the question on TCR-specificity by improved cross-referencing to the co-submitted  
2829 manuscript Dudek et al.. In fact, it turns out that the effect of CD8<sup>+</sup> T-cells is TCR-effector  
2830 function independent.

2831 Furthermore, we have performed combination therapy of 1) anti-TNF with/without PD-1  
2832 targeted immunotherapy; 2) anti-CD4 with/without PD-1 targeted immunotherapy; 3) anti-CD8  
2833 with PD-1 targeted immunotherapy and 4) PD-L1 targeted immunotherapy, to strengthen  
2834 hypotheses about TCR-independent mechanisms (included in **Figure 4, Extended Data 20-**  
2835 **23 and Rebuttal Figure 78, 79, 81, 83, 85, 86**).

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**Rebuttal Figure 80**

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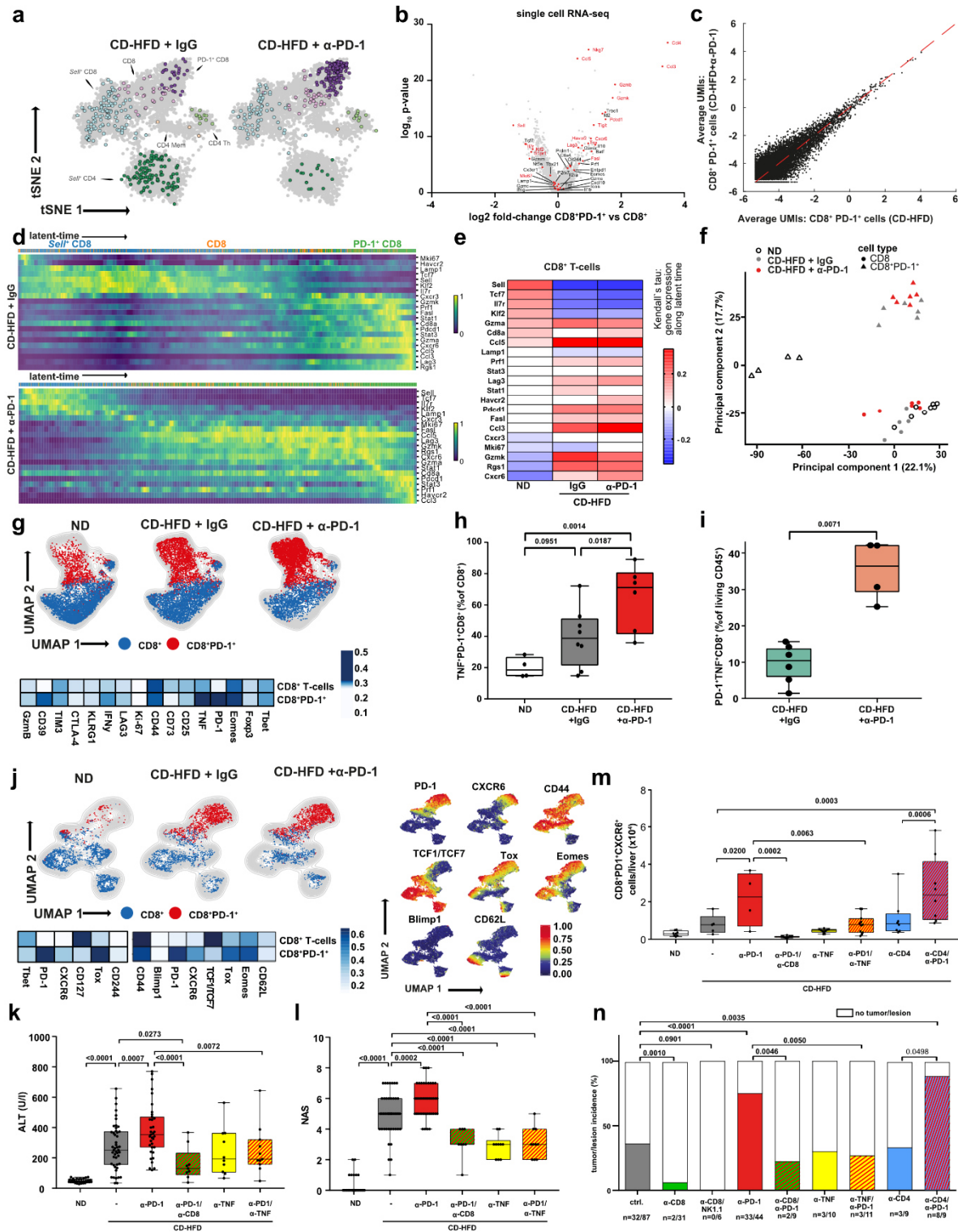
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(a) Histological staining of hepatic tissue by H&E of 3, 6 or 12 months ND, CD-HFD or WD-HTF-fed mice (H&E: 3 months: ND n = 5 mice; CD-HFD n = 5 mice; WD-HTF n = 3 mice; 6 months: ND n = 16 mice; CD-HFD n = 8 mice; WD-HTF n = 8 mice; 12 months: ND n = 9 mice; CD-HFD n = 12 mice; WD-HTF n = 6 mice). Scale bar: 100  $\mu$ m. (b) Body weight of 3, 6 or 12 months ND, CD-HFD or WD-HTF-fed mice (3 months: ND n = 8 mice; CD-HFD n = 8 mice; WD-

2844 HTF n= 3 mice; 6 months: ND n= 14 mice; CD-HFD n= 8 mice; WD-HTF n= 8 mice; 12 months:  
2845 ND n= 8 mice; CD-HFD n= 8 mice; WD-HTF n= 6 mice). (c) ALT levels of 3, 6 or 12 months  
2846 ND, CD-HFD or WD-HTF-fed mice (3 months: ND n= 15 mice; CD-HFD n= 46 mice; WD-HTF  
2847 n= 23 mice; 6 months: ND n= 46 mice; CD-HFD n= 59 mice; WD-HTF n= 21 mice; 12 months:  
2848 ND n= 25 mice; CD-HFD n= 69 mice; WD-HTF n= 5 mice). (d) NAS evaluation by of 3, 6 or 12  
2849 months ND, CD-HFD or WD-HTF-fed mice (3 months: ND n= 5 mice; CD-HFD n= 5 mice; WD-  
2850 HTF n= 3 mice; 6 months: ND n= 16 mice; CD-HFD n= 8 mice; WD-HTF n= 8 mice; 12 months:  
2851 ND n= 9 mice; CD-HFD n= 12 mice; WD-HTF n= 6 mice). (e) UMAP representation showing  
2852 the FlowSOM-guided clustering of randomly chosen CD45+ cells and quantification of hepatic  
2853 immune cell composition by flow cytometry of 12 months ND or CD-HFD-fed mice (ND n= 4  
2854 mice; CD-HFD n= 8 mice). (f) CD8 and PD-1 staining of hepatic tissue by  
2855 immunohistochemistry of 12 months ND, CD-HFD or WD-HTF-fed mice (PD-1: n= 5  
2856 mice/group; CD8: ND n= 6 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice). Scale bar: 100  $\mu$ m.  
2857 (g) Immunofluorescence staining of PD-1, CD8 and CD4 of 12 months ND or CD-HFD-fed  
2858 mice (n= 3 mice/group). Arrowheads indicate CD8+ (red), PD-1+ (green) or CD4+ (ocher) cells.  
2859 Scale bar: 100  $\mu$ m. (h) UMAP representation of 63 parameters (serology, flow cytometry,  
2860 histology) indicating NASH pathology severity measured of 12 months ND or CD-HFD-fed mice  
2861 (ND n= 22 mice; CD-HFD n= 31 mice). (i) tSNE representation of TCR $\beta$ + cells and analyses  
2862 of (j) differential gene expression, (k) RNA velocity indicating transcriptional activity, gene  
2863 expression and the trajectory of CD8+ cells by scRNA-seq of 12 months ND or CD-HFD-fed  
2864 mice (n= 3 mice/group) 53. Root cells: yellow cells indicate root cells, blue cells indicate cells  
2865 farthest away from root by RNA velocity. End points: yellow cells indicate end point cells, blue  
2866 cells indicate cells farthest away from defined end point cells by RNA velocity. Latent time:  
2867 pseudo-time by RNA velocity, dark color indicate start of velocity, yellow color indicate end  
2868 point of latent time. RNA velocity flow: Blue cluster defined as start point, orange cluster as  
2869 intermediate, green cluster as end point. Arrows indicate the trajectory of cells.

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**Rebuttal Figure 81**2872 (a) ScRNA-seq analysis of hepatic TCR $\beta$ <sup>+</sup> cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 or  $\alpha$ -CD8 antibodies (n= 3 mice/group).2873 (b) Selected marker expression in hepatic CD8<sup>+</sup> T-cells by scRNA-seq comparing CD8<sup>+</sup> with CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-12874 antibodies (n= 3 mice/group). (c) Average UMI comparison of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n=

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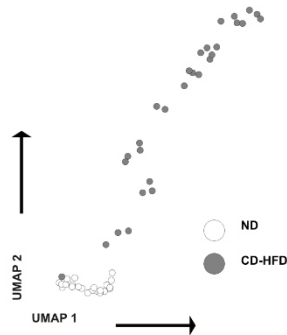
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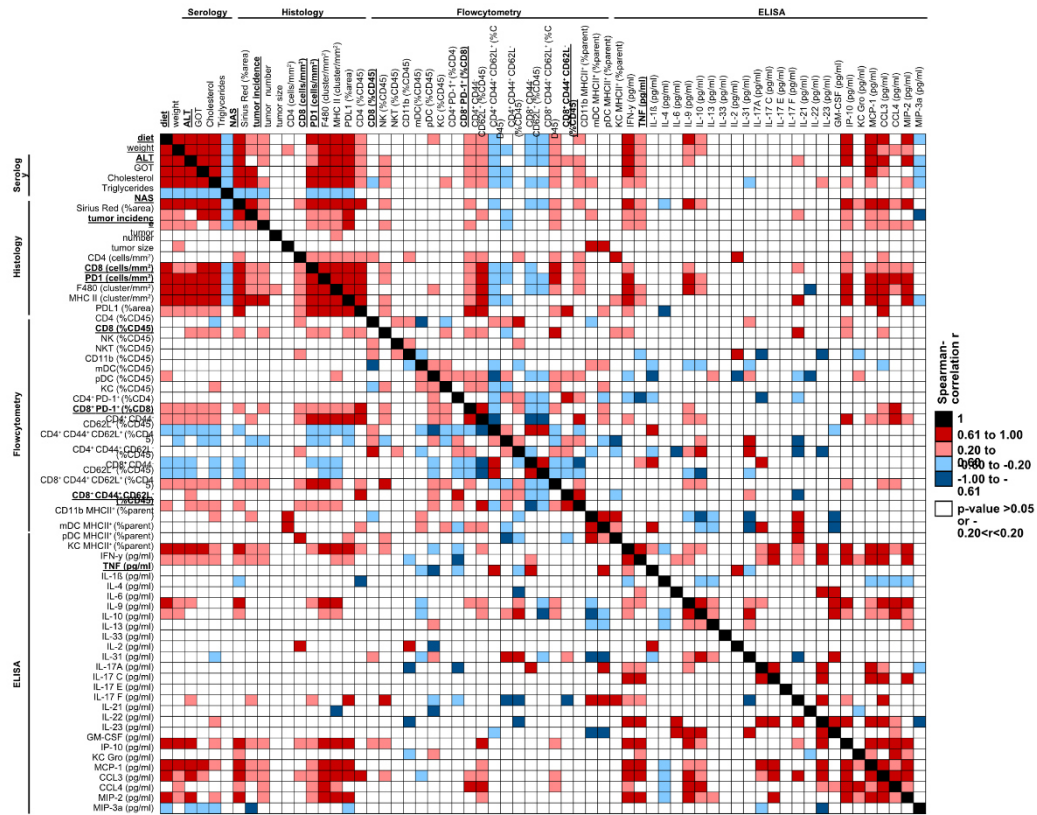
2878 3 mice/group). (d) RNA velocity analyses of scRNA-seq data showing expression and (e)  
2879 correlation of expression along the latent-time of selected genes along the latent-time (n= 3  
2880 mice/group). Root cells: yellow cells indicate root cells, blue cells indicate cells farthest away  
2881 from root by RNA velocity. End points: yellow cells indicate end point cells, blue cells indicate  
2882 cells farthest away from defined end point cells by RNA velocity. Latent time: pseudo-time by  
2883 RNA velocity, dark color indicate start of RNA velocity, yellow color indicate end point of latent  
2884 time. RNA velocity flow: Blue cluster defined as start point, orange cluster as intermediate,  
2885 green cluster as end point. Arrows indicate trajectory of cells. (f) PCA plot of hepatic CD8+ or  
2886 CD8+PD-1+ T-cells sorted TCR $\beta$ + cells by mass spectrometry of 12 months ND, CD-HFD or  
2887 CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD8+: ND n= 6 mice, CD-HFD  
2888 + IgG n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice; CD8+PD-1+: ND n= 4 mice, CD-HFD + IgG n=  
2889 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (g) UMAP representation showing the FlowSOM-guided  
2890 clustering, heatmap showing the median marker expression, and (h) quantification of hepatic  
2891 CD8+ T-cells of 12 months ND, CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  
2892  $\alpha$ -PD-1 antibodies (ND n= 4 mice; CD-HFD + IgG n= 8 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (i)  
2893 Quantification of CellCNN analyzed flow cytometry data of hepatic CD8+ T-cells of 12 months  
2894 CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD +  
2895 IgG n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice). (j) UMAP representation showing the FlowSOM-  
2896 guided clustering, the expression intensity of the indicated marker and heatmap showing the  
2897 median marker expression of flow cytometry data of hepatic CD8+PD-1+ T-cells of 12 months  
2898 ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 6 mice;  
2899 CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). (k) ALT and (l) NAS evaluation of 12 months  
2900 ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF, or  $\alpha$ -  
2901 PD-1/ $\alpha$ -TNF antibodies (ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice;  
2902 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF  
2903 n= 11 mice). (m) Quantification of hepatic CD8+PD-1+CXCR6+ T-cells ND, CD-HFD, CD-  
2904 HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4,  
2905 or  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35  
2906 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-  
2907 1/ $\alpha$ -TNF n= 11 mice); CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice). (n)  
2908 Quantification of tumor incidence of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks  
2909 treatment by  $\alpha$ -CD8,  $\alpha$ -CD8/NK1.1,  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or  
2910  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (tumor incidence: CD-HFD n= 32 tumors/lesions in 87 mice; CD-  
2911 HFD +  $\alpha$ -CD8 n= 2 tumors/lesions in 31 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 0 tumors/lesions in  
2912 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 33 tumors/lesions in 44 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 2  
2913 tumors/lesions in 9 mice; CD-HFD +  $\alpha$ -TNF n= 3 tumors/lesions in 10 mice; CD-HFD +  $\alpha$ -PD-  
2914 1/ $\alpha$ -TNF n= 3 tumors/lesions in 11 mice); CD-HFD +  $\alpha$ -CD4 n= 3 tumors/lesions in 9 mice; CD-  
2915 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 tumors/lesions in 9 mice).

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**a**



**b**



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**Rebuttal Figure 82**

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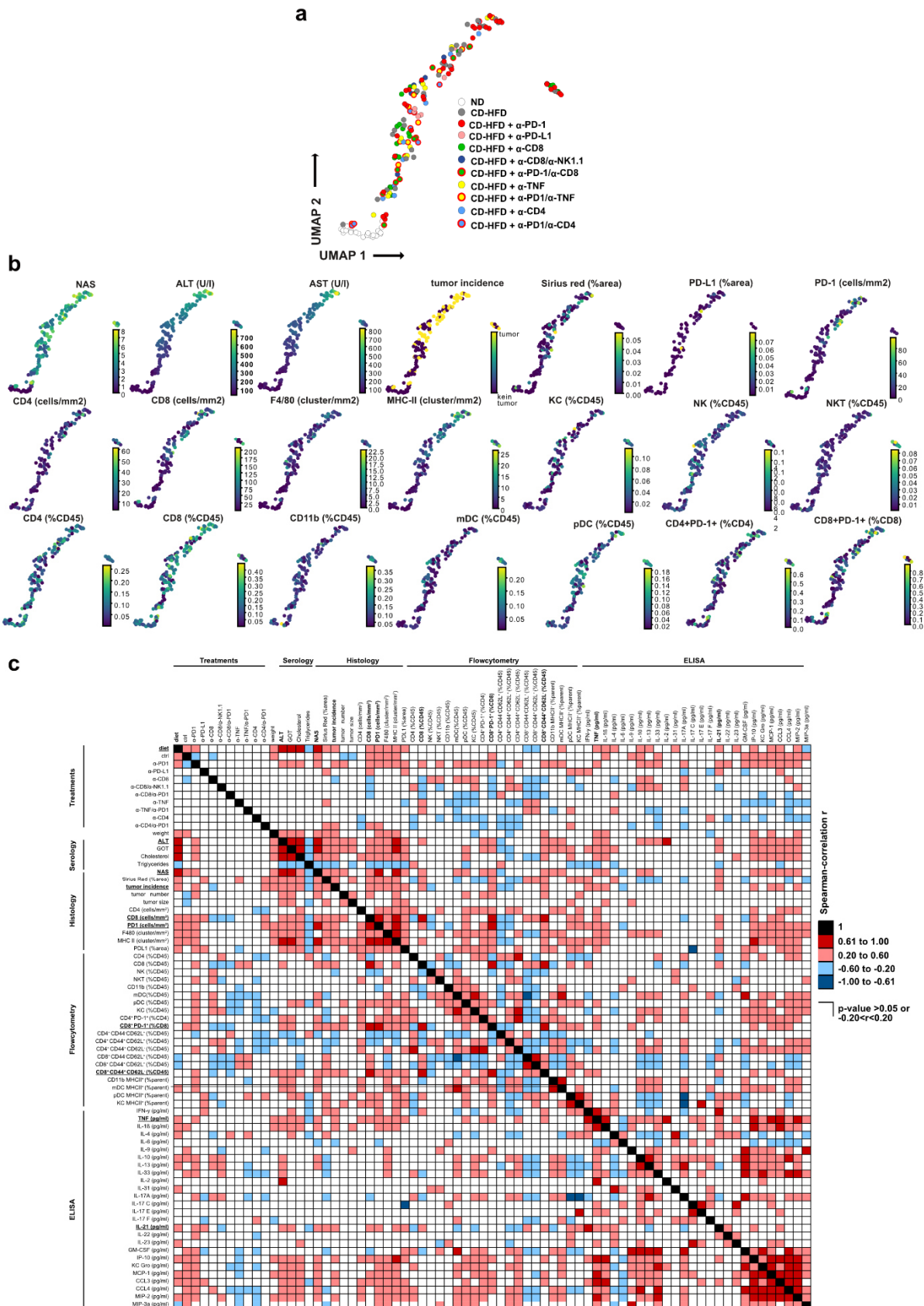
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(a) UMAP representation of 63 parameters (serology, flow cytometry, histology) indicating NASH pathology severity measured of 12 months ND or CD-HFD fed mice (ND n= 22 mice; CD-HFD n= 31 mice). (b) Data gathered from hepatic tissue analyses was binary correlated with each other of 6- or 12-months ND or CD-HFD fed mice (ND n= 47 mice; CD-HFD n= 72 mice).

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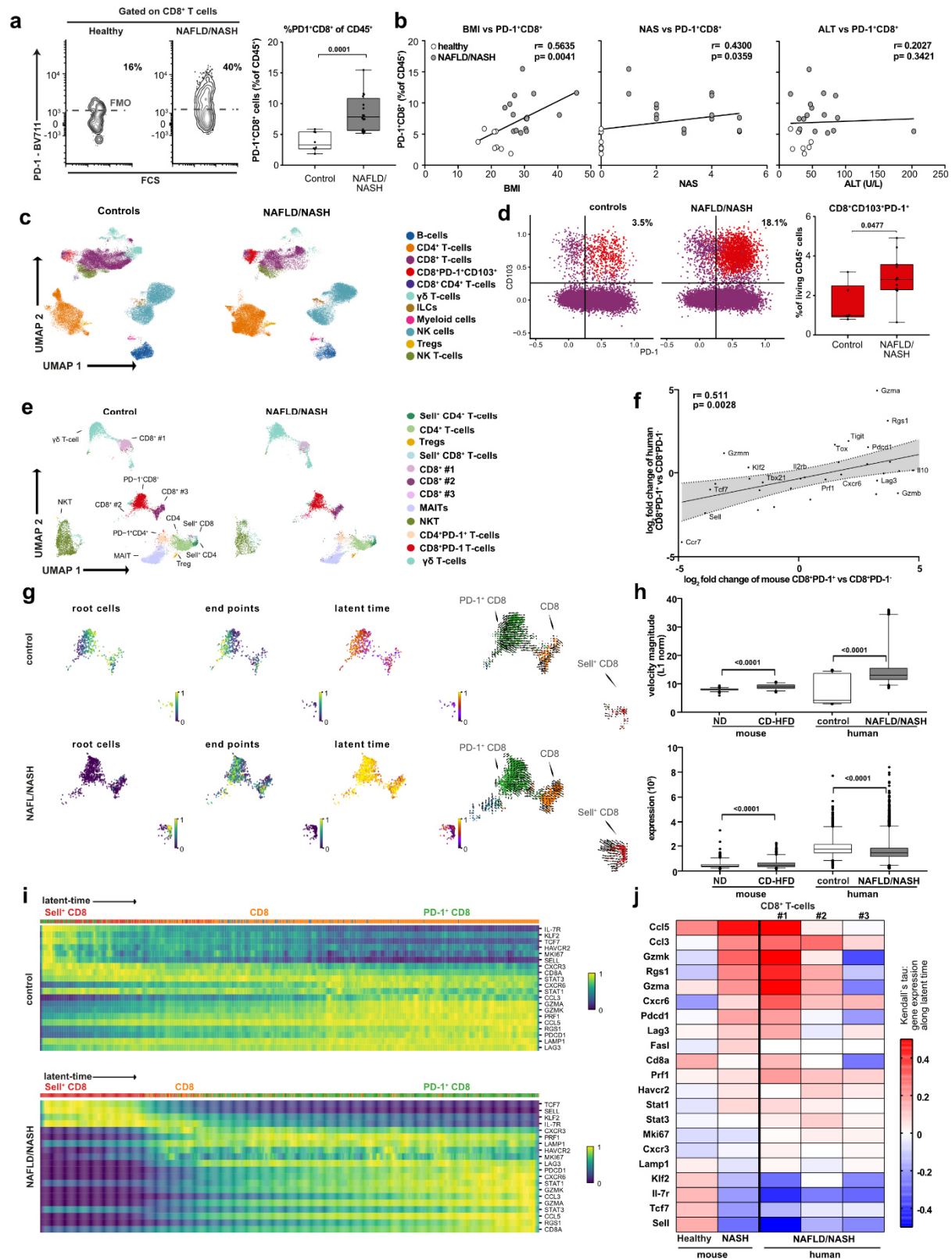
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**2925 Rebuttal Figure 83**

2926 (a) UMAP representation of 63 parameters (serology, flow cytometry, histology) and (b)  
2927 selected display of analyzed parameters indicating NASH pathology severity measured of 12  
2928 months ND or CD-HFD-fed mice (ND n= 22 mice; CD-HFD n= 31 mice; CD-HFD +  $\alpha$ -PD-1 n=  
2929 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; CD-HFD +  $\alpha$ -CD8 n= 24 mice; CD-HFD +  $\alpha$ -  
2930 CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice;  
2931 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4  
2932 n= 9 mice). (c) Data gathered from hepatic tissue analyses was binary correlated with each  
2933 other of 6- or 12-months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -CD8,  $\alpha$ -  
2934 CD8/ $\alpha$ -NK1.1;  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or  $\alpha$ -PD-1/ $\alpha$ -CD4 (ND  
2935 n= 47 mice; CD-HFD n= 72 mice; CD-HFD +  $\alpha$ -PD-1 n= 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6  
2936 mice; CD-HFD +  $\alpha$ -CD8 n= 29 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -  
2937 CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD  
2938 +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).

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**Rebuttal Figure 84**

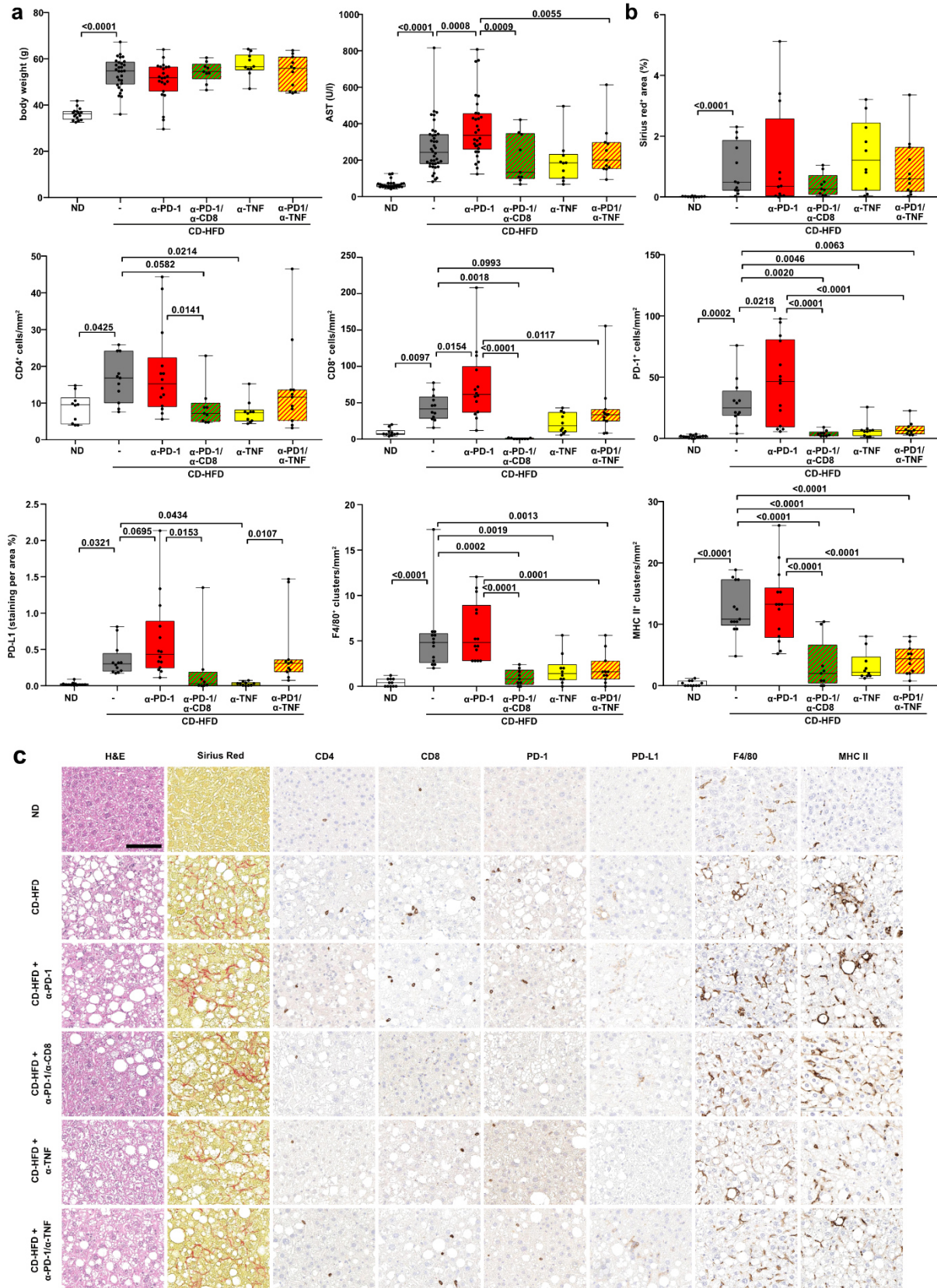
(a) Flow cytometry plots, quantification of patient-liver-derived PD-1+CD8+ T-cells, and (b) correlation of PD-1+CD8+ T-cells with BMI, NAS and ALT of healthy or NAFLD/NASH patients



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2944 (Supplementary Table 1: healthy n= 8 patients; NAFLD/NASH n= 16 patients). Fluorescence-  
2945 minus-one (FMO) defined in Extended Data 25. (c) UMAP representation showing the  
2946 FlowSOM-guided clustering of CD45+ cells and (d) flow cytometry plots and quantification of  
2947 CD8+PD-1+CD103+ derived from hepatic biopsies of control, or NAFLD/NASH patients  
2948 (Supplementary Table 2: control n= 6 patients; NAFLD/NASH n= 11 patients) Populations:  
2949 CD8+ (violet), CD8+PD-1+CD103+ (red). (e) UMAP representation of CD3+ cells and analyses  
2950 of differential gene expression by scRNA-seq of control, or NAFLD/NASH patients (control n=  
2951 4 patients; NAFLD/NASH n= 7 patients). (f) Correlation of significant differentially expressed  
2952 genes in liver-derived CD8+PD-1+ compared to CD8+PD-1- T-cells subsets of 12 months CD-  
2953 HFD-fed mice and NAFLD/NASH patients (mouse: n= 3 mice; human: n= 3 patients). (g)  
2954 Velocity analyses of scRNA-seq data showing (h) expression, transcriptional activity, (i) gene  
2955 expression and (j) correlation of expression along the latent-time of selected genes along the  
2956 latent-time of patient-liver-derived CD8+ T-cells of control, or NAFLD/NASH patients in  
2957 comparison to mouse-liver-derived CD8+ T-cells (patients: NAFLD/NASH n= 3 patients;  
2958 mouse: n= 3 mice/group). Root cells: yellow cells indicate root cells, blue cells indicate cells  
2959 farthest away from the root by RNA velocity. End points: yellow cells indicate end point cells,  
2960 blue cells indicate cells farthest away from defined end point cells by RNA velocity. Latent time:  
2961 pseudo-time by RNA velocity, dark color indicate start of RNA velocity, yellow color indicate  
2962 end point of latent time. RNA velocity flow: Blue cluster defined as start point, orange cluster  
2963 as intermediate, green cluster as end point. Arrows indicate the trajectory of cells.

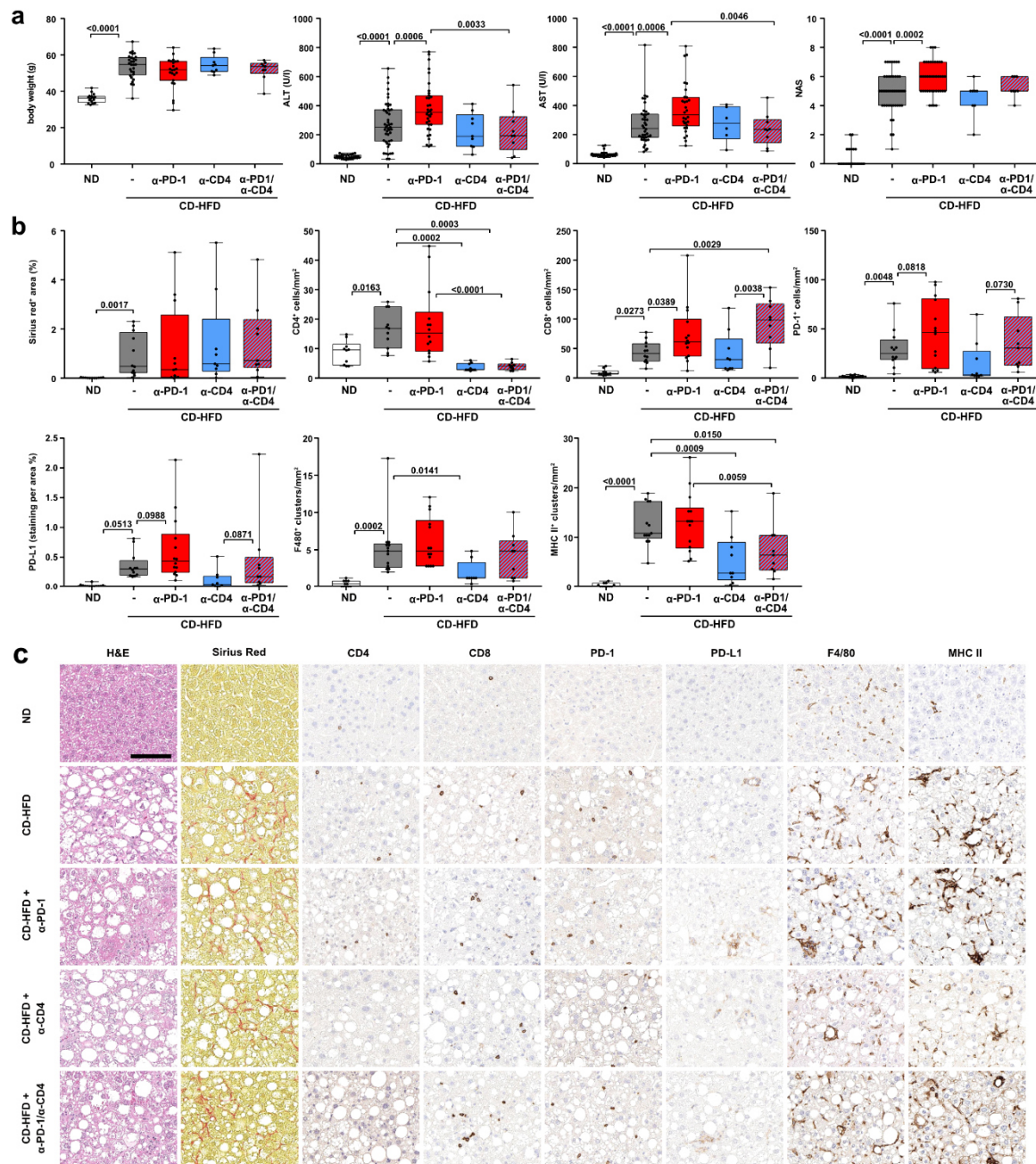
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**2965 Rebuttal Figure 85**

2966 (a) Body weight, AST, and histological evaluation by (b) Sirius red, CD4, CD8, PD-1, PD-L1,  
2967 F4/80, MHC-II and (c) staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  
2968  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF antibodies (body weight: ND n= 16 mice; CD-  
2969 HFD n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-  
2970 HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; AST: body weight: ND n= 30  
2971 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
2972 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; Sirius red: ND n=  
2973 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
2974 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD4: ND n= 10  
2975 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
2976 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8: ND n= 10  
2977 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-  
2978 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n=  
2979 11 mice; PD-1: ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD  
2980 +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 8 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 10  
2981 mice; PD-L1: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  
2982  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice;  
2983 F4/80: ND n= 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-  
2984 1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  
2985  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1  
2986 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  
2987  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). Scale bar: 100  $\mu$ m.

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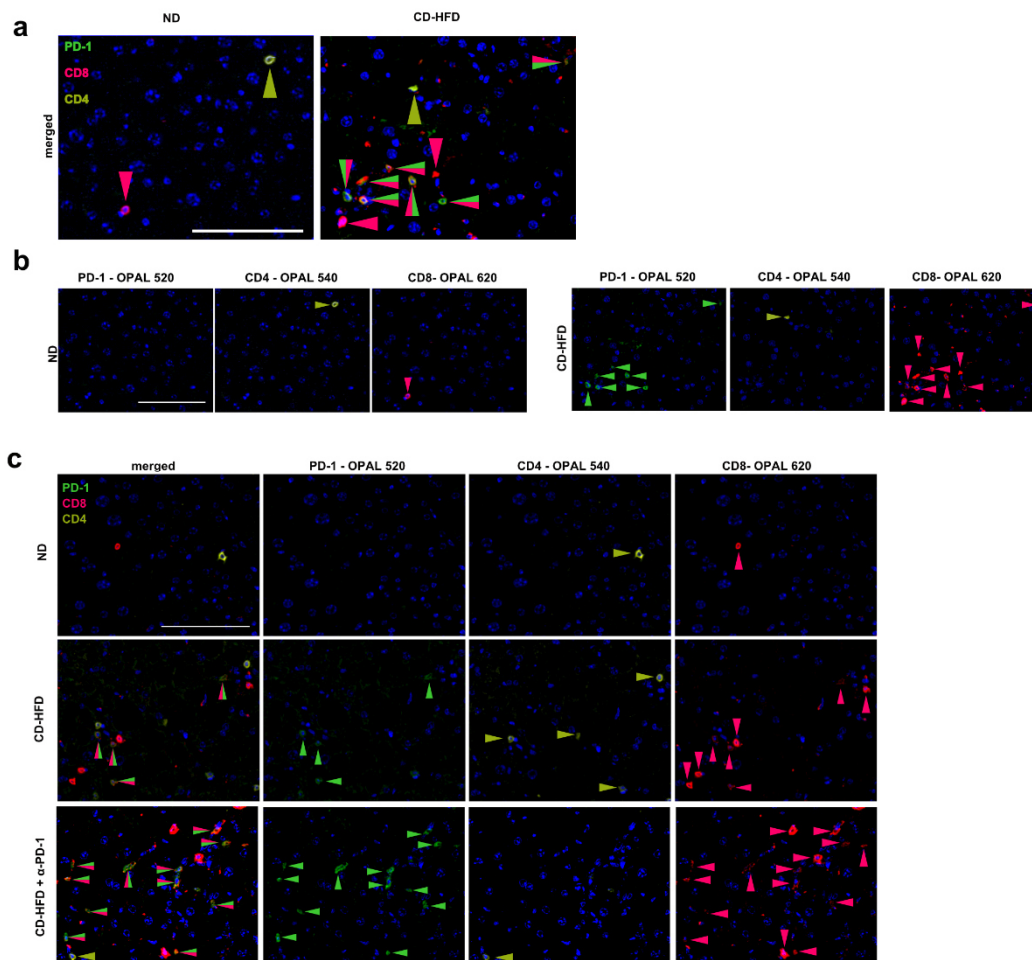


2991 **Rebuttal Figure 86**  
 2992 (a) Body weight, ALT, AST, NAS, and histological evaluation by (b) Sirius Red, CD4, CD8, PD-  
 2993 1, PD-L1, F4/80, MHC-II and (c) staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks  
 2994 treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (body weight: ND n= 16 mice; CD-HFD  
 2995 n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -  
 2996 CD4 n= 9 mice; ALT ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice; CD-  
 2997 HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; AST: ND n= 30 mice; CD-HFD  
 2998 n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -  
 2999 CD4 n= 9 mice; NAS: ND n= 31 mice; CD-HFD n= 46 mice; CD-HFD +  $\alpha$ -PD-1 n= 40 mice;  
 3000 CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice; Sirius red: ND n= 11 mice;  
 3001 CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  
 3002  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; CD4: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n=  
 3003 14 mice; CD-HFD +  $\alpha$ -CD4 n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 11 mice; CD8: ND n= 10

3004 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-  
 3005 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-1: ND n= 13 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -  
 3006 PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-L1:  
 3007 ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9  
 3008 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; F4/80: ND n= 11 mice; CD-HFD n= 13 mice; CD-  
 3009 HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice;  
 3010 MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -  
 3011 PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice). Scale  
 3012 bar: 100  $\mu$ m.  
 3013

3014 Figure 1f: The stains are both single stains. It should be possible to show a double staining  
 3015 CD8+PD1+ population and enumerate them as this seems like the critical part of the study.  
 3016

3017 We thank Referee #4 for pointing that out. We performed an additional double staining  
 3018 corroborating our flow cytometry data in **Figure 1**. In line, we have now included histological  
 3019 double staining in a revised manuscript (included in **Figure 1, Extended Data 3, 12,** and  
 3020 **Rebuttal Figure 87**). These data indicated that PD1+ expression is indeed associated with  
 3021 CD8+ staining.



3022

3023 **Rebuttal Figure 87**

3024 (a) Immunofluorescence staining of PD-1, CD8 and CD4 of 12 months ND or CD-HFD-fed  
3025 mice (n= 3 mice/group). Arrowheads indicate CD8+ (red), PD-1+ (green) or CD4+ (ocher) cells.  
3026 Scale bar: 100  $\mu$ m. (b) Immunofluorescence staining of single channel-staining PD-1, CD8 and  
3027 CD4 (ocher) of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). Arrowheads indicate  
3028 CD8+ (red), PD-1+ (green) or CD4+ (ocher) cells. Scale bar: 100  $\mu$ m. (c) Immunofluorescence  
3029 microscopy of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment of  $\alpha$ -PD-1  
3030 fed mice fed mice (n= 3 mice/group). Scale bar: 100  $\mu$ m.  
3031

3032 Figure 1j: One of the most upregulated genes in the PD1+ subset is IL-10. Do the authors have  
3033 any data on whether this is secreted by this subset. Although the subset is labelled as "PD1+"  
3034 it is not the top upregulated gene here (as above). A side-by-side broader functional study  
3035 would add a bit of resolution here and if they do secrete IL-10 this may impact on the overall  
3036 interpretation. The interpretations about function are all via the screening approaches so some  
3037 further specific back up by FACS/ELISA would be helpful in confirming functionality, especially  
3038 in the context of an "exhausted" phenotype – this would clarify the statement on line 199 about  
3039 "potential effector function". Such an experiment would also be valuable in the anti-PD1 treated  
3040 mice in later parts of the manuscript.

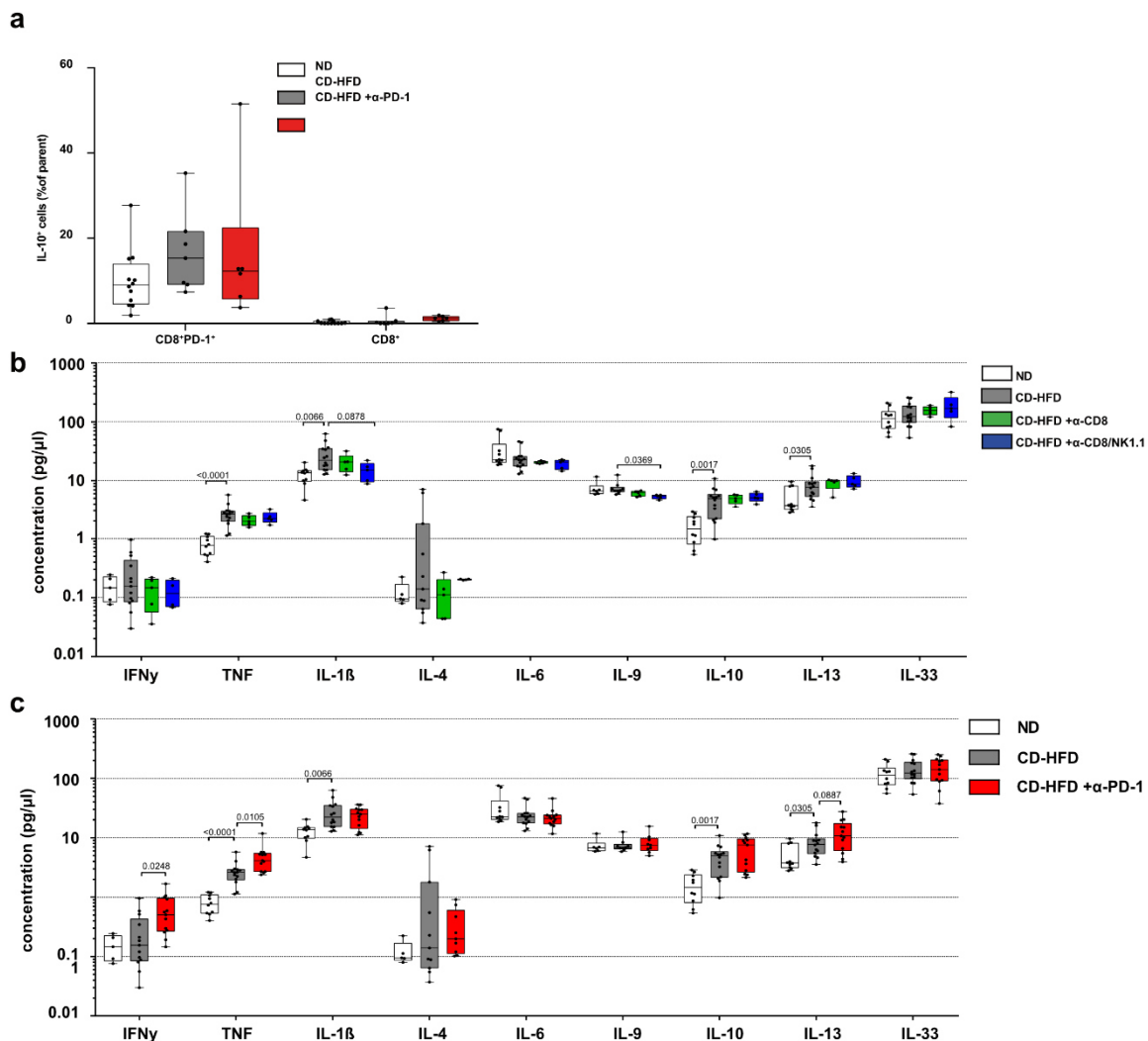
3041  
3042 We fully agree and thank Referee #4 for raising this important point of IL-10 expression, which  
3043 was also raised in a recent study (Breuer et al., 2020).

3044 We analyzed IL-10+ CD8+PD-1+ T-cells in our revised manuscript (included in **Extended Data**  
3045 **19** and **Rebuttal Figure 88a**).

3046 However, we did not see any changes in IL10+ CD8+PD1+ in comparison to CDHFD-fed and  
3047 control mice. Moreover, IL10 levels measured by ELISA did neither drop upon CD8-depletion  
3048 (included in **Extended Data 10** and **Rebuttal Figure 88b**) nor increase significantly upon anti-  
3049 PD1 treatment (included in **Extended Data 13** and **Rebuttal Figure 88c**). Thus, an increased  
3050 anti-inflammatory role by IL-10 expressing CD8+ T-cells upon PD1-targeted immunotherapy  
3051 could not be corroborated (included in **Extended Data 19** and **Rebuttal Figure 88a**) (Breuer  
3052 et al., 2020). Of note, in this publication diet-based NAFLD induction was achieved by feeding  
3053 either WD or CD-HFD for 8-10 weeks. This is in strong contrast to our experimental regime of  
3054 applying diet for 3, 6, or 12 months as we show, that the preclinical model presents different  
3055 stages of NASH pathology severity including hepatocarcinogenesis (data presented in **Figure**  
3056 **1** and **Rebuttal Figure 80**). Thus, in our opinion, CD8+PD1+ cells are the main effector  
3057 population driving liver inflammation and liver cancer – most likely independent of IL10 being  
3058 one of the most upregulated genes in this subset.

3059 In line with our mouse data scRNA-seq of CD8+PD1+ cells derived from control vs  
3060 NAFLD/NASH patients did not reveal increased IL10 expression. Besides in bulk RNA-seq of

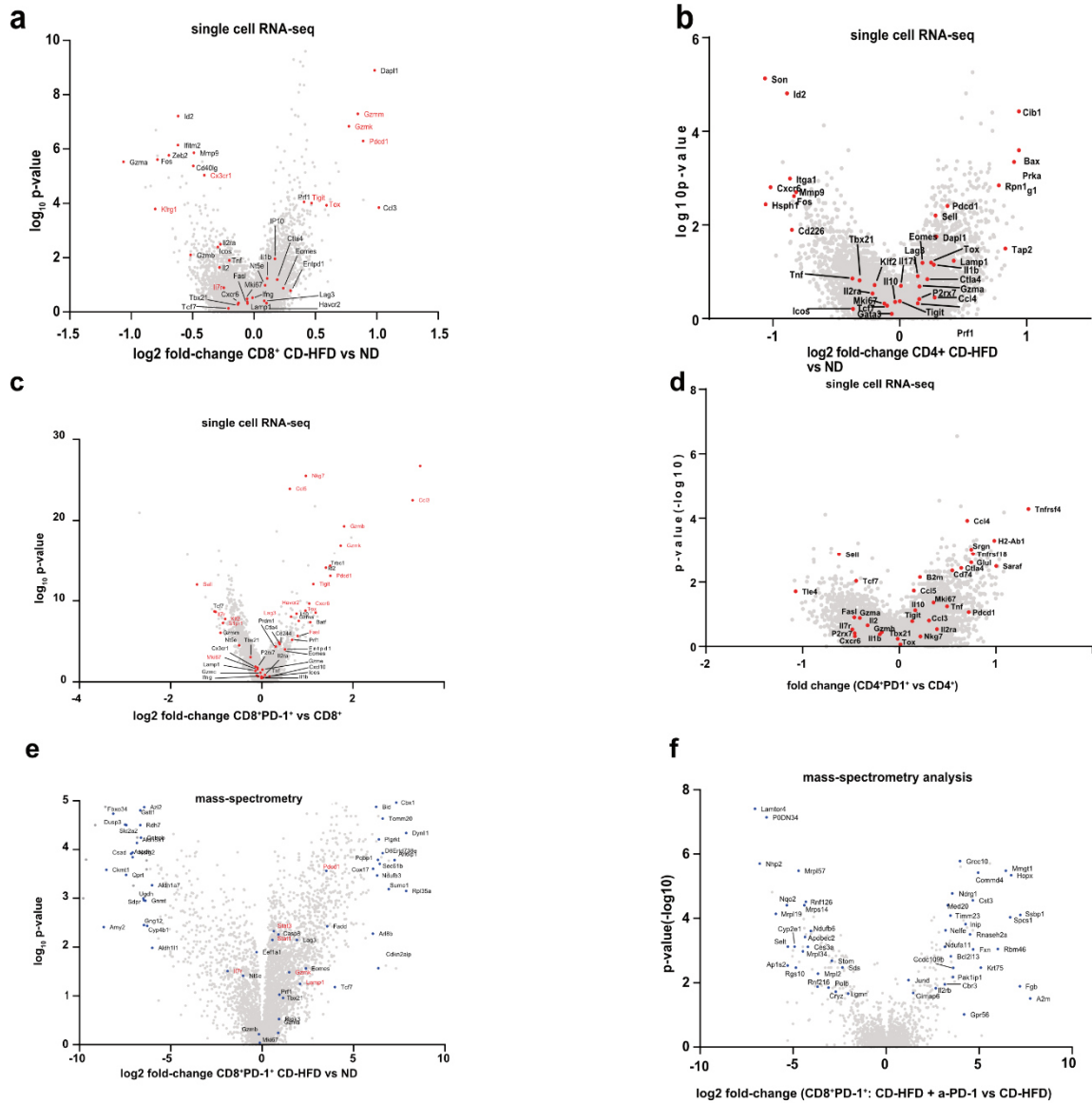
3061 human liver tissue, we observed a variable expression pattern depending on NASH pathology  
 3062 severity (included in **Figure 5, Extended Data 28** and **Rebuttal Figure , 77**).  
 3063



3064  
 3065 **Rebuttal Figure 88**

3066 Polarization by flowcytometry of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months ND, CD-HFD or CD-  
 3067 HFD-fed mice + 8 weeks treatment of α-PD-1 (ND n= 12 mice; CD-HFD n= 7 mice; CD-HFD  
 3068 + α-PD-1 n= 6 mice). (b) Multiplex ELISA concentrations of hepatic inflammation-associated  
 3069 cytokines of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment of α-CD8 or  
 3070 CD-HFD-fed mice + co-depletion of α-CD8/NK1.1 (ND n= 10 mice; CD-HFD n= 14 mice; CD-  
 3071 HFD + 8 weeks treatment of α-CD8 n= 5 mice; CD-HFD + co-depletion of α-CD8/NK1.1 n= 5  
 3072 mice). (c) Multiplex ELISA concentrations of hepatic inflammation-associated cytokines of 12  
 3073 months ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment of α-PD-1 (ND n= 10 mice; CD-  
 3074 HFD n= 14 mice; CD-HFD + α-PD-1 n= 13 mice).





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**Rebuttal Figure 89**

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(a) Selected average marker expression in T-cell subsets of CD8<sup>+</sup> and (b) CD4<sup>+</sup> sorted TCRβ<sup>+</sup> by scRNA-seq of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). (c) Selected marker expression in hepatic CD8<sup>+</sup> T-cells by scRNA-seq comparing CD8<sup>+</sup> with CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment of α-PD-1 fed mice (n= 3 mice/group). (d) Selected marker expression in hepatic CD4<sup>+</sup> T-cells by scRNA-seq comparing CD4<sup>+</sup> with CD4<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment of α-PD-1 (n= 3 mice/group). (e) Selected marker expression in hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells by mass- spectrometry of 12 months ND or CD-HFD-fed mice (ND n= 4 mice, CD-HFD n= 6 mice). (f) Selected marker expression in hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells sorted TCRβ<sup>+</sup> cells by mass- spectrometry of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment of α-PD-1 (n= 6 mice/group). Candidates developing steady in-/decrease from ND to CD-HFD to CD-HFD-fed mice + 8 weeks treatment of α-PD-1 are indicated in red. (n= 6 mice/group).

3091 Figure 2: It was not that clear why depleting CD8s had no impact on ALT, suggesting they are  
3092 not playing a role *in vivo*, while blocking PD1 had some impact (AST is not shown for the anti-  
3093 CD8 treatment).

3094

3095 We thank Referee #4 for highlighting that CD8+ T cell depletion in the context of NASH-HCC  
3096 transition had no or only minor impact on ALT reduction, an effect that has also come to our  
3097 attention and has puzzled us.

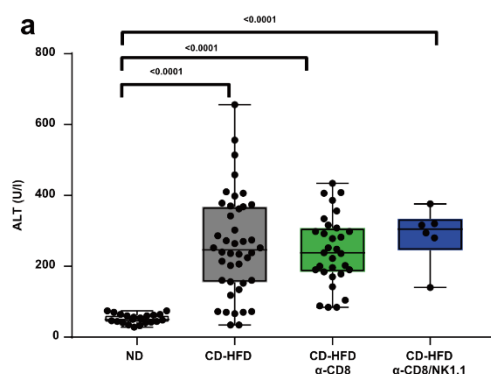
3098 On the other hand, we would like to note that in the context of anti-PD1-related immunotherapy  
3099 triggered liver damage CD8+ T cell depletion did lead to a significant reduction in liver damage  
3100 and NAFLD activity score. Thus, we believe that the anti-PD1 therapy-related damage in NASH  
3101 and NASH to HCC transition is mainly triggered by CD8+ T cells. In contrast, in the context of  
3102 NASH development without anti-PD1 antibody treatment, other cells than CD8+ T-cell also  
3103 contribute to liver damage – and that progressive NASH is characterized by multi-faceted,  
3104 collateral damage through myeloid cells, adaptive cells, and cell death.

3105 We think that CD8+ T-cells have an important *in vivo* role driving NASH to HCC transition, as  
3106 we strongly decreased or eliminated HCC by CD8+ T-cell depletion (both in NASH or NASH  
3107 with anti-PD1 treatment). In line, the co-submitted manuscript by Dudek et al., described  
3108 hepatocyte death by a CD8-dependent mechanism.

3109 Notably, ALT can be elevated as a result of the chronic metabolic environment and/or as a  
3110 result of the still ongoing hepatic inflammation independent of CD8+ or NK1.1+ cells (included  
3111 in **Extended Data 9** and **Rebuttal Figure 90**).

3112 Further, it can be that actually at late time points of co-existence of tumors and NASH – the  
3113 collateral damage might be mainly triggered by non-CD8+ T-cells. We have confirmed the  
3114 efficient depletion of the CD8 T-cells in our models, excluding that this might be a reason.

3115 AST levels are included in our AI-based analysis (included in **Figure 1 and 4**, **Extended Data**  
3116 **4 and 24** and **Rebuttal Figure 80-83**), indicating no change upon CD8 depletion as well.



3117

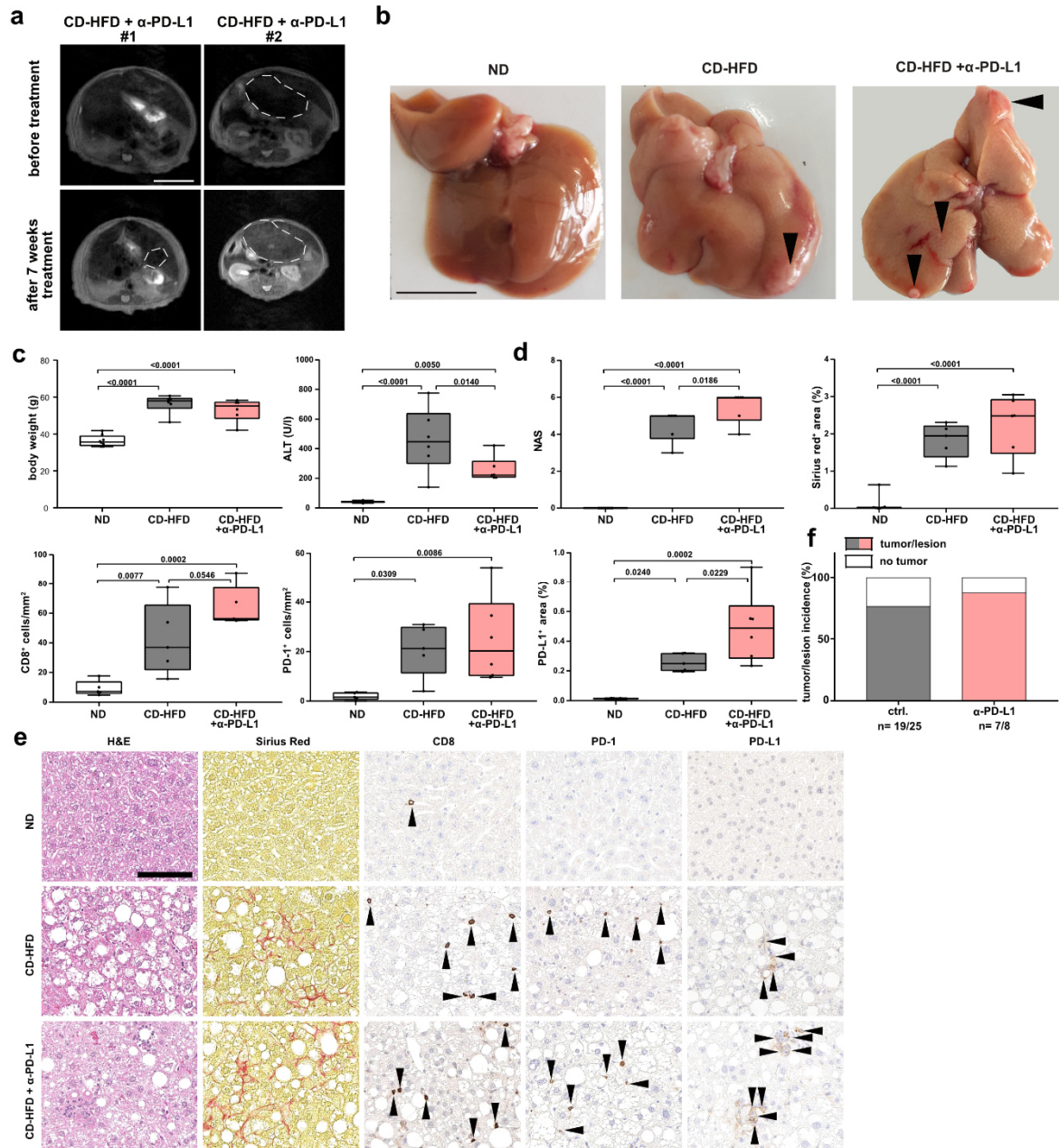
3118 **Rebuttal Figure 90**

3119 (a) ALT levels of 12 months ND, CD-HFD, CD-HFD + 8 weeks treatment of  $\alpha$ -CD8 or CD-HFD  
3120 + 8 weeks co-depletion of  $\alpha$ -CD8/NK1.1 (ALT: ND n= 22 mice; CD-HFD n= 42 mice; CD-HFD  
3121 +  $\alpha$ -CD8 n= 31 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 6).  
3122

3123 Line 202 – lack of impact of anti-PD1. Is there a control for this experiment? The implication is  
3124 that this lack of impact is etiology-specific but it may also be that the intervention does not work  
3125 well in other HCC models.  
3126

3127 We thank Referee #4 for highlighting the etiology-dependent potential outcome of PD-1-  
3128 targeted immunotherapy against HCC. We agree with Referee #4, that there might be  
3129 bivalence in other HCC models and, more importantly, only a subset of HCC patient react to  
3130 PD-1 targeted immunotherapy (El-Khoueiry et al., 2017; Hage et al., 2019). Thus, we have  
3131 also performed anti-PD-L1 targeted immunotherapy in CDHFD-fed mice with and without  
3132 established liver cancer (included in **Extended Data 7** and **Rebuttal Figure 91**).

3133 The data of our study indicate that similar to anti-PD1 - anti-PDL1-treatment does not induce  
3134 an anti-liver cancer effect for NASH-induced HCC but rather induces similar to anti-PD1  
3135 treatment a pro-inflammatory and pro-carcinogenic effect. These data further suggest that in  
3136 the preclinical NASH models used, both PD1- or PDL1-targeted immunotherapy induces  
3137 adverse effects. This is corroborated by our increased, retrospective cohort HCC-patients of  
3138 different etiologies under PD(L)1-targeted immunotherapy, in which multivariate analysis  
3139 results in NAFLD/NASH being an independent negative factor for overall survival (included in  
3140 **Figure 6** and **Rebuttal Figure 92**). Furthermore, we corroborated our hypothesis of non-viral  
3141 (NASH-related) HCC being less responsive to immunotherapy by a meta-analysis including  
3142 1656 patients of the three most important clinical trials, identifying immunotherapy vs control  
3143 for viral HCC as favorable treatment (HR(viral)= 0.64), in contrast, non-viral-HCC showed less  
3144 benefit (HR(non-viral)= 0.92) for immunotherapy (included in **Figure 6**, **Extended Data 30-32**,  
3145 **Supplementary Table 9** and **Rebuttal Figure 93, 94**)).



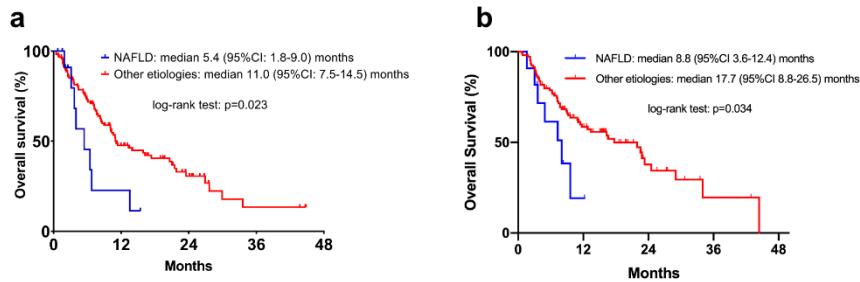
3146

3147

### Rebuttal Figure 91

3148 (a) MRI pictures of liver of mice after 13 months CD-HFD followed by 7 weeks treatment to  
 3149 CD-HFD or CD-HFD-fed mice + 7 weeks by  $\alpha$ -PD-L1 antibodies (CD-HFD n= 6 mice; CD-HFD  
 3150 +  $\alpha$ -PD-L1 n= 8 mice). Lines indicate tumor nodule. Scale bar: 10 mm. (b) Macroscopy of liver  
 3151 of ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-L1 antibodies. Arrowheads  
 3152 indicate tumor/lesions. Scale bar: 10 mm. (c) Body weight, ALT levels ND, CD-HFD or CD-  
 3153 HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-L1 antibodies (Body weight, ALT, : ND n= 8 mice;  
 3154 CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice) (d) and (e) NAS evaluation by H&E, fibrosis  
 3155 quantification (Sirius Red), quantification of CD8, PD-1 and PD-L1 staining of hepatic tissue  
 3156 by immunohistochemistry of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks  
 3157 treatment by  $\alpha$ -PD-L1 antibodies (NAS: ND n= 7 mice; CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-  
 3158 L1 n= 6 mice; Sirius Red: ND n= 7 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice ;  
 3159 CD8, : ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 5 mice; PD-1, PD-L1: ND

3160 n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice). Scale bar: 100  $\mu$ m. (f)  
 3161 Tumor/Lesion incidence in CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-L1  
 3162 antibodies (CD-HFD n= 19 tumors/lesions in 25 mice; CD-HFD +  $\alpha$ -PD-L1 n= 7 tumors/lesions  
 3163 in 8 mice). Arrowheads indicate specific staining positive cells.



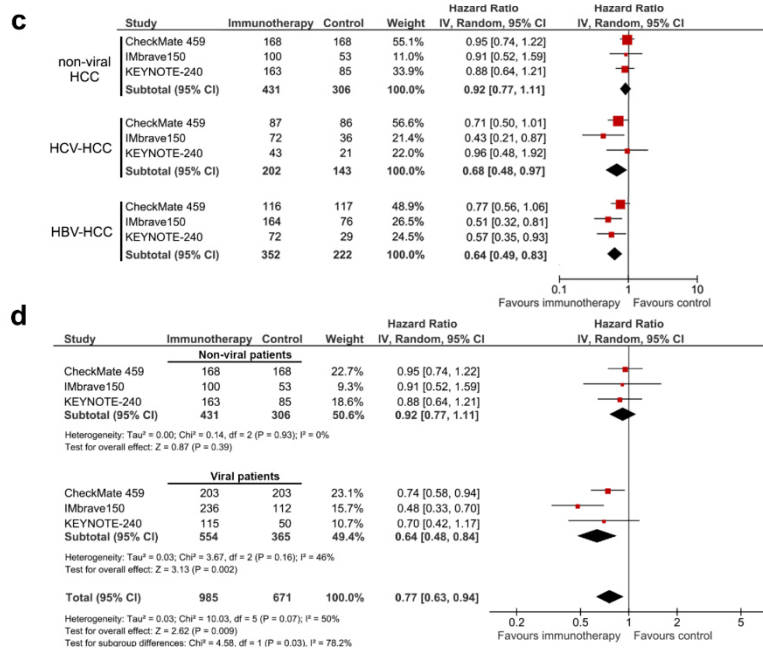
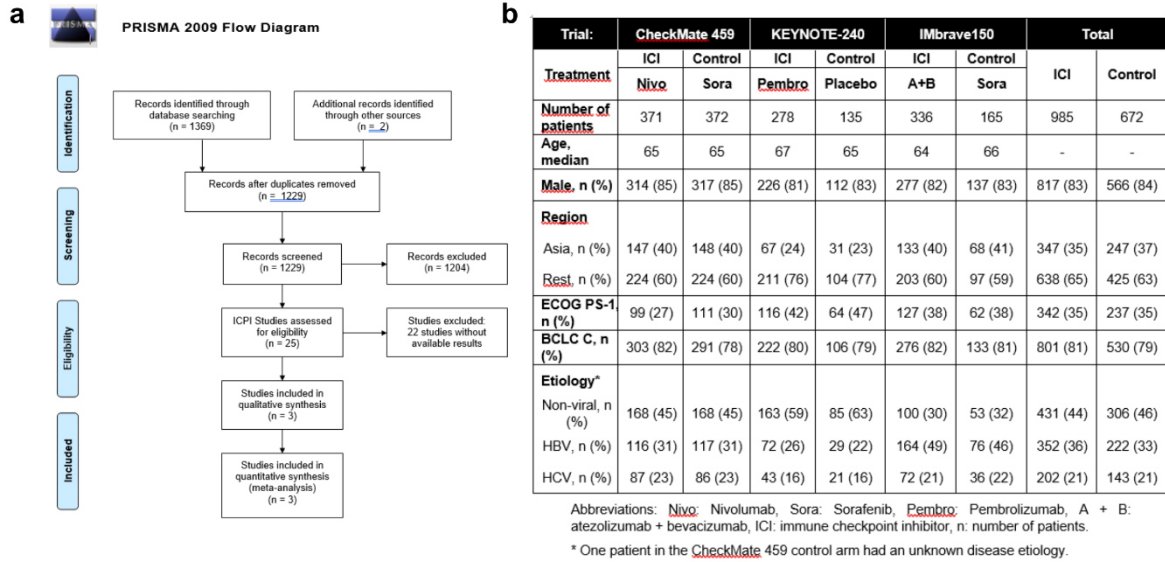
**C**

		Overall survival		
		HR	95% CI	p-value (Cox regression)
Etiology	Other etiologies	1		0.017
	NAFLD	2.6	1.2-5.6	
Performance Status	0	1		0.049
	$\geq 1$	1.7	1.0-2.8	
Macrovascular invasion	Absent	1		0.016
	Present	1.8	1.1-3.0	
Extrahepatic metastases	Absent	1		0.121
	Present	0.7	0.4-1.1	
Alpha-fetoprotein (ng/ml)	$\leq 200$	1		0.019
	$> 200$	1.8	1.1-2.9	
Child-Pugh class	A	1		0.075
	B	1.6	1.0-2.6	

3164  
 3165 **Rebuttal Figure 92**

3166 (a) Nonalcoholic fatty liver disease (NAFLD) is associated with a worse outcome in patients  
 3167 with hepatocellular carcinoma (HCC) treated with PD-(L)1-targeted immunotherapy. A total of  
 3168 130 patients with advanced HCC received PD-(L)1-targeted immunotherapy (Supplementary  
 3169 Table 8). Kaplan-Meier curve display overall survival of patients with NAFLD vs. those with  
 3170 any other etiology; all 130 patients were included in these survival analyses (NAFLD n=13, any  
 3171 other etiology n=117). (b) Validation cohort of patients with HCC treated with PD-(L)1-targeted  
 3172 immunotherapy. A total of 1180 patients with advanced HCC received PD-(L)1-targeted

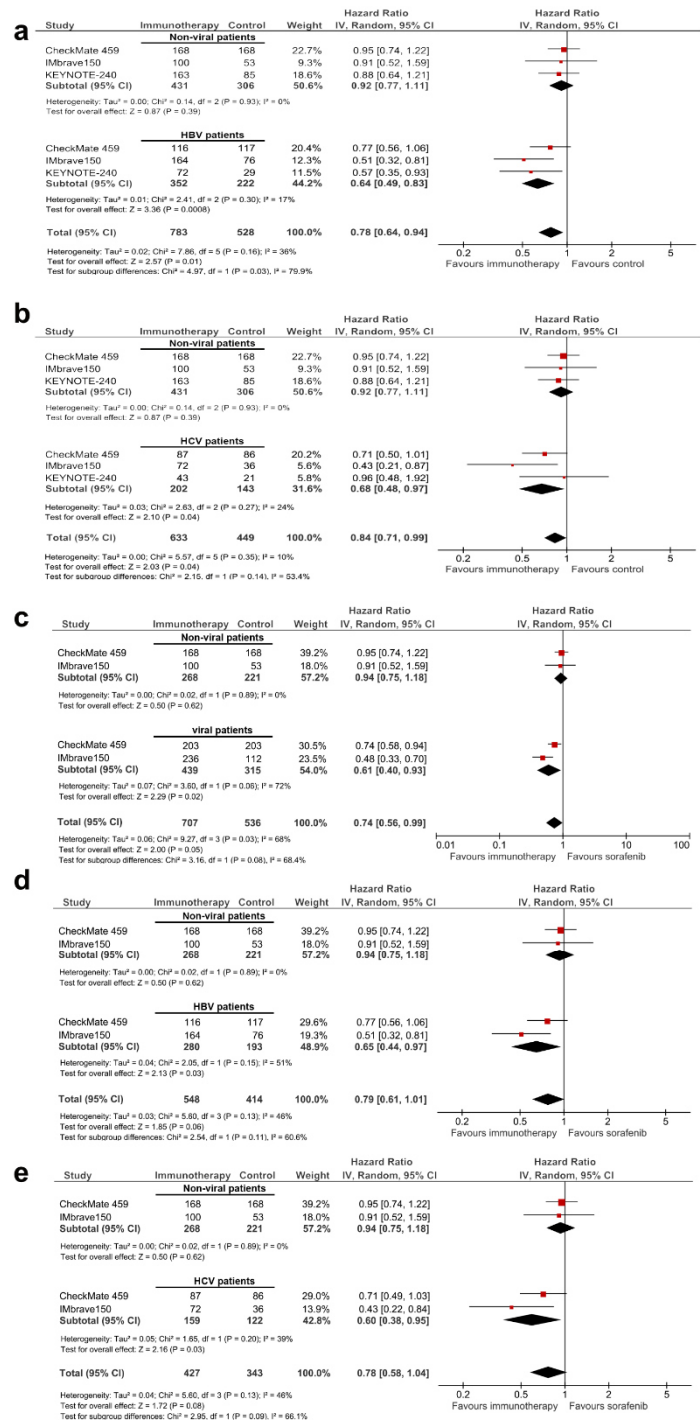
3173 immunotherapy (Supplementary Table 10). Kaplan-Meier curve display overall survival of  
 3174 patients with NAFLD vs. those with any other etiology; all 118 patients were included in these  
 3175 survival analyses (NAFLD n=11, any other etiology n=107). (c) Multivariate analysis of  
 3176 prognostic factors in HCC patients treated with anti-PD-(L)1-based immunotherapy



3177  
 3178 **Rebuttal Figure 93**

3179 (a) Selection of articles assessing the clinical outcome of immune checkpoint inhibitors in  
 3180 advanced HCC for inclusion in the systematic review and meta-analysis. ICPI: Immune  
 3181 checkpoint inhibitor. (b) Pooled baseline characteristics of the patients included in the meta-  
 3182 analysis (total n= 1656). (c) A total of 1656 patients were included in all three randomized trials,  
 3183 and 985 patients received a checkpoint inhibitor (Supplementary Table 7). (c) Separate meta-  
 3184 analyses were performed for each of the three etiologies: non-viral (including mostly NASH  
 3185 and alcohol intake), HCV and HBV. (d) HCV and HBV were pooled into a separate category,  
 3186 termed "viral", and a subsequent meta-analysis comparing viral (n=919) and non-viral,

3187 including mostly NASH and alcohol intake (n=737) was performed. Hazard ratios for each trial  
 3188 are represented by squares, the size of the square represents the weight of the trial in the  
 3189 meta-analysis. The horizontal line crossing the square represents the 95% confidence interval  
 3190 (CI). The diamonds represent the estimated overall effect based on the meta-analysis random  
 3191 effect of all trials.



3192  
 3193  
 3194

**3195 Rebuttal Figure 94**

3196 A total of 1656 patients were included in all three randomized trials, and 985 patients received  
3197 a checkpoint inhibitor. Subgroup analysis was performed to study the specific effects of  
3198 immunotherapy comparing non-viral etiologies (n=737) with (a) HBV (n=574) or (b) HCV  
3199 (n=345). Hazard ratios for each trial are represented by squares, the size of the square  
3200 represents the weight of the trial in the meta-analysis. The horizontal line crossing the square  
3201 represents the 95% confidence interval (CI). The diamonds represent the estimated overall  
3202 effect based on the meta-analysis random effect of all trials.

3203 A total of 1243 patients were included in two first-line trials comparing PD-1 or PD-L1 targeted  
3204 immunotherapy to sorafenib. 707 patients received an immune checkpoint inhibitor (either PD-  
3205 1 or anti-PD-1). (c) HCV and HBV were pooled into a separate category, termed "viral", and a  
3206 subsequent meta-analysis comparing viral (n=754) and non-viral (n=489), mostly NASH and  
3207 alcohol intake, was performed. A subgroup analysis studying the specific effects of non-viral  
3208 etiologies (n=489) on the magnitude of effect of immunotherapy are presented, when  
3209 compared to (d) HBV (n=473) or (e) HCV (n=281). Hazard ratios for each trial are represented  
3210 by squares, the size of the square represents the weight of the trial in the meta-analysis. The  
3211 horizontal line crossing the square represents the 95% confidence interval (CI). The diamonds  
3212 represent the estimated overall effect based on the meta-analysis random effect of all trials.

3213

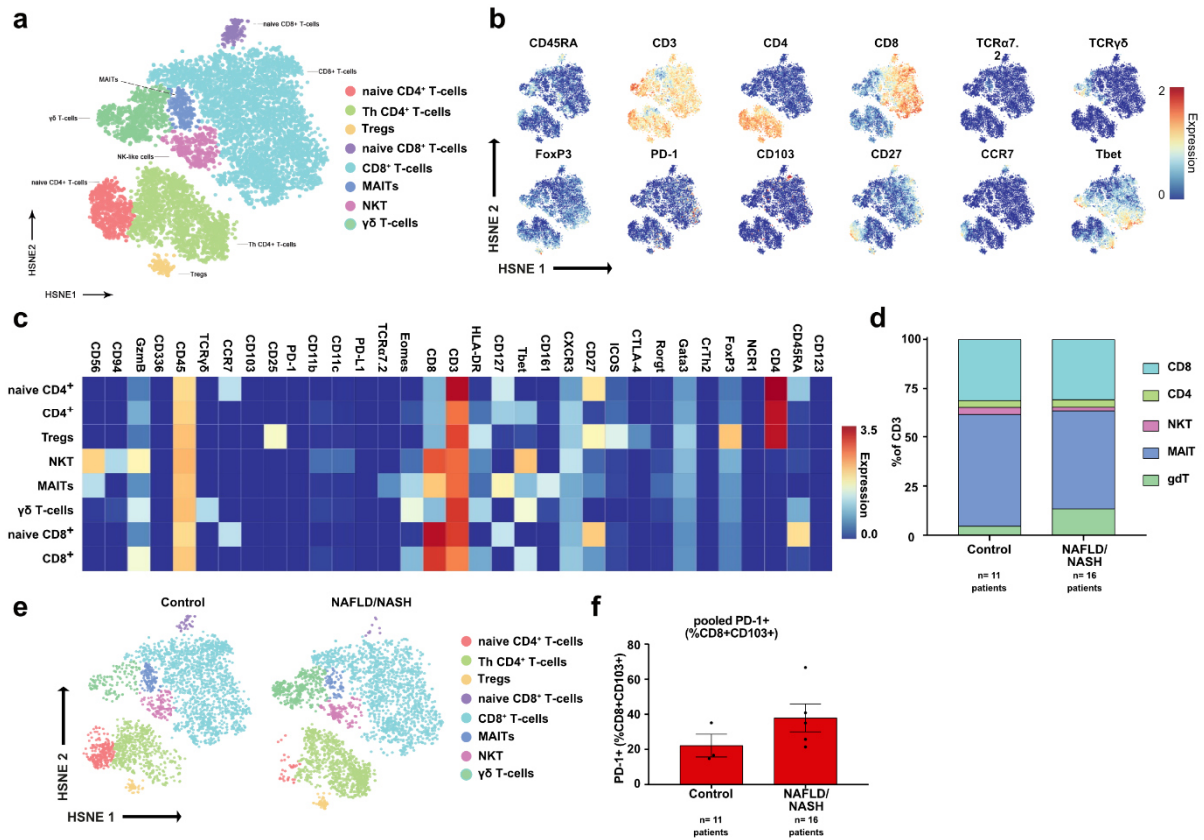
3214 Figure 5b and the text are presented in a slightly confusing way. It would be easier to  
3215 understand the disease associations of %CD8 (of CD3), and % PD1+ (or MFI) of CD3+CD8+  
3216 first. The association of CD103 with tissue residency in the liver is not as good as other tissues,  
3217 so a broader look at the CD8+PD1+ population by flow would be better as well as some caution  
3218 in interpretation.

3219

3220 We agree with this comment and thank Referee #4 for highlighting this problem. Inline, we  
3221 have now improved our manuscript as suggested by Referee#4 (included in **Extended Data**  
3222 **25 and 27** and **Rebuttal Figure 75, 76**). Moreover, we corroborated the association of NASH  
3223 patients and CD103 in a second patient cohort using CYTOF (included in **Figure 5** and  
3224 **Rebuttal Figure 95**).

3225





3226

3227

## Rebuttal Figure 95

3228 (a) tSNE representation, (b) marker expression, (c) average marker expression of defined T-cell subsets of patient-liver-derived T-cells analyzed by CyTOF of control and NAFLD/NASH patients (control n= 11 patients pooled in 3 analyses; NAFLD/NASH n= 16 patients pooled in 5 analyses). (d) Composition, (e) HSNE representation of defined T-cell subsets and (f) quantification of CD8<sup>+</sup>CD103<sup>+</sup>PD-1<sup>+</sup> cells of of patient-liver-derived T-cells analyzed by CyTOF of control and NAFLD/NASH patients (control n= 11 patients pooled in 3 analyses; NAFLD/NASH n= 16 patients pooled in 5 analyses).

3232

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3235

3236 Figure 5e could include some study of CD4s as well for reference. That subset has been linked

3237 to NASH pathogenesis as well. As above, it should be possible to perform some dual CD8 and

3238 PD1 staining to map the subset of interest.

3239

3240 We thank Referee #4 for highlighting this point, that CD4 T-cells and their expression of PD-1

3241 might play a crucial role in the observed phenotype and thus included an in detail analysis of

3242 CD4 T-cells to the majority of our experiments (e.g. **Extended Data 3** and **Rebuttal Figure**

3243 **96**). However, in the preclinical model the magnitude of effects observed in CD4<sup>+</sup> T-cells is

3244 minor when compared to CD8<sup>+</sup> T-cells (e.g. **Extended Data 11** mean (CD8<sup>+</sup>CD62L-

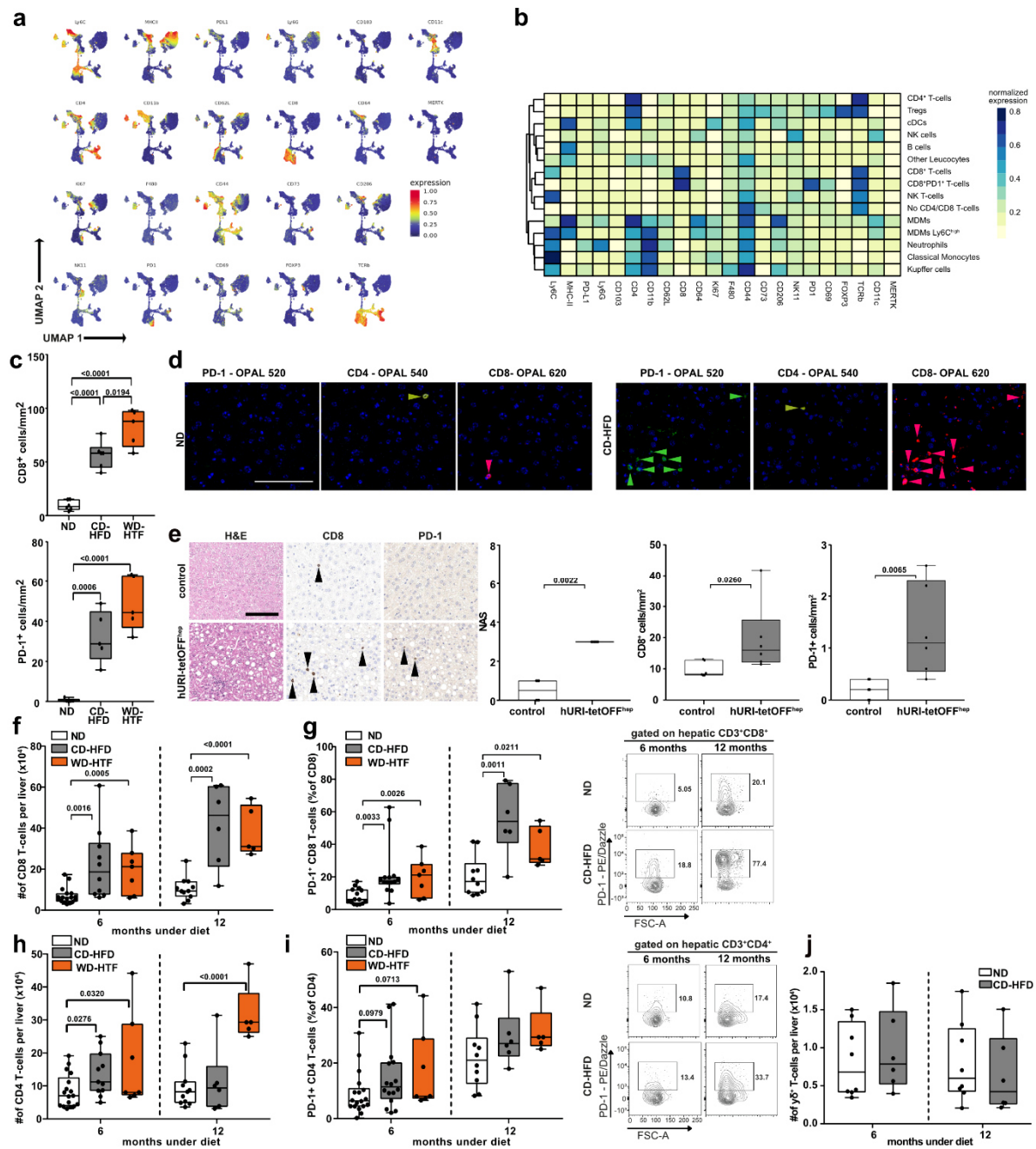
3245 CD44<sup>+</sup>CD69<sup>+</sup>) ~12% (%of CD45<sup>+</sup>) vs mean(CD4<sup>+</sup>CD62L-CD44<sup>+</sup>CD69<sup>+</sup>) ~4% (%of CD45<sup>+</sup>)

3246 upon PD-1 targeted immunotherapy).

3247 Data obtained from CD4 depletion with/without PD1-targeted immunotherapy indicate, that the  
3248 increased hepatocarcinogenesis in the context of anti-PD1 related immunotherapy is  
3249 independent of hepatic abundance of CD4+ T-cells in the preclinical NASH/HCC model  
3250 (included in **Figure 4, Extended Data 22 and 23** and **Rebuttal Figure 79, 81, 86**). However,  
3251 CD4+ T-cells might have a diverse set of effector functions (e.g. interpreting tumor incidence  
3252 in anti-CD8/anti-PD1 treated animals: in the absence of CD8+ T-cells but immunotherapy, thus  
3253 CD4+ T-cells might be responsible for baseline tumor incidence; or the trends of increased  
3254 tumor incidence upon anti-CD4/anti-PD1 co-treatment in **Figure 4** and **Rebuttal Figure 81n**).  
3255 To allow a wider interpretation of a potential effect of CD4+ T-cells in our preclinical model, we  
3256 integrated and correlated the variety and potential changes upon 12 months of diet-feeding in  
3257 the AI-based analyses correlating disease parameters with cellular abundance and  
3258 polarization (included in **Figure 1, Extended Data 4 and 24** and **Rebuttal Figure 82, 83**).  
3259 These data further strengthens that CD4+ T-cells play a minor role, as we see no significant  
3260 correlation of CD4-depleted animals with histological, or serological markers.

3261 Of note, CD4+ T-cells are also significantly changed in the human situation by classical flow  
3262 cytometry, but in the light of the results obtained in the preclinical model, we decided to not  
3263 investigate this result extensively (included in **Extended Data 27** and **Rebuttal Figure 75**). Of  
3264 note, CD4+ T-cells are also significantly changed in the human situation and have also  
3265 analyzed human CD4+ cells a by scRNASeq (included in **Extended Data 26** and **Rebuttal**  
3266 **Figure 75, 76, 89, 97**). In addition, we have performed a velocity analyses of the scRNA Seq  
3267 data of mouse and human CD4 T cells (see Rebuttal letter below). In mouse, no significant  
3268 velocity flow was detected in 12 months CD-HFD-fed mice, indicating, that CD4 cells are not  
3269 transcriptionally activated and driven by NASH-conditions or PD-1-targeted immunotherapy in  
3270 NASH. However, we want to point out, that in the mouse NASH model CD8 T-cells increase  
3271 statistically significant and thus CD4 are relatively fewer cells compared to CD8. Therefore,  
3272 the velocity analysis of mouse CD4 T-cells need to be taken with caution, because we included  
3273 300-500 cells only per described subset. As consequence, we included the negative CD4 T-  
3274 cell data not in the manuscript but in the Rebuttal letter. Velocity analyses on human CD4 lead  
3275 to comparable problems like seen in mouse. As a consequence, we included the negative CD4  
3276 T-cell data not in the manuscript but in the Rebuttal letter as **Rebuttal Figure 97**.

3277 However, we discuss the potential role of CD4+ T-cells in greater detail in the main text.



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**Rebuttal Figure 96**

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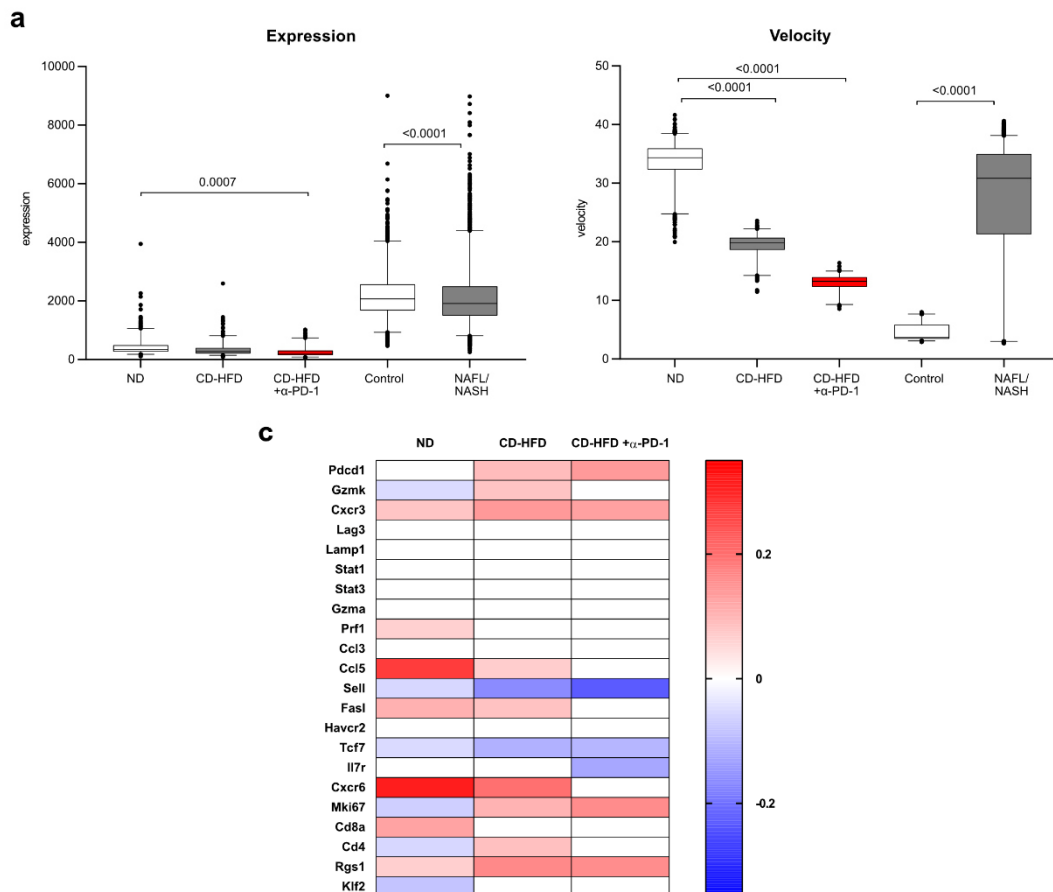
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(a) Analysis of 5000 randomly chosen CD45+ cells by flow cytometry to define distinct marker expression of 12 months ND or CD-HFD-fed mice (ND n= 4 mice; CD-HFD n= 8 mice). (b) Average marker expression of defined CD45+ subsets of 5000 randomly chosen CD45+ cells by flow cytometry of 12 months ND or CD-HFD-fed mice (ND n= 4 mice; CD-HFD n= 8 mice). (c) Quantification of hepatic CD8+ cells and PD-1+ expressing cells by immunohistochemistry of 12 months ND, CD-HFD or WD-HTF-fed mice (PD-1: n= 5 mice/group; CD8: ND n= 6 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice). (d) Immunofluorescence staining of single channel-staining PD-1, CD8 and CD4 (ocher) of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). Arrowheads indicate CD8+ (red), PD-1+ (green) or CD4+ (ocher) cells. Scale bar: 100  $\mu$ m. (e) H&E, CD8 and PD-1 staining, evaluation by NAS and quantification of CD8+ cells and PD-1+ expressing cells by immunohistochemistry of 32-weeks old hURI-tetOFF<sup>hep</sup> and non-transgenic litter control mice (n=6 mice/group). Arrowheads indicate specific staining positive

3292 cells. Scale bar: 100  $\mu$ m. (f) Quantification of abundance, (g) PD-1 expression and flow  
 3293 cytometry plots of hepatic CD8<sup>+</sup> T-cells by flow cytometry of 6 or 12 months ND or CD-HFD-  
 3294 fed mice (abundance of CD8: 6 months: ND n= 17 mice; CD-HFD n= 10 mice; WD-HTF n= 7  
 3295 mice; 12 months: ND n= 11 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice; PD-1 expression  
 3296 in CD8<sup>+</sup> T-cells: 6 months: ND n= 15 mice; CD-HFD n= 14 mice; WD-HTF n= 7 mice; 12  
 3297 months: ND n= 10 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice). (h) Quantification of  
 3298 abundance, (i) PD-1 expression and flow cytometry plots of hepatic CD4<sup>+</sup> T-cells by flow  
 3299 cytometry of 6 or 12 months ND or CD-HFD-fed mice (abundance of CD4: 6 months: ND n=  
 3300 17 mice; CD-HFD n= 10 mice; WD-HTF n= 7 mice; 12 months: ND n= 11 mice; CD-HFD n= 6  
 3301 mice; WD-HTF n= 5 mice; PD-1 expression in CD4<sup>+</sup> T-cells: 6 months: ND n= 15 mice; CD-  
 3302 HFD n= 14 mice; WD-HTF n= 7 mice; 12 months: ND n= 10 mice; CD-HFD n= 6 mice; WD-  
 3303 HTF n= 5 mice).  
 3304



### 3305 **Rebuttal Figure 97**

3306 (a) RNA velocity analyses of scRNA-seq data showing expression, and (b) velocity of patient-  
 3307 liver-derived CD4<sup>+</sup> T-cells of control, or NAFLD/NASH patients in comparison to mouse-liver-  
 3308 derived CD4<sup>+</sup> T-cells (patients: NAFLD/NASH n= 3 patients; mouse: n= 3 mice/group).  
 3309 (c) Correlation of expression along the latent-time of selected genes along the latent-time  
 3310 (mouse: n= 3 mice/group).  
 3311

3312 Figure 5f is not really that convincing of a relationship with TNF – the r-squared value would  
3313 be better to illustrate and would be very low. If the authors think TNF secretion is critical it  
3314 would be possible to explore this further in the mouse model.

3315

3316 We thank Referee #4 for highlighting this point. Although TNF is correlated significantly with  
3317 PD1 abundance, the correlation is weak as indicated by the r-value and therefore moved the  
3318 data to the Extended Data. Moreover, we fully agree with this Referee that further experiments  
3319 were needed to underline the role of TNF in NASH/HCC transition in the context of anti-PD1  
3320 related immunotherapy.

3321 Thus, we have performed an anti-TNF treatment with or without PD-1- targeted immunotherapy  
3322 in the context of NASH/HCC. Anti-TNF treatment without PD1-targeted immunotherapy led to  
3323 liver cancer formation comparable to control-treated CD-HFD-fed mice. However, anti-TNF  
3324 treatment in the context of PD1-targeted immunotherapy leads to a significant reduction of  
3325 tumor incidence compared to anti-PD1 treated CD-HFD-fed mice, indicating that TNF exerts  
3326 key functions of the observed adverse effects triggered by PD1-targeted immunotherapy,  
3327 namely the increased NAS, liver damage, and hepatocarcinogenesis (included in **Figure 4,**  
3328 **Extended Data 20 and 21** and **Rebuttal Figure 78, 81, 85**).

3329 Moreover, the combination of anti-PD1 therapy with anti-CD8 – also ablating the adverse and  
3330 pro-carcinogenic effects of CD8+ T-cells emphasize that CD8+ T-cells are a major cell  
3331 population mediating increased hepatocarcinogenesis in a TNF-dependent mechanism upon  
3332 PD1-targeted immunotherapy (included in **Figure 4, Extended Data 20 and 21** and **Rebuttal**  
3333 **Figure 78, 81, 85**).

3334 Importantly, by comparing classical flow cytometry, CYTOF, and on scRNA-seq level of  
3335 mouse-human of CD8+ T-cells isolated from liver tissue of NASH mice or patients, we identified  
3336 similar populations and transcriptional activation of CD8+ PD1+ in a total of three independent  
3337 center patient cohorts (included in **Figure 5, Extended Data 25 and 27** and **Rebuttal Figure**  
3338 **75, 76, 84**). These data indicate that results obtained and hypotheses built from the preclinical  
3339 NASH model and are in line with published results, where TNF blockade uncouples mediated  
3340 toxicity in dual CTLA-4 and PD-1 immunotherapy (Perez-Ruiz et al., 2019).

3341

3342 For Figure 5G some disease controls would be valuable.

3343

3344 We thank Referee #4 for his/her comment for pointing out the lack of appropriate control groups  
3345 (e.g. NASH-HCC vs different etiology-induced HCC under Sorafenib/different multi-kinase  
3346 inhibitors as a second/third-line therapy). Although of extreme interest for public health and

3347 public knowledge, we described this important issue in our discussion and to the best of our  
3348 knowledge there are no NASH-HCC treated cohorts available (apart from, possibly, inside of  
3349 the big pharma-industry), which would allow an adequate control arm. Thus, we evaluated  
3350 potential disease controls in the manuscript by performing a meta-analysis including 1656  
3351 patients of the three major clinical trials (Imbrave 150; Checkmate 459; Keynote 240). Here  
3352 we could identify immunotherapy vs control for viral HCC as favorable treatment ( $HR(viral)=$   
3353  $0.64$ ), in contrast non-viral-HCC showed less benefit ( $HR(non-viral)= 0.92$ ) for immunotherapy  
3354 (included in **Figure 6, Extended Data 30-32, Supplementary Table 9** and **Rebuttal Figure**  
3355 **93, 94**)).

3356 Furthermore, we toned down the conclusions of our retrospective cohort in the manuscript and  
3357 would like to point out, that bigger cohorts and prospective clinical trials are of utmost  
3358 importance for the scientific community.

3359

3360 Line 493+: This sentence is perhaps overstating the data, which were not significant in all those  
3361 parameters. It is likely quite hard to make the firmest comparisons, especially in such a  
3362 retrospective analysis, where the heterogeneous group of patients with eg viral aetiologies will  
3363 be on effective therapies - the actual aetiologies were not obvious in the supplementary data.  
3364 This interpretation could be a bit more cautious throughout (eg. it is in the abstract).

3365

3366 We would like to thank Referee #4 for the important comment and agree. Thus, we toned down  
3367 the wording and interpretation of our data. As described previously, we recruited additional  
3368 patients to increase the number of patients in our initial clinical cohort from 65 to 130 HCC  
3369 patients under anti-PD(L)1-targeted immunotherapy, which we validated in a second cohort  
3370 (included in **Figure 6** and **Rebuttal Figure 92**).

3371 We agree with Referee #4, that the presented retrospective PD-(L)1 targeted immunotherapy  
3372 treated NAFLD/NASH-associated HCC cohort - although unique for Europe and treatment not  
3373 officially licensed and thus reimbursement - is still small, although we would like to point out,  
3374 that prominent trends or effects can be seen in small retrospective cohorts as well. Thus, our  
3375 analyses of BCLC-C NAFLD/NASH-HCC vs other-etiological-HCC patients indicated, that  
3376 NAFLD/NASH-HCC has significantly reduced overall survival compared to other-etiological-  
3377 HCC in this small retrospective cohort. Of note, multivariate analyses identified NAFLD/NASH  
3378 as an independent factor for treatment response and thus identifying NAFLD/NASH as a  
3379 negative predictor for HCC immunotherapy (included in **Supplementary Table 9** and **Rebuttal**  
3380 **Figure 92**).



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3381 Like previously mentioned, we corroborated our hypothesis of non-viral (NASH-related) HCC  
3382 being less responsive to immunotherapy by a meta-analysis including 1656 patients of the  
3383 three most important clinical trials (IMbrave 150; Checkmate 459; Keynote 240), identifying  
3384 immunotherapy vs control for viral HCC as favorable treatment ( $HR(viral)= 0.64$ ), in contrast,  
3385 non-viral-HCC showed less benefit ( $HR(non-viral)= 0.92$ ) for immunotherapy (included in  
3386 **Figure 6, Extended Data 30-32, Supplementary Table 7 and Rebuttal Figure 93, 94**)).  
3387 Thus, we toned down the conclusions of our retrospective cohort in the manuscript and again  
3388 would like to point out, that bigger cohorts and prospective clinical trials are of utmost  
3389 importance for the scientific community.  
3390


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**Author Rebuttals to Initial Comments**

**SHORTENED AUTHOR REBUTTAL**

**(please note that the authors have quoted the reviewers in black and responded in blue)**



1 **Referee #1 (Remarks to the Author):**

2 Using two different mouse models of NASH-induced HCC as well as data from patients with NASH-  
3 associated HCC, the authors suggest the concept that CD8+PD1+ T-cells promote NASH development  
4 and that treatment with checkpoint inhibitors may release the brake in these NASH-promoting cells,  
5 resulting in disease exacerbation and more HCC, which they proposed is confirmed by their findings of  
6 absent response to checkpoint inhibitors Nivolumab and Pembrolizumab in patients with NASH-  
7 associated HCC but not in patients with HCC due to other causes. While the analyses are carefully  
8 performed and raise the question of harmful effects of checkpoints in NASH-associated HCC, both the  
9 mouse and patient studies have major limitations, and it cannot be excluded that this paper sends the  
10 wrong message to the community and will negatively impact the field.

11 We thank Referee #1 for appreciating that our experiments have been “carefully performed”  
12 experiments as well as for outlining the potential clinical impact of our study on PD-1 targeted  
13 immunotherapy in HCC. Also, we thank Referee #1 for pointing out the current limitations of  
14 the applied mouse models and clinical cohorts of our study, which we have taken utmost  
15 seriously and improved both. Statements on the role of checkpoint inhibitors in non-viral  
16 etiologies in HCC have been tempered, but nonetheless reflect the results of the meta-  
17 analysis, which is aligned with the pre-clinical findings.

18 **(i)** We have added a third preclinical mouse model of NASH with NASH to HCC transition  
19 (Gomes et al., 2016; Tummala et al., 2014). Analysis of this model corroborated the link  
20 between CD8+PD1+ T-cells and NASH development

21 **(ii)** We have extended our preclinical experiments with six novel treatment groups and  
22 performed in detail analyses on the mechanism and functional link of liver damage,  
23 inflammation, and responsiveness to anti-PD1-targeted immunotherapy in liver cancer.

24 **(iii)** We have added human clinical data sets (with 1656 HCC patients on immunotherapy  
25 involving the important clinical trials - IMbrave 150; Checkmate 459; Keynote 240), enlarged  
26 our initial retrospective clinical cohort, and validated results obtained from this cohort in a  
27 second cohort of HCC patients under immunotherapy. Moreover, we corroborated our findings  
28 of CD8+PD1+ increasing by NASH in now in total 3 independent patient cohorts across Europe  
29 by flow cytometry or single-cell RNA-seq. Furthermore, we have performed CYTOF and  
30 scRNA Seq analysis of lymphocytes from livers derived from human NAFLD/NASH and  
31 steatosis and compared these data with our preclinical models - corroborating our data.

32 In particular, we have now added a meta-analysis including 1656 HCC patients with different  
33 underlying etiologies (viral and non-viral) treated with immunotherapy derived from three large  
34 clinical trials (**Figure 6, Extended Data 30-32, Supplementary Table 7 and Rebuttal Figure**  
35 **1d,e and 2-4**). (Total number of patients in the combined cohort: 1656. One patient in the  
36 CheckMate-459 had unknown etiology, and could therefore not be included in the quantitative  
37 meta-analysis). We conducted this meta-analysis to support the experimental data suggesting



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38 that anti-PD1/anti-PDL1 checkpoint inhibitors would have a distinct effect in non-viral (NASH-  
39 related) HCC as opposed to viral-related HCC (**Figure 6, Extended Data 30-32** and  
40 **Supplementary Table 7** and **Rebuttal Figure 1d,e and 2-4**). Out of eight studies identified in  
41 the search, only three fulfill the pre-established criteria (**Extended data 30** and **Rebuttal**  
42 **Figure 2**), including a total of 1656 HCC patients.

43 These randomized controlled trials (RCT) included **A**) CheckMate-459 (Yau et al., 2019), a  
44 first-line, randomized, sorafenib-controlled trial testing nivolumab (an anti-PD1 monoclonal  
45 antibody) in monotherapy (n=742), **B**) IMbrave150 (Finn et al., 2020), a first-line, randomized,  
46 sorafenib-controlled trial testing the combination of atezolizumab (an anti-PD-L1 monoclonal  
47 antibody) and bevacizumab (an anti-VEGF-A monoclonal antibody) (n=501), **C**) KEYNOTE-  
48 240 (Finn et al., 2019), a second-line, randomized, placebo-controlled trial testing  
49 pembrolizumab (an anti-PD1 monoclonal antibody) monotherapy. All three trials reported a  
50 subgroup analysis of survival data stratified according to disease etiology: hepatitis B virus  
51 (HBV), hepatitis C virus (HCV), and non-viral, including both NASH and alcohol intake.

52 **First**, we analyzed whether checkpoint inhibitors were effective in each of three etiologies  
53 (HBV, HCV, and non-viral) and then compared the efficacy by categorizing patients with viral  
54 vs non-viral etiology HCC in all three phase III studies including a total of 1656 patients.  
55 Immunotherapy was superior to the control arm in both HBV (n= 574; p=0.0008) and HCV-  
56 related HCC patients (n= 350; p=0.04), **but not in non-viral** HCCs (n=737; p=0.39). The  
57 magnitude of the benefit with checkpoint treatment according to etiology was significantly  
58 better in viral etiology (pooled HBV and HCV cases) [HR: 0.64; 95%CI 0.48-0.94] than non-  
59 viral etiology [HR: 0.92; 95%CI 0.77-1.11]; p of interaction= 0.03 (**Rebuttal Figure 1e**). Then,  
60 we dissected the specific effect by each viral type in a subgroup analysis. Comparison of  
61 magnitude of effect was significant comparing HBV vs. non- viral etiology (n=1311; p  
62 interaction= 0.03), and there was a non-significant trend for HCV vs. non-viral etiology  
63 (n=1082; p of interaction=0.14) (**Rebuttal Figure 3**).

64 **Second**, considering that two out of three RCT were conducted in first-line treatment of  
65 advanced HCC with a homogeneous control arm (sorafenib), we conducted a subgroup  
66 analysis specifically with these two studies (n= 1234). This approach allowed us to control for  
67 biases related to the study population and distinct control arms. Immunotherapy was superior  
68 to sorafenib in both HBV (n= 473; p=0.03) and HCV-related HCC patients (n= 281; p=0.03),  
69 but not in non-viral HCC (n=489; p=0.62). (**Rebuttal Figure 4**). The magnitude of the  
70 checkpoint treatment effect vs sorafenib according to etiology showed a non-significant trend  
71 favoring viral etiology (n=754; HR: 0.61 (95%CI 0.40-0.93)] when compared to non-viral  
72 etiology [n=489; HR: 0.94 (95%CI 0.75-1.18)] (p of interaction= 0.08) (**Rebuttal Figure 4a**). As  
73 a result, we have included these data in the resubmitted manuscript (**Figure 6**).



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74 Based on these data we want to point out that it is - as indicated by Referee#1 - of the highest  
75 importance to us to specifically define/tone down appropriately the message of our manuscript:  
76 Our manuscript does not indicate that immunotherapy is not beneficial for HCC patients, rather  
77 demonstrates that HCC patients with viral etiologies do respond well and achieve survival  
78 benefits - however, that patients with non-viral etiologies (e.g. NASH) do not achieve a  
79 significant outcome benefit. We propose to stratify HCC patients who are very likely to profit  
80 from immunotherapy and strengthen the argumentation to use immunotherapy in specific  
81 cohorts of HCC patients. We agree with Referee#1 that this needs to be articulated  
82 appropriately, not to deliver wrong messages but to be very specific.

83  
84 **Specific points:**

85 1. The NASH-HCC mouse models represent a major weakness of this paper and may lead to premature  
86 conclusions on the effect of PD-1 therapy in NASH-associated HCC. While the employed mouse models  
87 may be among the best to study various aspects of NASH, several limitations preclude them from  
88 serving as useful preclinical models for HCC:

89 We thank Referee #1 for appreciating the used NASH-HCC models as “among the best to  
90 study various aspect of NASH”, and we agree in general that studies in preclinical models have  
91 their limitations, especially in the context of chronic inflammation-induced cancer. These  
92 limitations of preclinical models are pronounced if mouse models are not used chronically (e.g.  
93  $\geq 1$  year). However, we would like to point out that the model(s) used in our paper reflect  
94 sporadic liver cancer development with similar immune cell signature, pathophysiology, and  
95 the heterogeneous genetic landscape found in humans (Ma et al., 2016; Malehmir et al., 2019;  
96 Wolf et al., 2014 - and the data reported in this manuscript). In response to Referee #1, we  
97 have performed synteny analyses comparing HCC nodules from individual mice with human  
98 HCC (**Extended Data 6** and **Rebuttal Figure 5a,b**). These data indicated no significant  
99 changes in genomic aberrations between human HCC and mouse liver tumors.

100  
101 1a. Many mouse models of cancer are simply not responsive to checkpoint inhibition because of low  
102 mutational load and lacking tumor antigens/neo-antigens. The authors do not provide evidence that the  
103 employed models have a mutational load that is at least as high as in that seen in HCC patients.

104 We thank and agree with Referee #1 for pointing out the possible unresponsiveness of clinical  
105 models to checkpoint inhibition due to low mutational load. The mutational load HCC of most  
106 conventional preclinical models is indeed very low, or lower compared to human HCC. This is  
107 the case, in particular when taking into account liver cancer models triggered through  
108 transgenesis, e.g. c-myc transgenic mice or preclinical mouse models with hydrodynamic tail  
109 vein injection (HTDVi) of oncogenic drivers and tumor suppressors. In those models, pre-  
110 existing genetic drivers and tumor suppressor deficiencies can be a major drawback  
111 concerning additional mutations and increased mutational load.



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112 In a chronic model of liver inflammation, we could show that mutational load increases over  
113 time - comparing 9, 12, and 15 months (Finkin et al., 2015). Our chronic, spontaneous NASH-  
114 HCC models develop liver cancer in the absence of specific genetic drivers – but rather through  
115 chronic liver damage triggering DNA instability, ER and mitochondrial stress, accumulating  
116 genetic hits over time stochastically triggering liver cancer formation, like has been shown in  
117 human NASH (Boege et al., 2017).

118 In light of the important question of Referee #1, we have now included a further genetic  
119 screening of 19 mouse HCC nodules in our revised manuscript and compared them to human  
120 HCC nodules and their mutational landscape (**Extended Data 6** and **Rebuttal Figure 5a,b**).  
121 Data from this study confirm that quality, degree of heterogeneity, and load of chromosomal  
122 aberrations (gains and deletions) of the used NASH to HCC mouse model is similar to human  
123 HCC (Wolf et al., 2014 and this manuscript). Furthermore, we would like to point out, that  
124 overall in human HCC so far a responder rate of 17-20% for PD-1-targeted monotherapy was  
125 observed, potentially due to a generally low amount or lack of broad-scale tumor antigens in  
126 HCC (El-Khoueiry et al., 2017; Zhu et al., 2018).

127  
128 1b. The mouse model - albeit taking over a year - is not comparable to HCC development in patients,  
129 which takes decades and mostly occurs in the setting of advanced fibrosis or cirrhosis (even though a  
130 subset of NASH-associated HCC patients do not have cirrhosis, most of them have advanced fibrosis).  
131 Importantly, in most of these patients, the underlying NASH is much less activate than in earlier disease  
132 stages/burnt out - meaning that the risk of increasing NASH activity and thereby worsening not only  
133 NASH but also increasing NASH-HCC is much lower and possibly not even relevant. The authors'  
134 conclusions would be relevant if one employed checkpoint inhibitors for HCC prevention but are likely  
135 not applicable to patients except for those, in whom HCC develops in the absence of cirrhosis and with  
136 high NAS.

137 We thank Referee #1 to point out the limitations of preclinical models in comparison to patient-  
138 derived data. We agree that preclinical models do not take decades to develop HCC (averages  
139 mouse life-time ~ 2 years). However, mouse models have helped in the identification of  
140 molecular and cellular mechanisms leading to liver cancer (Ringelhan et al., 2018) - and if used  
141 in a long term fashion - up to 2 years - they do recapitulate in part the chronicity of inflammatory  
142 etiologies driving liver cancer. Moreover, mouse liver cancer occurs in age comparable to the  
143 life-span of patients (we applied 12 - 15 months of NASH-diet feeding months from 2 months  
144 of age onwards), which is comparable with the 4<sup>th</sup> to 5<sup>th</sup> life decade in humans regarding the  
145 age of HCC onset/HCC disease (Llovet et al., 2016). We would like to highlight, that preclinical  
146 models implemented in our study develop fibrosis to different degrees (mostly mild peri-cellular  
147 fibrosis to periportal streets and cirrhosis (Malehmir et al., 2019; Wolf et al., 2014)).

148 Thus, we agree with Referee #1, that the preclinical model might represent a patient subgroup  
149 developing HCC in the background of fibrosis. We agree with Referee#1, that underlying NASH  
150 in HCC patients might be less activated compared to earlier stages and burnt-out.



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151 Of note, clinical state-of-the-art care includes the use of corticosteroids for the treatment of  
152 adverse effects (Weiler-Normann and Lohse, 2016), which can also induce NASH-like  
153 pathologies. Thus, understanding mechanisms of underlying NASH in NASH-HCC in  
154 preclinical models is of vital interest. Furthermore, current studies explore checkpoint inhibitors  
155 for HCC as prevention of recurrence (Kudo, 2018).

156 We take this point of Referee #1 utmost seriously and devised importance for this critique in  
157 the discussion section. We toned down our interpretations from human cohorts analyzed in a  
158 retrospective design, although we believe the points raised in our manuscript address  
159 important topics like a potential stratification for etiology, the need for biomarkers, and clinical  
160 awareness of potential unfavorable side-effects of checkpoint inhibitor usage (Kim et al., 2020).  
161 In line with the suggestion of Referee #1 to explore the limitations of our mouse models and to  
162 understand the link between liver inflammation and tumor development better, we have re-  
163 analyzed our mouse data sets to dissect potential correlations of fibrosis, tumor size, tumor  
164 nodule number, flow cytometry data of livers, ALT, NAS, CD8, and PD-1 expression using  
165 artificial intelligence, machine learning and neuronal networking (**Figures 1 and Extended**  
166 **Data 4 and 24 and Rebuttal Figure 6 and 7c,d**). Moreover, we have added a third NASH-  
167 HCC mouse model, which corroborates the link between the amount of CD8+, PD1+ T-cells,  
168 and NASH (**Extended Data 3i and Rebuttal Figure 8i**).

169 Of note, we now underlined that our preclinical NASH models recapitulate in part the alterations  
170 of hepatic immune cells in NASH by performing correlative analyses and machine learning of  
171 liver-derived lymphocytes of NASH patients by CYTOF, classical flow cytometry, and scRNA-  
172 seq (**Figure 5, Extended Data 25-27 and Rebuttal Figure 9-12**). These analyses demonstrate  
173 that the pro-tumorigenic T cell population found in livers of preclinical NASH mouse models  
174 (CD8+PD1+CXCR6+) are also found in / and correlate with NASH in human livers  
175 (CD8+PD1+CD103+).

176  
177 2. In relation to above-described limitations of the model, the paper does not sufficiently focus on dual  
178 functions of CD8+PD1+ T-cells, promoting NASH but possibly also restricting HCC. These functions are  
179 likely to occur at different stages in patients.

180 We thank Referee #1 for this important concern. We agree that the effects of CD8+PD1+ cells  
181 are executed at different time points. However, we would like to draw attention to the point that  
182 immunotherapy is considered to boost pre-existing inflammation (determined e.g. by  
183 evaluation of liver infiltration by immune cells using immunohistochemistry or flow cytometry  
184 for CD3, CD8, and PD-L1). Our data rather indicate that this certain population has no impact  
185 in restricting HCC development - in the context of NASH - and even immunotherapy. In fact,  
186 we show that depletion of CD8+ T-cells in NASH prevents NASH to HCC transition.

187 Thus, CD8+PD1+ T cells drive NASH, which is exacerbated in the context of anti-PD1-related  
188 immunotherapy. We have now pointed this out more clearly, executed novel experiments to





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189 underline this point of early (NASH) and late time points (NASH to HCC transition), analyzed  
190 these cells in the context of human NASH and further discussed this in the discussion section.  
191 To mirror the clinical status of the majority of patients at the time of diagnosis, we performed  
192 PD-1-targeted checkpoint inhibition in mice with pre-existing liver tumors (**Extended Data 6**  
193 **and 7** and **Rebuttal Figure 5 and 13**) and performed now MRI-guided follow up.  
194 Our data clearly show, that anti-PD1 or anti-PDL1-related immunotherapy does not stop or  
195 revert tumor burden but rather supports further tumor abundance. In contrast, when anti-CD8  
196 antibody therapy was applied, it decreased tumor incidence and thus development (**Figure 2,**  
197 **Extended Data 8** and **Rebuttal Figure 14a-g,q** and **15**). Furthermore, we underlined the  
198 importance of hepatic CD8+ T-cells abundance driving NASH-induced hepatocarcinogenesis  
199 by antibody-based treatments in our mouse model (anti-CD8/anti-NK1.1, anti-CD4, anti-TNF;  
200 **Figure 2 and 4, Extended Data 8, 9, 20-23** and **Rebuttal Figure 14, 15, 16k-n, 17-21**), as  
201 well as cross-referencing to the co-submitted manuscript Dudek et al., which describes  
202 molecular mechanisms of CD8+ T-cell-mediated liver damage. Additionally, we dissected  
203 CD8+ T-cell mediated mechanisms driving NASH-induced hepatocarcinogenesis in PD1-  
204 targeted immunotherapy by antibody-based treatments (anti-CD8/anti-PD1, anti-TNF/anti-  
205 PD1, anti-CD4/anti-PD1; **Figure 4, Extended Data 20-23** and **Rebuttal Figure 16k-n, 17-21**).  
206 These data indicated that the abundance of CD8+ T-cells, as well as CD8+ T-cell-derived TNF  
207 plays an important role in boosting liver cancer in the context of NASH/HCC related  
208 immunotherapy. Of note, velocity analyses of scRNA-seq for transcriptional activation, or  
209 proteome analyses of sorted cells could not detect different phenotypes between CD8+PD1+  
210 T-cells derived from mice fed CDHFD with NASH or CDHFD treated with an anti-PD1 related  
211 therapy in the context of HCC development, indicating that the main proportion of CD8+PD1+  
212 T-cells in our preclinical models drive hepatocarcinogenesis and do not restrict HCC (**Figure**  
213 **4, Extended Data 4 and 24** and **Rebuttal Figure 6, 7 and 16**).  
214 Further, our data show that anti-PDL1 therapy lead (**Extended Data 7** and **Rebuttal Figure**  
215 **13**) to the same effects as observed in the anti-PD1 therapy (**Extended Data 6** and **Rebuttal**  
216 **Figure 5**) or in the context of our analyses using PD1 knock-out mice developing NASH/HCC  
217 (**Figure 3, Extended Data 14** and **Rebuttal Figure 22a,b** and **23**).  
218 Data that have not been included in the initial submission of the manuscript indicate that PD-1  
219 targeted immunotherapy-induced hepatic inflammation triggers the enrichment of central  
220 memory-like cells (CD44+CD62L+CD8+) but not T-cells with a naïve character  
221 (CD62L+CD8+) (**Extended Data 6** and **Rebuttal Figure 5n**). This enrichment of memory-like  
222 CD44+CD62L+CD8+ T-cells can be explained by one of two options: these cells might be  
223 expanded and infiltrate the liver upon the anti-PD-1 targeted immunotherapy to either drive  
224 hepatic inflammation or these memory-like T-cells might be indicative of a subset of T-cells  
225 reactive to tumor-associated antigens and thus of CD8+ T-cells of a dual role (**Extended Data**



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226 **6 and Rebuttal Figure 5n**). In respect of the co-submitted manuscript Dudek et al., CD8+ T-  
227 cells drive liver damage and liver cancer in NASH in an antigen-independent manner. Thus,  
228 enrichment of memory-like CD44+CD62L+CD8+ T-cells upon PD-1 targeted immunotherapy  
229 might argue in favor of a dual role of CD8 T-cells. However, tumor size, tumor number per  
230 liver, and tumor incidence are not affected by increased CD44+CD62L+CD8+ T-cells, arguing  
231 against a tumor restricting function of CD8 T-cells in this context. We have improved cross-  
232 referencing of the revised manuscript with the co-submitted manuscript (Dudek et al.).

233 Data described in this manuscript demonstrate that the NASH-induced microenvironment  
234 drives hepatic inflammation in a TCR-independent manner and thus rather describes a  
235 mechanism that activates CD8+T-cells downstream of the TCR through environmental  
236 signaling (e.g. acetate, IL21 signaling), arguing against a tumor antigen-specific CD8+ T-cells  
237 mediated HCC restriction in the context of NASH. It is exactly these CD8+ T-cells which –  
238 altered by the NASH liver microenvironment acquired a pro-tumorigenic phenotype – we can  
239 detect also by analysis of the ICF signature. The latter is predictive of inflammation triggered  
240 liver cancer in humans. Notably, CD8 depletion eliminates this signature, strongly underlining  
241 that CD8 T cells are the main source of driving the pro-tumorigenic environment.

242 3. The data on the NASH- and NASH-HCC-promoting role of CD8+ T-cells is similar to a previous study  
243 from the last author (Wolf et al, Cancer Cell). Hence a number of the findings presented in this  
244 manuscript are incremental with, adding PD1 into this context, with somewhat expected results, as well  
245 as novel techniques such as scRNA-seq.

246 We thank Referee #1 for the opinion on the progress we tried to achieve with this manuscript  
247 as a follow-up study (Wolf et al., 2014). We politely disagree with the statement of Referee #1  
248 – that indicates “...are incremental with, adding PD1 into this context, with somewhat expected  
249 results, as well as novel techniques such as scRNA-seq.”, because:

250 **(i)** Our presented data show for the first time that CD8+PD1+ T-cells and their behavior in  
251 the context of immunotherapy and metabolic syndrome affect liver cancer in an unexpected  
252 manner – CD8+PD1+ T cells are pro-tumorigenic in this context – which very likely has clinical  
253 implications. Identification of increased hepatic abundance of unconventional activated  
254 resident-like CD8+PD-1+ (e.g. CXCR6+, TOX+, TNF+), but not a change of quality in these  
255 cells are the hepatocarcinogenesis-driver in the context of NASH is novel – and can be found  
256 also in the human situation (e.g. two IHC-cohorts across Europe comparing viral vs.  
257 NAFLD/NASH-HCC, one IHC cohort dissecting the abundance of cells depending on NASH  
258 pathology severity; also comparing control vs NAFLD/NASH patient samples by scRNA Seq,  
259 CYTOF and flow cytometry).

260 **(ii)** Our data expand current knowledge of NASH pathology-associated mechanisms (e.g.  
261 auto-aggression in a TCR-independent manner with the co-submitted manuscript Dudek et al.,  
262 corroborating the data in total 3x preclinical models of NASH). Furthermore, we tested this



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263 mechanism hypothesis on a functional level by various antibody-based treatments (PD-L1-  
264 targeted immunotherapy; combination therapy of anti-TNF/anti-PD-1, anti-CD4/anti-PD-1, anti-  
265 CD8/anti-PD1) and now identify that it indeed is TNF and CD8 T cells that promote liver cancer  
266 in the context of PD1-related immunotherapy.

267 **(iii)** Novel comparison/corroboration and in-depth analysis of T-cell populations in human  
268 and mouse NASH by scRNA, flow cytometry and CYTOF. We did not expect a link between  
269 resident-like CD8+PD1+ cells in the progression of NASH pathology and NASH-induced  
270 hepatocarcinogenesis, as well as the correlation of preclinical model to patient data, identifying  
271 NASH as an etiology of unfavorable predictor of response (e.g. the meta-analysis of 1656  
272 patients corroborates non-viral (NASH-related) HCC compared to viral-HCC as less  
273 responsive to immunotherapy (**Figure 6, Extended Data 30-32 and Rebuttal Figure 1d,e and**  
274 **2-4**), as well as our own small retrospective NASH-HCC vs other-etiological-HCC cohort, which  
275 was validated in a second validation cohort of HCC-patients under immunotherapy (**Figure 6,**  
276 **Supplementary Table 9 and Rebuttal Figure 1f,g**).

277 4. The human data are based on a very small and poorly analyzed cohort of patients with NASH-  
278 associated HCC (n=10-11). While the underlying question is important, pairing data from this small  
279 cohort with the data from the mouse model with its above-described limitations and confounders may  
280 send a wrong and potentially deleterious message to the community, and much more careful analysis  
281 as well as larger cohorts are needed to put the provided message on a solid scientific foundation: The  
282 authors should analyze outcomes for NASH-HCC patients with or without cirrhosis to account for the  
283 possibility of worsened NASH in patients without cirrhosis (for which the cohort is much too small).

284 We thank Referee #1 and fully agree, that the presented retrospective  
285 Nivolumab/Pembrolizumab-treated NAFLD/NASH-associated HCC cohort - although unique  
286 for Europe where treatment is not officially licensed - is too small for subgroup analysis for  
287 patients. We have taken this point raised utmost seriously. Thus, we have strengthened our  
288 hypothesis of non-viral (NASH-related) HCC being less responsive to immunotherapy by a  
289 meta-analysis including patients of the three most important clinical trials (1656 patients,  
290 **Figure 6, Extended Data 30-32 and Rebuttal Figure 1d,e and 2-4**).

291 Moreover, we have increased the number of patients in our initial clinical cohort from 65 to 130  
292 HCC patients under anti-PD(L)1-targeted immunotherapy and validated our results in a second  
293 cohort of 118 HCC patients under PD(L)1-targeted immunotherapy (**Figure 6, Supplementary**  
294 **Table 9 and Rebuttal Figure 1f,g**).

295 A disadvantage by nature of a retrospective analysis of cohort across multiple centers is, that  
296 clinical material that would have the potential to characterize in patient subgroups (e.g.  
297 worsened NASH) was not sampled. Furthermore, no paired biopsies or other biological  
298 materials (e.g. blood or serum) before/after immunotherapy were taken in this cohort for HCC  
299 patients, making characterization of treatment response at the single patient resolution and  
300 thus subgroups impossible in this retrospective cohort. Therefore, we decided to investigate



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301 the outcomes for BCLC-C NAFLD/NASH-HCC vs other-etiology-HCC patients with cirrhosis  
302 and observed, that NAFLD/NASH-HCC have significantly reduced overall survival compared  
303 to other-etiology-HCC in this retrospective study. Of note, multivariate analyses identified  
304 NAFLD/NASH as an independent factor for treatment response (**Supplementary Table 9**).  
305 We validated these results in a second independent cohort of 118 under PD1-targeted  
306 immunotherapy based in North America, which included additional n= 11 patients with NASH-  
307 HCC under immunotherapy, corroborating that NASH/NAFLD is a negative predictor to  
308 immunotherapy (main text). We now have toned down the conclusions of our retrospective  
309 cohort in the manuscript and would like to point out, that larger cohorts and prospective clinical  
310 trials are of utmost importance for the scientific community.

311 A. A cohort of n=10-11 NASH-associated HCC patients is unacceptable. Many of the parameters such  
312 as PFS are not significant and it cannot be excluded that inclusion of a larger number of NASH-HCC  
313 patients may change the data significantly.

314 We agree with Referee #1, however we would like to point out attention, that prominent trends  
315 or effects can also be seen in small retrospective cohorts as well. Although unique for Europe,  
316 where treatment is not officially licensed yet, the complete cohort we have gathered is too small  
317 for subgroup analysis for patients.

318 We decided to leave out the non-significant data of TTP and PFS in our manuscript. Moreover,  
319 upon recruiting the validation cohort of 118 HCC-patients under immunotherapy we decided  
320 to not show TTP and PFS, but instead the multivariate analysis (**Supplemental Table 9**).  
321 However, we are in line, that an increased patient cohort allows a more sophisticated analysis.  
322 Thus, as mentioned in the previous comment, we increased our patient cohort (from 65 HCC-  
323 patients to 130 HCC-patients) and validated the results in the second cohort of 118 HCC-  
324 patients under PD(L)1-targeted immunotherapy. Furthermore, we would like to highlight the  
325 message from the performed meta-analysis of 1656 patients, also pointing towards identifying  
326 NAFLD/NASH as a negative predictor of immunotherapy response in HCC. Still, the cohorts  
327 are small, and thus, we toned down the conclusions drawn from this retrospective cohort  
328 analyses (added in the main text, **Figure 6**).

329  
330 B. The authors do not answer the question whether the differences in survival are due to failed  
331 checkpoint therapy or due to other differences between the two cohorts. Most likely, the differences in  
332 survival would persist if the authors removed all responders from the "other etiologies" group. Control  
333 groups that did not receive checkpoint inhibitors are missing to determine if survival is different between  
334 NASH and non-NASH HCC in patients who did not receive checkpoint inhibitors.

335  
336 We thank Referee #1 for raising this important point of potential differences in survival due to  
337 potential confounders. To address these issues, we have submitted our data to multivariate  
338 analyses, which we included in an updated **Supplementary Table 9**. When we excluded  
339 patients with a complete or partial response from the 112 patients with at least one follow-up



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340 imaging, 86 patients were available for analysis (NAFLD, n=9; other etiologies, n=77). Median  
341 OS was significantly shorter in the NAFLD group (5.4 (95%CI, 1.7-9.1) months vs. 10.3  
342 (95%CI, 8.2-12.4) months; p=0.006), as was median TTP (2.4 (95%CI, 2.1-2.7) months vs. 3.9  
343 (95%CI, 2.5-5.4) months; p=0.008), and median PFS (2.4 (95%CI, 1.9-3.0) months vs. 3.7  
344 (2.3-5.1) months; p=0.035). These data suggest that the improved outcome of non-NAFLD  
345 patients is not only driven by the better response rate observed in these patients. However,  
346 the interpretation of these data due to the size of the underlying cohorts needs to be taken with  
347 caution. Like mentioned before, we have now included a meta-analysis with appropriate  
348 control cohorts, identifying immunotherapy vs control for viral HCC as favorable treatment  
349 (HR(viral)= 0.64), in contrast, non-viral-HCC show less benefit (HR(non-viral)= 0.92). In this  
350 meta-analysis patients with NASH-HCC and Non-NASH HCC who did not receive checkpoint  
351 inhibitors are included as receiving either sorafenib (in RCT of front-line) or placebo (in RCT in  
352 second-line). We thank Referee #1 for pointing out the lack of appropriate control groups (e.g.  
353 NASH-HCC vs. different etiology-induced HCC under Sorafenib/different multi-kinase  
354 inhibitors as a second/third-line therapy). Although of extreme interest for public health and  
355 public knowledge, we described this important issue in our discussion and to the best of our  
356 knowledge there are no NASH-HCC treated cohorts available (apart from, possibly, inside of  
357 the big pharma-industry), which would allow an adequate control arm. Available cohorts (El-  
358 Khoueiry et al., 2017; Finn et al., 2019, 2020) are only differentiating between viral vs. non-  
359 viral etiologies, which combine ASH and NASH-induced HCC.

360

361 C. Is there any indication of increase NASH activity in patients receiving Pembro or Nivo?

362 We thank Referee #1 for this important comment. We have added baseline AST and ALT in  
363 the pre-existing and novel cohorts (**Supplementary Table 8**). Like previously mentioned, the  
364 character of the retrospective studies did not allow to obtain paired biopsies before/after  
365 immunotherapy, and bigger cohorts of prospective clinical trials are needed.

366 D. There is no proper analysis of confounding factors.

367 We thank Referee #1 for pointing out this lack of analyses in our initial submission. We have  
368 now performed multivariate analyses, which we included in the main text and in an updated  
369 **Supplementary Tables 8 and 9**. In short: Macrovascular invasion, a negative prognostic  
370 factor in HCC, was less frequent in NAFLD patients (23% vs 49%). NAFLD patients received  
371 immunotherapy more often as first-line therapy (46% vs. 23%), and the proportion of patients  
372 receiving the combination of atezolizumab plus bevacizumab, the only immunotherapy-based  
373 treatment that has succeeded in a phase III trial of advanced-stage HCC so far, was higher in  
374 the NAFLD cohort (23% vs. 5%). Despite these more favorable characteristics, immunotherapy  
375 was less effective in patients with NAFLD, which translated into a worse overall survival (OS)  
376 for the NAFLD cohort: 5.4 (95%CI, 1.8-9.0) months vs. 11.0 (95%CI, 7.5-14.5) months



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377 (p=0.023). Adjusting for other well-known prognostic factors (Child-Pugh class, macrovascular  
378 invasion, extrahepatic metastases, performance status, and alpha-fetoprotein (AFP)), NAFLD  
379 remained independently associated with worse survival (HR 2.6 (95%CI, 1.2-5.6; p=0.017).  
380 These data indicate that PD-1-targeted immunotherapy in HCC patients with concomitant  
381 NASH might lead to unfavorable effects.

382  
383 E. Another problem is mixing Pembro and Nivo groups. Even though the target is the same, the authors  
384 need to provide subgroup analysis for this and increase the number far beyond what they have to make  
385 any meaningful conclusions in these subgroups.

386 We thank Referee#1 for this comment. Nivolumab and pembrolizumab are mostly considered  
387 comparable in solid tumors. Performing a subgroup analysis based on Nivolumab and  
388 pembrolizumab is simply not feasible nor realistic in HCC, even more so in NASH-HCC.

389 We would like to draw attention to other studies performed in solid tumors (NSCLC (Cui et al.,  
390 2020), and Melanoma (Moser et al., 2020)) that show a similar efficacy (although the overall  
391 level of evidence is low): We agree with this point of Referee #1, which we so far have not  
392 been able to make clear. Similar to the previous point (4A.), our retrospective analyses of the  
393 patient cohorts is too small to address these concerns in an in-depth manner.

394 We agree with Referee #1, that both Nivolumab and Pembrolizumab are targeting the molecule  
395 PD-1, with similar response rates of 17-20% as monotherapy in HCC (El-Khoueiry et al., 2017;  
396 Zhu et al., 2018). The consensus in the literature is to combine both PD-1 targeting antibodies  
397 and pool their results. Moreover, we validated these results in the second cohort of 118 treated  
398 immunotherapy treated HCC-patients, including n= 11 NASH-HCC patients.

399 F. Characterization of patients is insufficient - how were other liver diseases excluded, including ALD,  
400 which is not trivial, and especially important in such small cohorts?

401 We thank Referee #1 for raising this important point and would like to draw the attention, that  
402 criteria for the retrospective patient cohort are described elsewhere (Scheiner et al., 2019).

403 We have especially analyzed the parameters to identify NAFLD/NASH from viral (e.g. patient  
404 history, liver histology, MRI, obesity). It should be indicated that the differences between NASH  
405 and BASH are indeed difficult to account for – less so when differentiating between NASH and  
406 ASH. Furthermore, we toned down our statement regarding the effects of immunotherapy in  
407 our patient cohorts/case reports in the revised manuscript.

408  
409 5. Do the authors get the same results when blocking CTLA-4 - which was, even though not approved  
410 for HCC - the first approach and published study to show efficacy of checkpoint inhibitors in HCC?

411 We thank Referee #1 for this important question and would like to draw the attention to a phase  
412 II trial combining TACE with Tremelimumab that did not differentiate between underlying  
413 etiology for the patient outcome or immune population (Agdashian et al., 2019; Duffy et al.,  
414 2016). This phase II trial showed a similar response rate (21-26%) compared to the 17-20%



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415 response rate for PD-1 targeted monotherapy (El-Khoueiry et al., 2017; Zhu et al., 2018).  
416 Clinical consensus for immunotherapy indicates increased hepatotoxicity of CTLA-4-  
417 compared to PD-1-targeting immunotherapy (Zen and Yeh, 2018), arguing in favor of PD-  
418 1/PD-L1-targeting immunotherapies for the future.

419 Although we observed in human Tregs cells CTLA-4 positivity by scRNA-seq and flow  
420 cytometry, in our manuscript CTLA-4 expression was not identified as significantly different  
421 between treatments as shown by scRNA-seq (**Figure 1**: CTLA-4 expression in CD8+ T-cells  
422 comparing ND vs CD-HFD: FC= 0.1894, p= 0.0642; **Extended Data 5**: CTLA-4 expression in  
423 CD4+ T-cells comparing ND vs CD-HFD: FC= 0.2173, p= 0.1431; **Figure 4 and Extended**  
424 **Data 18**). In our mass spectrometry-based data set, we found no significant change of CTLA-  
425 4 abundance (**Extended Data 5 and 18** and **Rebuttal Figure 24e** and **25e**), corroborating our  
426 flow cytometry-based analysis, which had also low CTLA-4 expression in mouse or human  
427 (**Extended Data 18 and 25** and **Rebuttal Figure 10d,e** and **25h**). Thus, we believe that the  
428 application of CTLA-4-targeted immunotherapy is unlikely to cause a positive effect in our  
429 preclinical model. We have discussed the potential use of targeting rather T-cell activation  
430 (anti-CTLA-4) than exhaustion (anti-PD-1 or anti-PD-L1) in combination, or together with a  
431 potential generation of tumor antigens by ablation strategies (e.g. TACE).

432



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**433 Referee #2 (Remarks to the Author):**

434 In their manuscript, Pfister and colleagues aim to show that CD8+PD-1+ T-cells expand during  
435 progressing, diet-induced NAFLD and, upon treatment with anti-PD-1 antibodies, that these cells can  
436 promote carcinogenesis by establishing an inflammatory tumor microenvironment in a diet-induced,  
437 murine model of advanced NAFLD. Additionally, the authors observe a similar, intratumoral  
438 CD8+CD103+PD-1+ T-cell subset in NASH-induced human HCC patients and claim that patients with  
439 NASH-induced HCC respond worse to anti-PD-1 therapy compared to HCC of other origin. While the  
440 seminal observation in this paper is intriguing, namely that anti-PD-1 treatment can exacerbate  
441 tumorigenesis in a murine model of NASH-induced HCC, the authors fail to demonstrate clear causal  
442 relationships between the implicated cell types, liver inflammation and tumor development in the vast  
443 amount of the data they present, which therefore remain largely correlative. I will highlight my major  
444 concerns below.

445 We thank Referee #2 for the concise and detailed comments and understanding of our aimed  
446 key points to be delivered in the manuscript. Also, we thank Referee #2 for pointing out the  
447 limitations of our study of correlative data interpretation rather than functional dissection. We  
448 appreciate Referee`s #2 opinion, that our human cohort results lead to indications of a worse  
449 response rate of NAFLD/NASH-induced HCC compared to non-NAFLD/NASH-HCC upon PD-  
450 1 targeted immunotherapy. We would like to address the referee`s concerns in the following  
451 section point-by-point:

452  
453 1. In the reporting summary, the authors state that “Exclusion criteria was pre-established and the CD-  
454 HFD fed mice which did not show the NASH phenotype, high ALT, AST and body weight, were excluded  
455 from the analysis”. I fail to understand why this decision was taken as these mice offer valuable insight  
456 in the author’s proposed mechanism. Do CD-HFD mice without overt signs of NASH have reduced  
457 CD8+PD-1+ T-cells? Do these mice also less frequently grow tumors upon anti-PD-1 blockade? Do the  
458 T-cells in the livers of these mice fail display an enhanced effector phenotype? Aside from the valuable  
459 experimental insights that could be gained from these mice, the decision to exclude these CD-HFD but  
460 non-NASH mice from analysis also invalidates any claim that links a given diet to a given phenotype  
461 since mice that did not fit the authors’ desired phenotype were excluded.

462  
463 We thank Referee #2 for the above questions. All mice were included in the respective  
464 treatment – as stated in the paper, indicated by the large mouse data sets in **Figure 1-4** in  
465 NAS, ALT, AST, and body weight. Thus, the statement “Exclusion criteria ....” is inappropriate  
466 and a mistake made on our side and is corrected in an updated Reporting Summary. We fully  
467 agree with Referee #2 that these mice “offer valuable insight in the proposed mechanism” and  
468 this is actually why we have included all of them in our analyses.

469 To display the experimental range of mice fed 12 months CD-HFD, we have now performed  
470 correlations of a large number of integrated parameters of each mouse (e.g. tumor incidence,  
471 tumor size, tumor nodule number, immune-histochemistry, serology, flow cytometry data;  
472 **Figures 1 and 4, Extended Data 4 and 24 and Rebuttal Figure 6, 7c-e, 16, and 26**): In more





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473 detail, we have re-analyzed our data sets to dissect the potential correlations of CD8+ T-cells,  
474 PD-1+ T-cells, ALT, fibrosis, NAS, tumor incidence, tumor nodule size, and effector phenotype  
475 - by artificial intelligence and machine learning clustering.

476 We did not analyze the hepatic environment at time points 10, but after 12 months under diet,  
477 after treatment finished, thus a paired analysis of mice with reduced CD8+PD-1+ T-cells and  
478 their reaction to PD-1-targeted immunotherapy is not possible. In 12 months, CD-HFD-fed  
479 mice CD8 (%CD45) and effector CD8 cells (CD8+CD44+CD62L-) correlate positively with  
480 markers of severity of NASH pathology (e.g. ALT, AST, NAS), as well as tumor incidence  
481 (**Extended Data 4** and **Rebuttal Figure 6**). In 12 months CD-HFD-fed mice polarization by  
482 PD-1 of these CD8+ T-cells (CD8+PD-1+(%CD8)) correlate positively with ALT, AST, but not  
483 significantly with NAS or tumor incidence, indicating that the hepatic abundance of CD8+PD-  
484 1+ cells is important for NASH (e.g. CD8+PD-1+ (%CD45) correlates (Spearman correlation  
485  $r= 0.3844$ ,  $p= 0.0058$ ) with NAS, not reported in the paper).

486 Correlation data included in **Extended Data 24** and **Rebuttal Figure 7c-e** shows, that PD-1-  
487 targeted immunotherapy correlates positively with markers of severity of NASH pathology (e.g.  
488 ALT, AST, NAS), with tumor incidence and tumor numbers per liver, and hepatic CD8 T-cells  
489 (e.g. by histology and flow cytometry), effector CD8 cells (CD8+CD44+CD62L-), as well as the  
490 polarization of CD8+PD-1+(%CD8). These data indicate similar to the Referee's comment,  
491 that mice with reduced hepatic CD8 T-cells and thus also less effector CD8 cells  
492 (CD8+CD44+CD62L-) develop fewer tumors, and that in our data set reduced numbers of  
493 hepatic CD8+PD1+ T-cells result in lower NAS and lower tumor incidence upon PD-1-targeted  
494 immunotherapy (**Extended Data 24** and **Rebuttal Figure 7c-e**).

495 We agree with Referee #2, that these data allowed us to gain valuable insights understanding  
496 the phenotype, why some mice develop milder NAFLD/NASH when compared to experimental  
497 controls submitted to similar times of diet feeding, and how this affected PD-1 blockade. We  
498 would like to point out that mice develop NAFLD/NASH at 12 months post-diet start with an  
499 incidence of 100% (please also see **Figures 1** and **Rebuttal Figure 26a-d**).

500  
501 2. The data presented by the authors fail to demonstrate clear causal relationships. As an example, the  
502 authors note in lines 341-343 that a pro-inflammatory hepatic environment is created by TNF upon anti-  
503 PD-1 treatment, yet fail to show supporting evidence that this indeed drives "necro-inflammation" and  
504 accelerated hepatocarcinogenesis. The authors should neutralize TNF in their in vivo models to  
505 determine whether this molecule is indeed required for their phenotype, i.e., inflammatory  
506 microenvironment, liver damage and increased tumorigenicity.

507 We thank Referee #2 for this very important point. We agree with the comment of Referee #2  
508 and therefore have performed anti-TNF treatment in NASH mice with/or without PD-1 targeted  
509 immunotherapy (**Figure 4**, **Extended Data 20 and 21** and **Rebuttal Figure 16k-n, 18 and 19**).



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510 Of note, data from these experiments demonstrate that TNF, derived from CD8+ T-cells is the  
511 main driver of the pro-tumorigenic effects of T-cells in the context of immunotherapy in NASH  
512 (**Figure 3** and **Rebuttal Figure 22e**).

513 Furthermore, we would like to highlight, that our manuscript correlates increased hepatic  
514 abundance of CD8+PD-1+ T-cells upon PD-1-targeted immunotherapy as crucial for driving  
515 hepatocarcinogenesis. Besides, we have now performed additional scRNA-seq and velocity  
516 blot analyses from human patients with NAFLD/NASH or steatosis and compared those with  
517 mouse immune cells. These data demonstrate high similarities between CD8+ PD1+ T-cells  
518 derived from human and mouse NASH livers. Moreover, we would like to draw the attention of  
519 this Referee to the improved cross-referencing to the co-submitted manuscript Dudek et al., in  
520 which the authors also show that TNF is one key molecule driving increased CD8-dependent  
521 hepatic pathogenesis.

522  
523 3. Based on the authors' presented data, this problem can be further expanded. In Figure S9d and S9m,  
524 the authors show an increase in the number of antigen-presenting cells and increased MHC-II  
525 expression. Are these recruited upon liver inflammation? Are they required for liver inflammation?

526 We thank Referee #2 for raising the point about myeloid cells in the context of chronic  
527 inflammation and would like to interpret the data shown in **Extended Data 11** and **Rebuttal**  
528 **Figure 27** in comparison to **Extended Data 8** and **Rebuttal Figure 15**, which now indicates,  
529 that antigen-presenting cells and increased MHC-II expression are a result of increased liver  
530 inflammation upon PD-1 targeted immunotherapy. We would like to highlight our previous  
531 study (Malehmir et al., 2019), which demonstrated, that myeloid cells are correlated with liver  
532 inflammation and are recruited as a consequence of NASH development. Moreover, we have  
533 shown by depletion of antigen-presenting cells, including Kupffer cells (by chlodronate  
534 encapsulating liposomes) abrogates or prevents NASH development.

535 To address the point raised by Referee #2 more experimentally, we analyzed our mouse  
536 cohorts in total by AI, which indicates that hepatic MHCII+ cells correlate positively with NASH  
537 pathology (weight, NAS, ALT, AST, cholesterol, fibrosis by Sirius Red staining, hepatic  
538 concentrations of MCP-1, CCL3, MIP-2, and IL-21) and MHCII+ as a marker of myeloid  
539 activation on different subsets correlated predominantly in CD11b+CD11c+ (myeloid dendritic  
540 cells (CD11b+CD11c+) with ALT, GOT, NAS in 12 months CD-HFD-fed mice (**Extended Data**  
541 **4** and **Rebuttal Figure 6**). To dissect the Referees question in our experimental functional  
542 antibody-treatment experiments (**Extended Data 24** and **Rebuttal Figure 7c-e**). MHCII+ cells  
543 correlate positively with CD-HFD and CD-HFD+PD-1-targeted immunotherapy, as well as  
544 NASH pathology (weight, NAS, ALT, AST, cholesterol, fibrosis by Sirius Red staining, hepatic  
545 concentrations of MCP-1, CCL3, CCL4, MIP-2, and IL-21) in 12 months old mice. Moreover,  
546 MHCII+ as a marker of myeloid activation on different subsets correlated for CD11b+MHCII+  
547 and mDC+MHCII+ positive with PD-1-targeted immunotherapy, ALT, AST, NAS CCL4, and



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548 MIP-2. pDC+MHCII+ and KC+MHCII+ cells correlated negatively in CD8-depleted and  
549 CD8+NK1.1 co-depleted animals. The latter myeloid subset correlates positively with fibrosis  
550 and tumor incidence when pooling the data of all treatments.

551 We would like to highlight our previous study (Malehmir et al., 2019), which showed, that  
552 myeloid cells are correlated with liver inflammation and are recruited as a consequence of  
553 NASH development. However, a genetic study using CCR2<sup>-/-</sup> mice (impaired myeloid  
554 recruitment upon inflammation) developed NASH and NASH-induced tumors; in contrast,  
555 Rag1<sup>-/-</sup> mice with functional myeloid but impaired adaptive immune compartments were  
556 protected from NASH and NASH-induced tumors (Wolf et al., 2014). These data argue, that  
557 myeloid cells are recruited to the liver, extend, and fine-tune liver inflammation.

558  
559 4. In Figure S11 the authors show an increase in many inflammatory mediators upon anti-PD-1 therapy;  
560 which of these are required for the accelerated carcinogenesis? While the authors propose a mechanism  
561 based on liver inflammation leading to increased hepatocarcinogenesis upon anti-PD-1 blockade, they  
562 provide little if any conclusive evidence for this hypothesis.

563 We thank Referee #2 for asking this important question. We believe that the inflammatory  
564 mediators for increased hepatocarcinogenesis stem from the increase of CD8+ T-cells upon  
565 anti-PD1 immunotherapy. Importantly, by performing depletion experiments of different T-cell  
566 subsets – anti-CD8 or anti-CD4, we can demonstrate that the CD8+ T-cells but not CD4+ T-  
567 cells are needed for driving hepatocarcinogenesis and driving the pro-tumorigenic effect of  
568 anti-PD1-related immunotherapy (**Figure 4, Extended Data 20-23 and Rebuttal Figure 16,**  
569 **18-21**). Of note, PD-1-targeted immunotherapy increases the hepatic abundance of  
570 CD8+PD1+ T-cells in vivo (e.g. **Extended Data 11 and Rebuttal Figure 27d,e**), as well as  
571 increases the number of CD8+PD1+ cells in vitro (**Extended Data 18 and Rebuttal Figure**  
572 **25I**). To understand the nuances of the observed necro-inflammation, anti-PD1-related  
573 immunotherapy, and liver cancer formation, we perform correlations analysis of fibrosis, tumor  
574 nodule number, tumor size, ALT, NAS, CD8, and PD-1 expression by machine learning and  
575 neuronal networking (**Figures 1 and 4, Extended Data 4 and 24 and Rebuttal Figure 6, 7c-**  
576 **e, 16, 26h**).

577 We have analyzed the inflammatory environment looking into a specific signature (ICF) on the  
578 transcriptional level in NASH mice with and without anti-PD1-related immunotherapy (**Figure**  
579 **3 and Rebuttal Figure 22d**). This transcriptional ICF signature is a predictor of liver cancer  
580 formation triggered through inflammation in humans. It can be stated that the altered  
581 inflammatory signature of NASH livers in the context of anti-PD1-related immunotherapy  
582 overlaps with a signature that from human patients is known to have a bad prognosis and high  
583 correlation with inflammation triggered liver cancer. Importantly, upon CD8+ T cell depletion  
584 the intrahepatic ICF signature is downregulated - demonstrating that CD8+ T cell-derived  
585 inflammatory mediators might be linked with liver cancer formation.



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586 Moreover, to identify factors secreted in relation to CD8+ T-cells in NASH livers (as identified  
587 by their reduction upon anti-CD8 treatment) we have performed *in situ* RNA hybridization  
588 analyses for several cytokines. Further, we have performed flow cytometry and RNA-seq of  
589 hepatic tissues as well as scRNA-seq from human and mouse immune cells. Doing so, we  
590 have identified T-cell derived TNF as a possible, important candidate for increased  
591 hepatocarcinogenesis upon PD1-targeted immunotherapy.

592 To test this hypothesis on a functional level, we performed an anti-PD1/anti-TNF as well as an  
593 anti-TNF treatment alone. These experiments demonstrate that TNF is a functionally important  
594 cytokine contributing to the anti-PD1 antibody treatment mediated pro-carcinogenic effect.

595 Besides, we would like to draw attention to the improved cross-referencing to the co-submitted  
596 manuscript Dudek et al., which shows that TNF and IL-15, a target downstream of IL-21 - both  
597 upregulated upon anti-PD-1 therapy - are crucial mediators of CD8-mediated hepatic cell  
598 death. In line, literature highlights the crucial role of TNF for hepatocarcinogenesis (Nakagawa  
599 et al., 2014; Park et al., 2011; Pikarsky et al., 2004) and that anti-TNF treatment uncouples the  
600 toxicity of CTLA-4/PD-1-targeted immunotherapy (Perez-Ruiz et al., 2019).

601  
602 5. Some of the data the authors present seems internally inconsistent. As an example, the authors  
603 postulate that the pro-inflammatory hepatic environment is responsible for the increase in liver cancer  
604 incidence in anti-PD-1-treated mice, which they underscore by an increase in inflammatory cytokines in  
605 the liver microenvironment (Figure S11). However, they also show that upon CD8 depletion, which  
606 reduces cancer incidence, the inflammatory cytokines do not significantly reduce compared to the CD-  
607 HFD diet mice alone. This implies that the inflammatory microenvironment is not actually responsible  
608 for increased cancer incidence. How do the authors harmonize these findings?

609 We thank Referee #2 for his comment on the bivalence of cellular and micro-environmental  
610 induced cell death, inflammation, and liver cancer formation. However, we firmly state, that our  
611 data is not internally inconsistent, and have added several experiments that clarify the  
612 mechanisms of action. We state, that anti-PD-1 therapy induces an increased hepatic  
613 inflammatory microenvironment, indicated by a) increased abundance of hepatic immune cells  
614 (mainly CD8+ and CD8+PD-1+ cells) (**Figure 2 and Extended Data 11 and Rebuttal Figure**  
615 **14, 27**); b) by increased inflammation-associated cytokines (e.g. IFN $\gamma$ , TNF, IL-21, IP10, MCP-  
616 1, CCL3) (**Extended Data 13 and Rebuttal Figure 28**); c) on mRNA expression levels we  
617 actually clearly see the increase in all pathways relevant for inflammation induced liver cancer  
618 - as analyzed by the ICF-signature (**Figure 3 and Rebuttal Figure 22d**). Thus, we think, that  
619 there are 2 components (first cells, like CD8+ T-cells and second, the inflammatory liver  
620 environment) responsible for (increased) liver cancer incidence.

621 We agree with Referee #2 that initially this appears not logic - but we believe that a liver tissue  
622 homogenate analysis cannot uncover the CD8+-T cell restricted cytokine changes, as other  
623 immune cells will still produce inflammatory immune cells. This is indicated for example in



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624 **Figure 3** and **Rebuttal Figure 22e**, which shows, that upon CD8 depletion TNF+ cells are  
625 significantly reduced by *in situ* hybridization. Again, effects of the CD8 depletion manifests  
626 strongly on mRNA expression level as pathways relevant for inflammation induced liver cancer  
627 are strongly reduced - as analyzed by the ICF-signature (**Figure 3** and **Rebuttal Figure 22d**).  
628 Moreover, as stated by the Referee it appears that anti-CD8 treatment alone did not reduce,  
629 but anti-CD8/anti-PD-1 did reduce several chemokines indicative of a hepatic inflammatory  
630 environment on protein level, that are responsible for myeloid cell attraction like MCP-1, CCL2,  
631 CCL3, MIP-3a, or alarmins like IL-33 when compared to anti-PD1 alone (**Extended Data 10**  
632 and **21** and **Rebuttal Figure 19c-e** and **29**).

633 Moreover, we want to point out that our data are also confirmed by the co-submitted manuscript  
634 Dudek et al., revealing that the mechanisms of CD8+ T-cell mediated cell death is 1) CD8+ T-  
635 cell dependent, 2) TCR independent, and 3) TNF is a crucial cytokine sensitizing the CD8+ T-  
636 cell to get auto-aggressive and thus starts to mediate cell death.

637 We demonstrate that TNF is a marker of a pro-inflammatory, pro-carcinogenic hepatic  
638 environment and that it is increased upon PD-1-targeted immunotherapy and remains high in  
639 CD8+ depleted mice (**Extended Data 10** and **Rebuttal Figure 29**). However, CD8 depleted  
640 mice lack tumor development (**Figure 2** and **Rebuttal Figure 14q**). In line with Referee #2 and  
641 the co-submitted manuscript Dudek et al., we think, that the presence of CD8+ T-cells is  
642 essential to drive hepatocarcinogenesis. We thus have performed the above mentioned CD8  
643 depletion combined with PD-1 targeted immunotherapy to underline that CD8+ T-cells are  
644 essential for increased hepatocarcinogenesis upon PD-1-targeted immunotherapy compared  
645 to control mice under CDHFD diet (**Figure 4** and **Extended Data 20+21** and **Rebuttal Figure**  
646 **16, 18** and **19**).

647 We have functionally strengthened data shown by Dudek et al. that TNF - as a marker of the  
648 inflammatory environment - is crucial for sensitizing the hepatic microenvironment to CD8 T-  
649 cell -mediated cell death by performing anti-TNF with/without PD-1-targeted immunotherapy.  
650 This has allowed the interpretation and has been experimentally demonstrated that only an  
651 inflammatory environment combined with the presence of CD8 T-cells drives increased  
652 hepatocarcinogenesis upon PD-1-targeted immunotherapy (**Figure 4, Extended Data 20+21**  
653 and **Rebuttal Figure 16, 18** and **19**).

654 Furthermore, to shed new light on potential compensatory immunological mechanisms of  
655 CD4+PD-1+ T-cells in the context of PD-1-targeted immunotherapy, we have performed CD4  
656 depletion with/without PD-1-targeted immunotherapy (**Extended Data 22 and 23** and **Rebuttal**  
657 **Figure 20** and **21**). Notably, these experiments indicate that in contrast to CD8+ T-cells CD4+  
658 T-cells do not play a major effector role in comparison to CD8+ T-cells in anti-PD1 related liver  
659 cancer formation in the context of NASH and anti-PD1 treatment (**Figure 16n**).



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660 6. Crucially, and related to my previous point, the authors also did not perform CD8 depletion  
661 in the context of anti-PD-1 treatment to show that CD8 cells are indeed the cells that are  
662 responsible for increased carcinogenesis upon anti-PD-1 therapy.

663  
664 We thank Referee #2 for this important comment and fully agree that anti-PD-1 treatment in  
665 the context of CD8 depletion is crucial for data interpretation and we included this experiment  
666 in a revised manuscript (**Figure 4, Extended Data 20 and 21 and Rebuttal Figure 16, 18 and**  
667 **19**). The combined anti-CD8/anti-PD-1 treatment has allowed an understanding on a functional  
668 level, that indeed increased the hepatic abundance of CD8+PD-1+ T-cells upon PD-1-targeted  
669 immunotherapy is crucial for driving hepato-carcinogenesis. Notably, this treatment reduced  
670 NAS, liver damage and some cytokines (e.g. MCP-1, CCL2, CCL3, MIP-3a) that affect the  
671 pathway of CD8+ T-cell activation by the liver environment (e.g. IL33, IL21).

672  
673 7. At times, the authors are (highly) selective in the data they choose to discuss and interpret. As an  
674 example, regarding Figure 1i, the authors describe the CD8+ T-cells in CD-HFD mice to demonstrate  
675 profiles of cytotoxicity and effector function because of increased expression of GzmK/M and Pdcd1.  
676 However, in the same plot shows that these cells have reduced expression of GzmA/B, Klrg1, Il2ra, TNF  
677 and Il2; all markers of effector/cytotoxicity. How do the authors harmonize these observations?

678  
679 We thank Referee #2 for asking this important question. As Referee #2 highlighted in the  
680 example of **Figure 1**, we think it is of vital importance to display the observed profile of CD8 T-  
681 cells on a broad scale. We believe that this particular character of T cells – that initially appears  
682 to be exhausted (e.g. TOX expression) is actually hyperactivated with a particular pattern of  
683 expression.

684 Thus, the single-cell technology allows dissecting the expression profile of CD-HFD-fed CD8+  
685 T-cells into a combination of cytotoxicity/exhaustion expression, indicative of a unconventional  
686 activation/effector. To not lose single-cell resolution and how the data translates into proteins,  
687 we have corroborated these data by mass-spectrometry. These data corroborated the scRNA-  
688 data of **Figure 1** with enrichment for effector function (e.g. T-cell activation, T-cell  
689 differentiation, and NK mediated cytotoxicity) in CD-HFD-fed CD8+PD-1+ T-cells (**Extended**  
690 **Data 5 and Rebuttal Figure 24**). Thus, we decided to display a wide variety of markers of  
691 effector function/cytotoxicity allowing the reader a more sophisticated view into the phenotype.  
692 Moreover, we have compared this pattern with human NASH and indeed could find that  
693 patients with NASH do resemble a similar pattern.

694 To test this unconventional activation/exhaustion phenotype on a functional level, we  
695 performed all the treatments described in **Figures 2-4** in the absence or in the presence of  
696 anti-PD1-related immunotherapy (anti-CD8, anti-CD8/anti-NK1.1, anti-CD8/anti-PD1, anti-  
697 PD1, anti-PDL1, anti-TNF, anti-TNF/anti-PD1, and as control experiment anti-CD4 and anti-  
698 CD4/anti-PD1), as well as the corroboration with the human data.



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699 For example, an increased anti-inflammatory role by IL-10 expressing CD8+ T-cells upon PD1-  
700 targeted immunotherapy could not be corroborated (**Extended Data 19** and **Rebuttal Figure**  
701 **30k**) (Breuer et al., 2020). Of note, in this publication diet-based NAFLD induction was  
702 achieved by feeding either WD or CD-HFD for 8-10 weeks. This is in strong contrast to our  
703 experimental regime of applying diet for 3, 6, or 12 months as we show, that the preclinical  
704 model presents different stages of NASH pathology severity including hepatocarcinogenesis  
705 (**Figure 1** and **Rebuttal Figure 26a-d**).

706 Furthermore, we would like to draw attention to the improved cross-referencing to the co-  
707 submitted manuscript Dudek et al., which confirmed a CD8 profile of effector  
708 function/exhaustion/cytotoxicity on a functional level (e.g. TNF sensitizing, high Granzyme  
709 expression, TCR-independent mediated cell death). Moreover, we tried to improve the  
710 discussion on recent literature on the role of CD8 T-cells in metabolic diseases.

711  
712 8. Regarding Figure 1e, the authors state that CD-HFD contain a significantly altered immune  
713 composition that mainly affects the CD8+ T-cell compartment. However, this finding was not  
714 significant ( $p=0.09$  for CD8+PD-1+ T-cells and ns for CD8+ T-cells). In this plot, the authors  
715 do show significant differences in frequency of CD4+ T-cells ( $p<0.01$ ), classical monocytes  
716 ( $p<0.01$ ) and MDMs Ly6CHigh ( $p=0.01$ ). Why are these cell types not regarded as interesting?  
717 Are these cells responsible for the authors' proposed phenotype? In line 259 the authors state  
718 that there are only minor differences in the CD4 compartment, yet when looking at the data  
719 (Figure S9h and Figure S9f) the difference in the CD4 subset of CD62L-CD44+CD69+ upon  
720 anti-PD-1 blockade is as strong as, if not stronger than, in the same subset of CD8 T-cells,  
721 which the authors do deem interesting.

722  
723 We thank Referee #2 pointing out these details in our analysis. We agree with Referee #2, that  
724 immunological subsets represented in our data set are well described in the literature (e.g.  
725 reduction of CD4+ T-cells (Ma et al., 2016) and changes in the myeloid compartment, including  
726 classical monocytes and MDMs Ly6CHigh (Malehmir et al., 2019; Nakagawa et al., 2014),  
727 therefore the respective citations are included in our introduction and discussion.

728 We added new data and have re-analyzed the data displayed in **Figure 1e** according to  
729 Referee`s #4 comments also by highlighting NKT cells. These results, in CD8+PD1+ ( $p= 0.03$ ),  
730 significantly changed. Other changed cellular subsets after 12 months of CD-HFD feeding are  
731 CD4+ T-cells ( $p= 0.04$ ), classical monocytes ( $p< 0.01$ ), KC ( $p= 0.01$ ), MDMs ( $p=0.02$ ), MDMs  
732 Ly6C+ ( $p< 0.01$ ). We agree with Referee #2, that CD4 T-cells and their expression of PD-1  
733 might play a crucial role in shaping the liver micro-environment and in the observed phenotype  
734 and thus included analysis of CD4 T-cells to the majority of our experiments (e.g. **Extended**  
735 **Data 3** and **Rebuttal Figure 8c-h**).



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736 However, the magnitude of effects observed in CD4+ T-cells is minor when compared to CD8+  
737 T-cells (e.g. **Extended Data 11** mean (CD8+CD62L-CD44+CD69+) ~12% (%of CD45+) vs  
738 mean (CD4+CD62L-CD44+CD69+) ~4% (%of CD45+) upon PD-1 targeted immunotherapy).  
739 Data obtained from CD4 depletion with/without PD1-targeted immunotherapy indicate, that the  
740 increased hepatocarcinogenesis in the context of immunotherapy is independent of hepatic  
741 abundance of CD4+ T-cells in the preclinical NASH model (**Figure 4, Extended Data 22 and**  
742 **23 and Rebuttal Figure 16n, 20 and 21**).

743 However, CD4+ T-cells might have a diverse set of effector functions (e.g. interpreting tumor  
744 incidence in anti-CD8/anti-PD1 treated animals: although CD4 cells show trends for  
745 decreasing, CD4 are relatively increased in the absence of CD8+ T-cells but immunotherapy,  
746 thus CD4+ T-cells might be responsible for baseline tumor incidence in the context of  
747 immunotherapy (**Extended Data 22 and 23 and Rebuttal Figure 20 and 21**); or CD4 might  
748 have a tumor controlling role, as there are the trends of increased tumor incidence upon anti-  
749 CD4/anti-PD1 co-treatment (tumor incidence (anti-PD-1 mono-treatment)= 75% vs tumor  
750 incidence (anti-CD4/anti-PD1 co-treatment)= 88%) (**Figure 4 and Rebuttal Figure 16n**)).

751 Of note, CD4+ T-cells might also significantly changed in the human situation, and have also  
752 analyzed human CD4+ cells a by scRNA-Seq (**Extended Data 25c and Rebuttal Figure 10c**).  
753 In addition, we have performed RNA velocity analyses of the scRNA Seq data of mouse and  
754 human CD4 T cells. In mouse, no significant velocity flow was detected in 12 months CD-HFD-  
755 fed mice, indicating, that CD4 cells are not transcriptionally activated and driven by NASH-  
756 conditions or PD-1-targeted immunotherapy in NASH. However, we want to point out, that in  
757 the mouse NASH model CD8 T-cells increase statistically significant, and thus CD4 are  
758 relatively fewer cells compared to CD8. Therefore, the velocity analysis of mouse CD4 T-cells  
759 need to be taken with caution, because we included 300-500 cells only per described subset.  
760 As a consequence, we included the negative CD4 T-cell data not in the manuscript but in the  
761 Rebuttal letter as **Rebuttal Figure 31**. Velocity analyses on human CD4 lead to comparable  
762 problems like seen in mouse. As a consequence, we included the negative CD4 T-cell data  
763 not in the manuscript but in the Rebuttal letter as **Rebuttal Figure 31**.

764 Like previously mentioned in point 3 raised by Referee #2 concerning the myeloid cells, our  
765 presented data argue, that myeloid cells are recruited to the liver, extend and fine-tune liver  
766 inflammation. While we see MDMs Ly6C+ cells increased comparing 12 months ND vs CD-  
767 HFD-fed mice, our functional treatments (anti-PD-1, anti-CD8/anti-PD-1, anti-TNF, anti-  
768 TNF/anti-PD-1, anti-CD4 and anti-CD4/anti-PD-1) did not result in significant changes in  
769 CD11b+Ly6C+ cells, indicating a rather minor role in comparison to the changes we observed  
770 in the CD8 compartment (**Extended Data 4, 21, 23 and 24 and Rebuttal Figure 6, 7c-e, 19**  
771 **and 21**).





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772 Furthermore, we discuss the myeloid changes and potential role of CD4+ T-cells in greater  
773 detail in the main text.

774 Finally, we performed an anti-CD4 antibody treatment with or without the combination of anti-  
775 PD1-related immunotherapy. Anti-CD4 antibody treatment successfully depleted or strongly  
776 reduced intrahepatic CD4+ T cells in NASH. However, depletion of CD T cells did not reduce  
777 liver cancer incidence – which is in contrast to CD8+ T cell depletion. Rather, in contrast, CD4  
778 T cell depletion showed a trend in increase of tumor incidence – in line with published data by  
779 (Ma et al., 2016).

780  
781 9. Along these lines, in line 387 the authors state that consistent with previous results, effects on the  
782 CD4+PD-1+ T-cell compartment remained minor, yet the differences observed for matching analyses  
783 (i.e. S17a vs S17g, S17b vs S17f, S17i vs S17j) of CD4 and CD8 populations show similar, if not  
784 stronger, effects for the CD4 T-cell population. Why are these differences disregarded by the authors?

785  
786 We believe that the comment of Referee #2 is important and we are in line that the context of  
787 highlighting potential CD4-mediated effects in the context of PD-1-targeted therapy had to be  
788 investigated in detail (e.g. in **Extended data 5, 18** and **Rebuttal Figure 15** and **24**). In line with  
789 the comment of Referee#2, we set out to investigate the character and function of CD4+ T-  
790 cells by scRNA-seq analyses in human and mouse NASH livers, but like raised in point 8 of  
791 Referee #2 strongly suggest to take the velocity analysis of mouse CD4 T-cells with caution,  
792 because we included 300-500 cells only per described subset. Thus, we included these  
793 analyses in only in the **Rebuttal Figure 31**. Moreover, our experiments using an anti-CD4  
794 depleting antibody alone or in the context of anti-PD1-related immunotherapy indicate a minor  
795 role of the CD4 compartment in our model as well (**Extended Data 22, 23** and **Rebuttal Figure**  
796 **20** and **21**).

797 As mentioned in point 8 raised by Referee #2, we agree with Referee #2, that similar  
798 phenotypes can be observed when comparing effects in CD4+ and CD8+ T-cell subsets upon  
799 PD-1 targeting immunotherapy. We do not disregard the changes in the CD4 compartment but  
800 would like to draw attention to the magnitude of changes in the setting of chronic hepatic  
801 inflammation – and the functional experiments with anti-CD8, anti-CD8/anti-PD-1, anti-CD4,  
802 and anti-CD4/anti-PD1 antibodies.

803 We have also discussed the relevant literature as well as our data on CD4+ T cells in the  
804 discussion in detail. We, in addition, believe that the CD4+ T-cell depletion experiments  
805 with/without PD-1 targeted immunotherapy in mice have enabled us to strengthen our  
806 hypothesis on a more functional level: CD4 depletion alone or in the context of anti-PD1-related  
807 immunotherapy in NASH-induced HCC failed to revert/prevent liver cancer formation. In  
808 contrast, anti-CD8 depleting antibody treatment alone reverted/prevented liver cancer  
809 formation. The role of CD4+ T-cells in the context of immunotherapy remains to be defined in  
810 more detail, as CD4-depletion did not lead to a reversal of the pro-tumorigenic effects of anti-



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811 PD1 therapy in the context of NASH induced HCC. However, CD4+ T-cells might exert a  
812 protective/controlling role in the context of PD1-targeted immunotherapy and presence of  
813 CD8+ T-cells, as combinatorial treatment of anti-CD4 depletion and PD1-targeted  
814 immunotherapy led to an increase of tumor incidence compared to anti-PD1 treatment alone  
815 **(Figure 4, Extended Data 22 and 23 and Rebuttal Figure 16n, 20 and 21)**.

816  
817 10. Similarly, in Figure 5a, the authors claim that a CD8+PD-1+ T-cell population arises upon NASH.  
818 However, there is a, perhaps even stronger, depletion of an Eomes+ gamma-delta T-cell subset.  
819 Additionally, a very strong induction of a CD4+CD27+ population is observed in NASH samples. Why  
820 are these not discussed? Can these populations also be identified in the authors' murine models? Do  
821 these contribute to the authors' described phenotype? The authors should deplete CD4 T-cells and  
822 gamma-delta T-cells in their murine models, as these cell types may, at the very least, contribute to  
823 what occurs in patients.

824  
825 We thank Referee #2 for raising this important concern. Indeed, we have so far not discussed  
826 the loss of gamma-delta T-cell subsets or a potential increase of CD4+ T-cells and included  
827 this now thoroughly in the revised version of the manuscript **(Extended Data 3, 21, 23, 25 and**  
828 **26 and Rebuttal Figure 8, 19, 21 and 10, 11)**. In line with the comments of Referee#2, we  
829 have now described and discussed these populations in detail, by scRNA-seq and multicolor  
830 flow cytometry in mouse and three distinct human cohorts recruited from 3 different centers  
831 across Europe.

832 As mentioned in points 8 and 9 raised by Referee #2, we have depleted CD4 T-cells  
833 with/without PD-1 targeted immunotherapy. Of note, CD27 could not be detected in our  
834 scRNA-seq data set obtained from the preclinical mouse model as significantly changed. In  
835 human bulk RNA-seq CD27 expression increased, but CD4 expression decreases with the  
836 severity of pathology. CD27+CD4+ T cells did not reach statistical significance in our cohorts  
837 by flow cytometry **(Extended Data 25 and Rebuttal Figure 10)**. Of note, in our second cohort,  
838 CD4+ T-cells are significantly enriched in NAFLD/NASH patients by flow cytometry, however  
839 as this cohort was analyzed retrospectively, we could not analyze CD27 expression **(Extended**  
840 **Data 25)**. Furthermore, the abundance of CD4+CD27+ cells was not increased in our human  
841 scRNA cohorts **(Extended Data 27 and Rebuttal Figure 12)**.

842 As mentioned in point 8 we have performed a velocity analyses of the scRNA Seq data of  
843 mouse CD4 T cells (see Rebuttal letter below). In mouse, no significant velocity flow was  
844 detected in 12 months CD-HFD-fed mice, indicating, that CD4 cells are not transcriptionally  
845 activated and driven by NASH-conditions or PD-1-targeted immunotherapy in NASH.  
846 However, we again want to point out, that the velocity analysis of mouse CD4 T-cells need to  
847 be taken with caution because we included 300-500 cells only per described subset. As a  
848 consequence, we included the negative CD4 T-cell data not in the manuscript but in the  
849 Rebuttal letter. Velocity analyses on human CD4 lead to comparable problems as seen in



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850 mouse. As a consequence, we included the negative CD4 T-cell data not in the manuscript but  
851 in the Rebuttal letter as **Rebuttal Figure 31**.

852 We agree that  $\gamma\delta$  T-cells might be involved in underlying processes of NASH or NASH to HCC  
853 transition – also in the context of PD1-related immunotherapy. In humans, our data is not  
854 conclusive in all experiments, e.g. our data indicate for  $\gamma\delta$  T-cells, if we compare: bulk RNA-  
855 seq indicates a reduced expression in severe NASH pathology of EOMES, TRDC, and TRGC1  
856 (**Extended Data 28** and **Rebuttal Figure 32**), however, both flow cytometry cohorts and the  
857 scRNA-seq cohort indicate no change of either  $\gamma\delta+$  T-cells or  $\gamma\delta+$  Eomes+ T-cells comparing  
858 control vs NAFLD/NASH patients (**Extended Data 25, 27** and **Rebuttal Figure 10** and **12**).

859 Corroborating the human flow cytometry data in our mouse model upon NASH establishment,  
860 we detected no difference in hepatic abundance of  $\gamma\delta$ -T-cells between chow- or CD-HFD-fed  
861 control mice. Furthermore, data presented in **Figures 1 and 4** and **Extended Data 3** argues  
862 against the major contribution of gamma delta T-cells in the mouse model of NASH. Here, we  
863 did not observe significant differences in the “other leukocytes” subset. In the revised  
864 manuscript, we analyzed  $\gamma\delta$ -T-cells separately to strengthen the point, that these cells are not  
865 significantly changed upon diet feeding (included in **Extended Data 3, 20-23** and **Rebuttal**  
866 **Figure 8j, 18-21**).

867  
868 11. The patient data is not convincing, but also does not match their murine models. In Figure 5a, the  
869 authors show that CD8+GzmB+ cells are specifically lost in NASH samples which seems to counteract  
870 the claim made by the authors that inflammatory CD8 T-cells cause liver inflammation and associated  
871 carcinogenesis. The authors similarly show in S19a that IFN $\gamma$ , Ccl3 and PD-L1 are in fact reduced in  
872 advanced NASH samples; does the loss of these inflammatory genes not counteract the claims made  
873 in Figure 3g, S4d, S10, S11 and S13a?

874  
875 We thank Referee #2 for raising this important point and agree, that GzmB+CD8+ population  
876 is decreased as well as GzmB expression in bulk RAN-seq (**Extended Data 28** and **Rebuttal**  
877 **Figure 32a**), other populations, on the other hand, are increased. GzmB is a strong indication  
878 for inflammatory CD8+ T-cells. We would like to draw attention to the improved cross-  
879 referencing to the co-submitted manuscript Dudek et al., in which Gzmb along with other  
880 cytotoxic effector molecules (e.g. TNF) are key mediators of a hepatic inflammatory  
881 environment, but not the executing molecules driving hepatocarcinogenesis. However, we  
882 agree with Referee #2, that the data presented in **Figure 5** has limitations due to the small  
883 sample size, although we could reproduce the cellular abundance between healthy vs  
884 NAFLD/NASH patients in a second cohort from a second center (**Figure 5** and **Extended Data**  
885 **25** and **Rebuttal Figure 9** and **10**).

886 We agree with Referee #2, that certain inflammatory genes (e.g. Ifny, Ccl3, Cd274) show  
887 decreased expression along with NASH progression, however, how this translates into local  
888 hepatic proteins-expression remains elusive (e.g. for human gene expression vs



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889 immunohistochemical staining of Pdccl1 in NASH F1-3 (**Figure 6** and **Rebuttal Figure 1a,b**);  
890 or F0-F4 for CD4, or CD274 (**Extended Data 28** and **Rebuttal Figure 32**). As an example,  
891 human PD-L1 increases with NASH severity on IHC, which is corroborated by the preclinical  
892 model (**Extended Data 3, 20, 22** and **Rebuttal Figure 8k,l, 18** and **20**).

893 To shed more light on the phenomena, we focused on our human scRNA-seq on the analyses  
894 of CD8+ T-cells (**Figure 5, Extended Data 27** and **Rebuttal Figure 9** and **12**) and correlated  
895 these cells to the CD8+ T-cells analyzed from our preclinical model (**Figure 5** and **Rebuttal**  
896 **Figure 9f,j**). These data match each other very well, strengthening in our opinion hypotheses  
897 and conclusions drawn from the preclinical NASH-model. Therefore, we do not think the results  
898 of the bulk RNA-seq counteracts the claims of previous figures from the mouse model but  
899 allows an in-depth understanding of underlying inflammation in different NASH stages (e.g.  
900 Referee #1: decrease activity of NASH with disease progression to HCC).

901  
902 12. Lastly, the majority of patient data are not significant and show weak effect sizes; is it fair to draw  
903 strong conclusions on the basis of these data as the authors do?

904 We agree with Referee #2 and thus recruited additional patients to increase the number of  
905 patients in our initial clinical cohort from 65 to 130 HCC patients under anti-PD(L)1-targeted  
906 immunotherapy and validated our results in a second cohort of 118 HCC-patients under PD-  
907 1-targeted immunotherapy (**Figure 6** and **Rebuttal Figure 1f,g**).

908 We agree with Referee #2, that the presented retrospective PD(L)1 targeted immunotherapy  
909 treated NAFLD/NASH-associated HCC cohort - although unique for Europe and treatment not  
910 officially licensed and thus reimbursement - is still small, although we would like to point out,  
911 that prominent trends or effects can be seen in small retrospective cohorts as well. Thus, our  
912 analyses of BCLC-C NAFLD/NASH-HCC vs. other-etiology-HCC patients indicated, that  
913 NAFLD/NASH-HCC have significantly reduced overall survival compared to other-etiology-  
914 HCC in this small retrospective cohort. Multivariate analyses identified NAFLD/NASH as an  
915 independent factor for treatment response and thus identifying NAFLD/NASH as a negative  
916 predictor for HCC immunotherapy (**Supplementary Table 8**).

917 We corroborated our hypothesis of non-viral (NASH-related) HCC being less responsive to  
918 immunotherapy by a meta-analysis including 1656 patients of the three most important clinical  
919 trials, identifying immunotherapy vs control for viral HCC as favorable treatment ( $HR(viral)=$   
920  $0.64$ ), in contrast, non-viral-HCC showed less benefit ( $HR(non-viral)= 0.92$ ) for immunotherapy  
921 (**Figure 6, Extended Data 30-32, Supplementary Table 9** and **Rebuttal Figure 1-4**).

922 Based on these data we want to point out that it is - as indicated by Referee#2 - of the highest  
923 importance to us to specifically define/tone down appropriately the message of our manuscript:  
924 Our manuscript did not intend to indicate that immunotherapy is not beneficial for HCC patients.  
925 It rather demonstrates that HCC patients with viral etiologies do respond well and achieve



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926 survival benefits - however, that patients with non-viral etiologies (e.g. NASH) do not achieve  
927 a significant outcome benefit.

928 We thus propose to stratify HCC patients who are very likely to profit from immunotherapy and  
929 strengthen the argumentation to use immunotherapy in specific cohorts of HCC patients. We  
930 agree with Referee#1 that this information needs to be articulated in the paper appropriately  
931 not to deliver wrong messages but to be very specific. We truly believe that these are important  
932 clinical data, also providing the basis to test our hypotheses in prospective studies on non-  
933 significantly beneficial effects in terms of OS for immunotherapy in HCC patients with non-viral  
934 and NAFLD/NASH etiology, in particular. Moreover, we toned down the conclusions of our  
935 retrospective cohort in the manuscript and would like to point out, that bigger cohorts and  
936 prospective clinical trials are of utmost importance for the scientific community.

937 Minor points:

938 - Figure 1j lacks a color scale bar and proper description. How does one interpret the difference between  
939 ND and CD-HFD in this plot?

940 We thank Referee #2 for highlighting the lack of a color bar in this panel, we have added a  
941 color scale bar with a proper description. Figure 1j displays the median expression of selected  
942 genes in the different T-cell populations observed in our scRNA-seq data set (**Figure 1,**  
943 **Extended Data 5** and **Rebuttal Figure 24** and **26**) and serves as a supplement to the 2-  
944 dimensional tSNE plot. In this panel, we do not compare ND to CD-HFD rather simply allow  
945 the readers to view the gene signatures characterizing the different populations. A comparison  
946 of ND and CD-HFD is visualized using volcano plots in Figure 1. As this heatmap is rather a  
947 technical information, but does not condense scientific explanation in great detail, we decided  
948 to move this heatmap to **Extended Data 5**.

949  
950  
951 - Where is the ND + PD-1<sup>-/-</sup> in Figure 3b? Do these mice also get accelerated carcinogenesis?

952 We thank Referee #2 for highlighting this inconsistency. In line with the point raised by  
953 Referee#2 we have improved this in a revised manuscript including PD-1<sup>-/-</sup> mice on ND.  
954 Literature does not report accelerated hepatocarcinogenesis  
955 (<http://www.informatics.jax.org/allele/allgenoviews/MGI:4397682>) and we did not observe any  
956 hepatocarcinogenesis in PD1<sup>-/-</sup> under ND.

957  
958 - There is no color scale bar in Figure 3e.

959 We thank Referee #2 for highlighting this inconsistency and improved our manuscript by  
960 adding a scale bar.

961  
962 - In Figure 5k, shouldn't progression-free survival and time to progression plots yield the exact same  
963 data, but inversed? Why don't these curves match?



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964 We thank Referee #2 for this question. TTP and PFS are different endpoints. TTP is defined  
965 as the time from the date of treatment initiation until the date of first radiological tumor  
966 progression. PFS is a composite endpoint. It is defined as the time from the date of treatment  
967 initiation until radiological progression OR death, whatever comes first (Llovet et al., 2008). We  
968 decided to leave out the non-significant data of TTP and PFS in our manuscript. Moreover,  
969 upon recruiting the validation cohort of 118 HCC-patients under immunotherapy we decided  
970 to not show TTP and PFS, but instead the multivariate analysis (**Supplemental Table 9**).

971  
972 - In Figure S1i, what is the parent population?  
973

974 We thank Referee #2 for highlighting this inconsistency and improved our manuscript by  
975 adding the description of the parent population. In the case of **Extended Data 1** the parental  
976 populations are CD8+ (left) and respective CD4 or CD8 (right) T-cells.

977  
978 - In Figure S4a, how does one distinguish ND from CD-HFD mice? The y-axis lacks a label.

979 We thank Referee #2 for highlighting this inconsistency and improved our manuscript by  
980 adding the description of the y-axis.

981  
982 - Figure 5c is plotted in a confusing manner (as the z-score scale is red independent of whether it goes  
983 up or down), but it seems that the TNF signaling gene sets are actually decreasing in expression.

984  
985 We thank Referee #2 for highlighting this inconsistency. We decided after integration of the  
986 new data, to leave that graph out as it communicates similar information already included in  
987 **Extended Data 28**. Of note, if we change the labeling of z-score, it clarifies, that TNF is indeed  
988 an increased pathway (similar to **Extended Data 28**).

989  
990 - Why do the PD-1<sup>-/-</sup> mice still express PD-1 (Fig. S12e)?

991 We thank Referee #2 for highlighting this inconsistency and improved our manuscript by re-  
992 analyzing our flow cytometry data set (as gates have been set too loose – leading to a subset  
993 of around 1% PD1 expressing CD4+ and CD8+ T cells). Analyses revealed that PD1<sup>-/-</sup> ND-fed  
994 mice have no intrinsic higher immune cell abundance, or activation and hepatocarcinogenesis  
995 compared to ND-fed wt control mice at 6 months under diet (**Figure 3** and **Extended Data 14**  
996 and **Rebuttal Figure 22, 23**). Moreover, as indicated no PD1-expression can be observed.

997  
998 - In Figure S13k, the authors should present cleaved Caspase 3 and cleaved Caspase 8 if they want to  
999 conclude something about T-cell death, as total, uncleaved levels of these proteins do not indicate cell  
1000 death.

1001 We thank Referee #2 for highlighting this point. We have removed these plots and demonstrate  
1002 cleaved caspase 3 by immunohistochemistry, which has the advantage that we not only see  
1003 the Cleaved Caspase 3 directly but also which cells are undergoing apoptosis. These data are  
1004 now included in **Extended Data 16** and **Rebuttal Figure 33**.



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1005 - In Figure S16f, the FACS plot does not match the quantification on the left.

1006

1007 We thank Referee #2 for bringing this up and apologize for this inconsistency. We would like  
1008 to draw the attention, that in the flow cytometry plot the data is displayed as “%of CD8”, in  
1009 contrast in the box plot the data is displayed as “%of CD45” to give the reader a more  
1010 quantitative analysis.

1011

1012 - Regarding Figure S17b, the authors claim an increase in calcium levels in line 383 of their manuscript,  
1013 but this difference is not significant.

1014 We agree with Referee #2. Thus, we have performed additional experiments – supporting our  
1015 initial finding that upon PD1-targeted immunotherapy calcium levels were increased on CD8+  
1016 but not CD4+ T-cells. This inconsistency was improved our manuscript accordingly.

1017

1018 - In Figure S18b, how does one interpret the difference between healthy, borderline NASH or NASH  
1019 patients? There is no explanation of the color scale bar. Also, what are “randomly chosen CD45+ cells”  
1020 as mentioned in the corresponding Figure Legend?

1021 We thank Referee #2 for highlighting this inconsistency and improved our manuscript  
1022 accordingly by describing differences between patients and highlighting our analysis pipeline  
1023 for flow cytometric data according to (Brummelman et al., 2019). Moreover, we have added 2  
1024 more cohorts in the main Figure (**Figure 5**) and Extended Data and pooled borderline NASH  
1025 and NASH patient into one group of NAFLD/NASH patients after consultation with our  
1026 pathologists, who indicated that the difference between borderline NASH and NASH can be  
1027 regional – and thus is always is regarded as NASH (**Extended Figure 25** and **Rebuttal Figure**  
1028 **10**).

1029

1030 - Figure S19b is not legible.

1031 We thank Referee #2 for this comment. In line, we have now changed the graph size and font  
1032 size.

1033 - In lines 237-246 the authors describe that NK1.1-based depletion of immune populations did not result  
1034 in changed liver pathology, body weight, fibrosis ALT, hepatic cytokines and hepatic chemokines.  
1035 However, the animals who underwent this depletion also completely lacked liver cancer development.  
1036 How does this happen if the authors did not detect any changes? The authors should perform NK1.1  
1037 depletion by itself to see if NK1.1+ cells, potentially depending on CD8 cells, are in fact responsible for  
1038 the authors' phenotype.

1039

1040 We thank Referee #2 for highlighting this unprecise description of our data. We improved our  
1041 manuscript by highlighting differences between CD8 depletion and CD8/NK1.1 co-depletion.  
1042 We included additional GSEA analysis of RNA-seq data, which display changes in CD8/NK1.1  
1043 co-depleted in comparison to CD8 single depleted animals (CD8-single depleted animals  
1044 showed enrichment for “cholesterol homeostasis” (**Extended Data 9** and **Rebuttal Figure 17**)).



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1045 Furthermore, we would like to draw attention to a previous study (Wolf et al., 2014), in which  
1046 NKT-cells were responsible for metabolic changes and CD8 T-cells driving hepatic damage.  
1047 We think, that the lack of liver cancer incidence is a result of CD8 depletion and a reduction of  
1048 a pro-tumorigenic environment - e.g. including pro-tumorigenic TNF signaling, which is  
1049 similarly enriched (TNF signaling via NFKB) in CD-HFD-fed control animals (NES(CD8  
1050 depletion vs control)= -1.6718) and NES(CD8/NK1.1 co-depletion vs control)= -1.6538)  
1051 (**Extended Data 8 and 9 and Rebuttal Figure 15 and 17**). These data were also corroborated  
1052 by the analyses of the ICF signature which is strongly abrogated upon CD8 T cells depletion.  
1053 Thus, we dissected the role of NK1.1 cells in greater detail by including the GSEA analysis of  
1054 RNA-seq data comparing CD8-depleted and CD8/NK1.1 co-depleted animals. Furthermore,  
1055 we improved cross-referencing to the co-submitted study Dudek et al. to highlight, that CD8 T-  
1056 cells are driving hepatocarcinogenesis. In line, together with Dudek et al. we generated new  
1057 data using mouse strains with impaired NKT cells - namely  $J\alpha 18^{-/-}$  and  $CD1d^{-/-}$  - under NASH-  
1058 inducing diet. Both genetic knockout mouse models develop NASH (including systemic  
1059 obesity, fibrosis, ALT) and NASH-induced hepatocarcinogenesis similar to WT control animals  
1060 at 12-months diet-feeding. These data argue against an essential role of NKT-cells to drive  
1061 hepatocarcinogenesis at this time-point.

1062  
1063 - Sentence 289-292 is unclear.

1064 We thank Referee #2 for highlighting the imprecise description and have now improved this in  
1065 the main text of the revised manuscript. The sentence now reads as follows: "Next, we  
1066 investigated the mechanisms underlying the increased occurrence of liver cancer  
1067 incidence/liver tumor formation associated with anti-PD-1 treatment in the context of NASH."

1068  
1069 - When discussing GSEA, the authors frequently use the wording 'reduced enrichment (e.g. line 241)  
1070 when talking about enrichment in the opposite phenotype. This is incorrect, as the absolute amount of  
1071 enrichment is often similar just, as mentioned, in the opposite direction.

1072 We thank Referee #2 for highlighting this imprecise description. We altered this in the revised  
1073 manuscript. The changes read now as follows e.g.: "Gene set enrichment analysis (GSEA) of  
1074 RNA sequencing data from whole liver tissue of CD8<sup>+</sup> depleted mice revealed enrichment for  
1075 DNA repair, oxidative phosphorylation, complement, and TNF signaling compared to CD-HFD-  
1076 fed control)".

1077





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**1078 Referee #3 (Remarks to the Author):**

1079 This full article manuscript is novel, and the experimentation to support the conclusions is exhaustive  
1080 and solid for the most part. In essence, the findings indicate that, in NASH livers, there is an  
1081 accumulation/expansion of a pathogenic CD8 T-cell population that expresses PD-1 and exacerbates  
1082 NASH pathology and fosters hepatocellular carcinogenesis and progression. The inflammatory and  
1083 tissue-damaging functions of this pathogenic CD8 T-cells are repressed by PD-1 blockade that is  
1084 common clinical practice for second-line treatment of advanced HCC and is under clinical trials for earlier  
1085 stages of the disease. In fact, PD-L1 blockade plus anti-VEGF will soon become the standard of  
1086 treatment for advanced HCC in first line. According to the findings in this paper upon PD-1 blockade,  
1087 authors document an exacerbation of carcinogenesis and liver damage that questions the indication of  
1088 PD-1 blockade in NASH-associated liver cancer. A balanced presentation of preclinical and supportive  
1089 clinical results in patient specimens very much enhances the significance of this study.

1090

1091 [We thank Referee #3 for the positive feedback and the statement that our study is “novel, and](#)  
1092 [the experimentation to support the conclusions is exhaustive and solid for the most part”.](#) [We](#)  
1093 [would like to address his/her concerns in the following section point-by-point by presenting](#)  
1094 [new experimental data sets experiments, rephrasing, and re-analysis of the underlying data-](#)  
1095 [sets.](#)

1096

1097 Questions and comments:

1098 1. TNF seems to be an actionable therapeutic target for the observed harmful effects of this CD8 T-cell  
1099 population. It would be interesting to know if TNF could be blocked preserving anti-cancer immunity  
1100 (especially under checkpoint inhibition therapy) but preventing tissue damage and carcinogenesis  
1101 promotion.

1102 [We thank Referee #3 for raising this important concern and thus have performed anti-TNF](#)  
1103 [with/without anti-PD-1-related immunotherapy in the context of NASH/HCC. Anti-TNF](#)  
1104 [treatment alone - without PD1-targeted immunotherapy - leads to liver cancer formation](#)  
1105 [comparable to control-treated CD-HFD-fed mice. However, anti-TNF treatment in the context](#)  
1106 [of PD1-targeted immunotherapy leads to a significant reduction of tumor incidence \(tumor](#)  
1107 [incidence\(anti-PD-1\)= 75% vs tumor incidence\(anti-TNF/anti-PD-1\)= 25%, p= 0.0024\), liver](#)  
1108 [damage \(ALT\(anti-PD-1\)= 381.6 U/L vs ALT\(anti-TNF/anti-PD-1\)= 250 U/L, p= 0.0072\) and](#)  
1109 [NAFLD-activity score \(NAS\(anti-PD-1\)= 5.875 vs NAS \(anti-TNF/anti-PD-1\)= 3.1, p= <0.0001\),](#)  
1110 [when compared to anti-PD1 treated CD-HFD-fed mice alone. This indicates that TNF exerts](#)  
1111 [key functions of the observed adverse effects of PD1-targeted immunotherapy, namely](#)  
1112 [contributing to increased hepatocarcinogenesis \(\*\*Figure 4, Extended Data 20 and 21 and\*\*](#)  
1113 [Rebuttal \*\*Figure 16, 18 and 19\*\*\).](#)

1114 [Moreover, the combination of anti-PD1 therapy with CD8-T cell depleting antibodies fully](#)  
1115 [eliminated the adverse, NAS increasing and pro-carcinogenic effects of CD8+ T-cells. These](#)



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1116 data emphasize that CD8+ T-cells are a major cell population mediating increased  
1117 hepatocarcinogenesis through a TNF-dependent mechanism upon PD1-targeted  
1118 immunotherapy (**Figure 4, Extended Data 20 and 21 and Rebuttal Figure 16, 18 and 19**).  
1119 On one hand, the mechanisms could be executed by CD8 T-cell derived TNF itself or by  
1120 mechanisms that depend on TNF-signaling on other cells (e.g. myeloid cells). For example,  
1121 we see a drastic reduction of myeloid attracting chemokines but also cytokines of liver  
1122 inflammation (e.g. IL-17A, IL-10), all cytokines/molecules which might fuel liver inflammation  
1123 and thus hepatocarcinogenesis in PD-1-targeted immunotherapy in NASH mice.

1124 Importantly, comparing mouse-human of CD8+ T-cells isolated from liver tissue of NASH mice  
1125 or patients through classical flow cytometry, CYTOF, and on scRNA-seq level we identified  
1126 similar populations and transcriptional activation of CD8+ PD1+ in a total of three independent  
1127 center patient cohorts (**Figure 5, Extended Data 25-27 and Rebuttal Figure 9-13**). These  
1128 data indicate that results obtained and hypotheses built from the preclinical NASH model are  
1129 relevant for human disease and are in line with published results, where TNF blockade  
1130 uncouples mediated toxicity in dual CTLA-4 and PD-1 immunotherapy (Perez-Ruiz et al.,  
1131 2019).

1132  
1133 2. Would PD-L1 blockade enhance liver cancer and tissue damage as well? Which cells are expressing  
1134 PD-L1 in the system? This becomes important given the recent approval of atezolizumab +  
1135 bevacizumab.

1136 We agree with Referee #3 for raising the point that dissection of anti-PD-L1-targeted  
1137 immunotherapy is of major concern, especially in the light of the recent results of the  
1138 IMBrave150 study. Data we have received from RNA in situ hybridization and  
1139 immunohistochemistry indicate that PD-L1 is expressed with increased level over time – with  
1140 progression of NASH disease (in mice and men). In summary, PDL1 staining in the preclinical  
1141 model is mainly associated with inflammatory cells, positive cells can be observed in the  
1142 sinusoidal space as well (**Extended Data 3, 20, 22 and Rebuttal Figure 8, 18 and 20**). In  
1143 humans, PDL1 positivity was observed in aggregates of inflammatory cells in the parenchyma  
1144 and the portal tract area. Focally, positivity was also seen in sinusoidal lining cells (**Extended  
1145 Data 28 and Rebuttal Figure 32**).

1146 The cells expressing PD-L1 in NASH-affected mice are mainly lymphocytes but also some  
1147 parenchymal cells (**Extended Data 3+7, 20+22 and Rebuttal Figure 8, 13, 18 and 20**).

1148 In line with the comment of Referee #3, we have also performed anti-PD-L1 targeted  
1149 immunotherapy in mice with and without established liver cancer (**Extended Data 7 and  
1150 Rebuttal Figure 13**). Results from these experiments indicate that similar to anti-PD1 - anti-  
1151 PDL1-treatment does not induce an anti-cancer effect for NASH-induced HCC but induces -  
1152 similar to anti-PD1 treatment - a pro-inflammatory and pro-carcinogenic effect (e.g. increased  
1153 NAS, strong trend in increased hepatic CD8 abundance by IHC ( $p=0.0546$ ), cytokines like IL-



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1154 21 and CCL3) (**Extended Data 7+13** and **Rebuttal Figure 13** and **28**). These data indicate,  
1155 that in the preclinical NASH model both PD1 or PDL1-targeted immunotherapy induces  
1156 adverse effects. This is corroborated by our increased, retrospective cohort HCC-patients of  
1157 different etiologies under PD(L)1-targeted immunotherapy, in which multivariate analysis  
1158 results in NAFLD/NASH being an independent negative factor for overall survival and validated  
1159 these results in a second cohort of 118 HCC-patients (**Figure 6** and **Rebuttal Figure 1g,f**).  
1160 Furthermore, we corroborated our hypothesis of non-viral (NASH-related) HCC being less  
1161 responsive to immunotherapy by a meta-analysis including 1656 patients of the three most  
1162 important clinical trials, identifying immunotherapy vs control for viral HCC as favorable  
1163 treatment (HR(viral)= 0.64), in contrast, non-viral-HCC showed less benefit (HR(non-viral)=  
1164 0.92) for immunotherapy (**Figure 6, Extended Data 30-32, Supplementary Table 9** and  
1165 **Rebuttal Figure 1-4**)).

1166  
1167 3. Results on NASH in human samples are compelling and supportive of the relevance of the findings.  
1168 It would be interesting to know in such livers which cells express PD-L1.

1169 We thank Referee #3 for highlighting this important aspect of our data – and have consequently  
1170 performed PD-L1 expression analyses by immunohistochemistry in human specimens  
1171 described in the previous point raised by Referee #3. Although analysis by bulk RNA-seq of  
1172 liver tissue indicates a decrease of PDL1/CD274 expression with the severity of NASH  
1173 pathology, immunohistochemistry indicates an increase of PDL1 positivity with the severity of  
1174 NASH pathology. PDL1 positivity was observed in aggregates of inflammatory cells in the  
1175 parenchyma and the portal tract area. Focally, positivity was also seen in sinusoidal lining cells  
1176 (**Extended Data 28** and **Rebuttal Figure 32**).

1177  
1178 4. What do you think is the fibrogenic factor/s promoted by pathogenic CD8 cells? Any candidates from  
1179 the extensive transcriptomic analyses?

1180  
1181 We thank Referee #3 for pointing out, that the fibrogenic factor is of major concern to prevent  
1182 HCC in subgroups of NASH patients. Our transcriptomic data-set has so far not pointed  
1183 towards specific fibrogenic factors, indicating that the chronic inflammatory environment  
1184 correlating with pathogenic CD8 cells drives fibrosis in our mice. To strengthen this hypothesis  
1185 AI-based analyses of a broad range of parameters of our 12 months CDHFD-fed mice  
1186 revealed, that Sirius red staining correlates negatively within CD8 depleted animals, indicating  
1187 that CD8-associated inflammation or CD8-dependent mechanisms might be functionally linked  
1188 with fibrosis (included in **Figure 1, Extended Data 4 and 24** and **Rebuttal Figure 6, 7** and  
1189 **26**). Moreover, in 12 months CDHFD-fed mice fibrosis correlated positively with CD8 T-cells  
1190 abundance, CD8+PD-1+ (%CD8), pDC+MHCII+ polarization, and hepatic TNF concentration.  
1191 Therefore, we cannot point out one specific factor driving fibrosis on pathogenic CD8 cells.

1192



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1193 5. Are Kupffer cells involved in the CD8-dependent pathogenesis mechanisms?

1194 We thank Referee #3 for asking the important question about Kupffer cells (KC). A study  
1195 (Malehmir et al., 2019) reports, that KCs have a crucial role in the pathogenesis of NASH, but  
1196 activation of monocytes and myeloid-derived macrophages correlates with disease  
1197 progression. Data presented in **Extended Data 8 and 11** cannot exclude KC-dependent  
1198 mechanisms, however, they seem to have a minor role, especially concerning the co-submitted  
1199 manuscript Dudek et al. in which CD8+ cells drive pathogenesis in KC-independent ways.

1200 We have further performed analyses on how KC correlate with varying degrees of inflammation  
1201 induced by our antibody treatments (anti-CD8, anti-CD8/anti-NK1.1, anti-CD8/anti-PD1, anti-  
1202 PD1, anti-PDL1, anti-TNF, anti-TNF/anti-PD1, and as control experiment anti-CD4 and anti-  
1203 CD4/anti-PD1) by our AI-based analysis approach (**Figure 1, Extended Data 4, 20-24** and  
1204 **Rebuttal Figure 6, 18-21** and **26**). Under baseline conditions (12 months CD-HFD-fed animals  
1205 receiving no treatments) KC abundance does not correlate with any serological or histological  
1206 marker, but KC activation (measured by MHCII+ polarization) correlates strongly with tumor  
1207 size and IL-21 (**Extended Data 4** and **Rebuttal Figure 6**). However, when applying treatments  
1208 (e.g. PD-1-targeted immunotherapy) KC correlates with treatments as well as activation of  
1209 hepatic KC (measured by MHCII+) correlate positively with CD8+PD-1+ (%CD8), Sirius Red  
1210 staining, tumor incidence, tumor number, tumor size, and IL-21 (**Extended Figure 24** and  
1211 **Rebuttal Figure 7**).

1212 In summary, we believe in line with our own study (Malehmir et al., 2019) and recent literature  
1213 (Remmerie et al., 2020) that Kupffer cells are an important cell type on whose basis not  
1214 inflammatory pathologies are initiated and maintained, but also in end-stage disease fresh  
1215 KC/KC-like cells (attracted by cytokines e.g. MCP-1, CCL3, MIP-2 (**Extended 2, 13, 21** and  
1216 **23** and **Rebuttal Figure 19, 21, 28** and **34**) activation might be detrimental as indicated by our  
1217 correlation analysis. – laying the ground for adaptive immune cell reactions.

1218  
1219 6. Obesity and response to PD-1 associations have been reported (PMID: 30420753 and PMID:  
1220 30813970). According to these studies, obesity relates to T-cell dysfunction that PD-1 blockade  
1221 derepresses and results in better responsiveness. The models of NASH should suffer overweight as  
1222 well as perhaps the patients in the reported series. This point should be addressed if possible and at  
1223 least discussed. Authors may gain insight with their comparisons of the models with and without choline  
1224 in the diet. As a potential consequence, would it be the case that in HCC patients, obese patients  
1225 respond worse to treatment contrary to other indications? Of clinical note, advanced HCC patients  
1226 frequently experience cachexia but perhaps less frequently so those with presumed or documented  
1227 NASH etiology.

1228  
1229 We thank Referee #3 for highlighting these important studies of checkpoint inhibition in the  
1230 frame of obese cancer patients. (Wang et al., 2018) shows - similar to our study - convincingly  
1231 that increased PD-1 expression is a hallmark of diet-induced obesity, thus we cite the study in



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1232 our introduction and improved cross-referencing in our discussion. Potential differences in the  
1233 outcome of PD-1-targeted immunotherapy might be a consequence of the use of obesity-but,  
1234 not NASH-inducing high-fat diet, which we show is crucial to induce hallmarks of NASH by  
1235 comparing HFD with CD-HFD in **Extended Data 1**. Moreover, we would like to draw attention  
1236 to the different cancer entities, which potentially affect immunotherapy-responsiveness. Wang  
1237 et al. use subcutaneous tumor models of lung carcinoma (3LL) and melanoma (B16-F0), but  
1238 not spontaneously developed liver cancer in a chronic inflammatory metabolically challenged  
1239 hepatic microenvironment. Notably, obese animals have bigger tumor-volumes and anti-PD-1  
1240 reactive animals do not control tumor-volume to a smaller absolute tumor-volume compared  
1241 to non-obese controls (Figures 2 and 4 in (Wang et al., 2018)).

1242 The second study of (Cortellini et al., 2019) corroborates the preclinical data of (Wang et al.,  
1243 2018) nicely in lung-, renal-carcinoma, or melanoma patients, but not liver cancer. No grading  
1244 of obese patients was performed (e.g. we report in Supplementary Table 1: healthy/control  
1245 liver, NAFLD/NASH), which we show in **Figure 5** is crucial for hepatic CD8 and PD-1  
1246 abundance. Supporting our manuscript, (Cortellini et al., 2019) report significantly more  
1247 likelihood of obese patients experiencing immune-related-Adverse-Effects (irAEs) “compared  
1248 to non-overweight patients (55.6% vs. 25.2%,  $p < 0.0001$ )”. Unfortunately, no subgroup  
1249 analyses about differences of hepatic irAEs between obese/non-obese patients are shown.

1250 We included the study of (Cortellini et al., 2019) in our introduction/ discussion.

1251 Our NAFLD/NASH cohort without immunotherapy treatment indicate a correlation of BMI with  
1252 CD8+PD-1+ T-cells (**Figure 5** and **Rebuttal Figure 9**). In our conducted meta-analysis, no  
1253 BMIs were reported, thus statements about treatment response remain hypothetical.  
1254 Furthermore, our retrospective HCC-patient cohort under PD(L)1 immunotherapy was too  
1255 small for subgroup analysis, however, there was no significant difference in BMI between  
1256 NAFLD/NASH-HCC and other etiologies-HCC patients, indicative of obesity (**Supplementary**  
1257 **Table 7**).

1258  
1259 7. The retrospective series of patients with advanced HCC treated cannot be considered conclusive at  
1260 this point and only hypothesis-generating. The wording there needs to be carefully down-toned.

1261 We agree with Referee #3, that the presented retrospective PD-(L)1 targeted immunotherapy  
1262 treated NAFLD/NASH-associated HCC cohort – although unique for Europe and treatment not  
1263 officially licensed and thus reimbursement - is still small, although we would like to point out,  
1264 that prominent trends or effects can be seen in small retrospective cohorts as well.

1265 Thus, our analyses of BCLC-C NAFLD/NASH-HCC vs other-etiologies-HCC patients  
1266 indicated, that NAFLD/NASH-HCC has significantly reduced overall survival compared to  
1267 other-etiologies-HCC in this small retrospective cohort, which we validated in a second cohort  
1268 of 118 HCC patients under immunotherapy (included in **Figure 6** and **Rebuttal Figure 1f,g**).  
1269 Of note, multivariate analyses identified NAFLD/NASH as an independent factor for treatment



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1270 response and thus identifying NAFLD/NASH as a negative predictor for HCC immunotherapy  
1271 (included in **Supplementary Table 9**).

1272 We corroborated our hypothesis of non-viral (NASH-related) HCC being less responsive to  
1273 immunotherapy by a meta-analysis including 1656 patients of the three most important clinical  
1274 trials (IMbrave 150; Checkmate 459; Keynote 240), identifying immunotherapy vs control for  
1275 viral HCC as favorable treatment (HR(viral)= 0.64), in contrast, non-viral-HCC showed less  
1276 benefit (HR(non-viral)= 0.92) for immunotherapy (included in **Figure 6, Extended Data 30-32,**  
1277 **Supplementary Table 7 and Rebuttal Figure 1-4**).

1278 Based on these data we want to point out that it is - as indicated by Referee#3 - of the highest  
1279 importance to us to specifically define/tone down appropriately the message of our manuscript:  
1280 Our manuscript does not indicate that immunotherapy is not beneficial for HCC patients at all.  
1281 Our manuscript rather demonstrates that HCC patients with viral etiologies do respond well  
1282 and achieve survival benefits - however, that patients with non-viral etiologies (e.g. NASH) do  
1283 not achieve a significant outcome benefit.

1284 We thus propose to stratify HCC patients who are very likely to profit from immunotherapy and  
1285 strengthen the argumentation to use immunotherapy in specific cohorts of HCC patients. We  
1286 agree with Referee#1 that this information needs to be articulated in the paper appropriately  
1287 not to deliver wrong messages but to be very specific. We truly believe that these are important  
1288 clinical data, also providing the basis to test our hypotheses in prospective studies on non-  
1289 significantly beneficial effects in terms of OS for immunotherapy in HCC patients with non-viral  
1290 and NAFLD/NASH etiology, in particular. Moreover, we toned down the conclusions of our  
1291 retrospective cohort in the manuscript and would like to point out, that bigger cohorts and  
1292 prospective clinical trials are of utmost importance for the scientific community.

1293  
1294 8. An important message of this paper is that progression following PD-(L)1 treatment in NASH patients  
1295 could be the development of a second primary malignancy rather than from the same one. Can this  
1296 point be addressed in the models? Is multifocal cancer more common in those cases? The more CD8  
1297 pathogenic T-cells in the infiltrate, the more multifocal the tumors?

1298 We thank Referee #3 for asking this important question. In our opinion dissection of  
1299 primary/second primary malignancy is overstepping the limitation of the preclinical model,  
1300 indicated by the variability of immunohistochemical staining and by the similarity of genomic  
1301 aberrations (**Extended Data 16 and Rebuttal Figure 33**).

1302 We further have performed correlation analyses (e.g. CD8, PD-1, PD-L1, NAS, fibrosis, liver  
1303 damage, tumor size, and tumor load) to allow readers a more detailed description of the  
1304 presented data (**Figure 1, Extended Data 4+24 and Rebuttal Figure 6, 7c-e and 26**).

1305



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1306 9. The companion back to back paper shows more data on the physiology of the pathogenic CD8 T-  
1307 cells that I would otherwise ask to this article. Therefore, proper cross-reference of those findings is  
1308 needed at least in discussion.

1309  
1310 We thank Referee #3 for highlighting the importance of the co-submitted paper Dudek et al.  
1311 and therefore, we improved cross-referencing in the discussion.



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**1312 Referee #4 (Remarks to the Author):**

1313 This is an interesting and quite original study of the role of immunity in promoting liver cancer. There are  
1314 data from the mouse models presented which show that CD8+ T-cells can contribute to the pathology  
1315 of NASH and the risk of cancers. The implication is that checkpoint blockade which can accentuate the  
1316 function of CD8 populations can worsen disease. There are also some human data which are fairly  
1317 consistent with this idea. It is perhaps not surprising that checkpoint inhibition might worsen an  
1318 inflammatory condition, although inducing a cancer risk is very interesting. Overall the authors do a very  
1319 good job in describing the cellular responses and the impact of depletion/blockade. There seemed to be  
1320 a bit of a gap around defining the mechanisms in terms of how the CD8+ T-cell population induced  
1321 cancer. Also it was somewhat unclear what the specificity of these T-cells was and what was triggering  
1322 their initial responsiveness in NASH. So although a strong case is made for the pro-tumor role the actual  
1323 pathways to disease were less concrete.

1324  
1325 We thank Referee #4 for appreciating our study's originality in shedding new light on the role  
1326 of immunity promoting liver cancer, with fairly consistent human data correlating with the  
1327 findings in the preclinical model. We thank Referee #4 for pointing out the limitations of our  
1328 study which has helped us to increase the quality of our manuscript and address the respective  
1329 points. We would like to address the concerns of Referee #4 in the following section point-by-  
1330 point by newly performed experiments, re-phrasing, re-analysis of the underlying data-sets and  
1331 would like to draw attention to the improved cross-referencing to the co-submitted manuscript  
1332 Dudek et al., which dissect the molecular and cellular mechanism of CD8+ T-cell dependent  
1333 pathogenesis in NASH.

1334  
1335 Figure 1: There do not appear to be any iNKT-cells in the UMAP or tSNE plots – these are discussed  
1336 latter in the text. That seems a little surprising as they are quite dominant in the mouse liver and have a  
1337 clear transcriptional profile. Could the authors clarify where these cells lie. It would be also useful to  
1338 know whether other unconventional cell subsets including GD T-cells and MAIT-cells are incorporated  
1339 in this, although they are likely much rarer. The latter may be relevant even if rare as they have been  
1340 linked to liver fibrosis. The same questions would also apply to the scRNAseq of the human samples.

1341 We thank Referee #4 for raising this important point. We have now dissected mouse NK1.1+  
1342 cells in the revised version of our manuscript into NK1.1+TCRb+ as NKT and NK1.1+TCRb-  
1343 as NK cells (**Figure 1** and **Rebuttal Figure 26**). Similarly, we highlighted NKT-cells, MAITs,  
1344 and  $\gamma\delta$  T-cells in our patient-derived hepatic lymphocytes analysis by flow cytometry, newly  
1345 performed scRNA-seq, and CYTOF analysis (**Figure 5**, **Extended Data 25-27** and **Rebuttal**  
1346 **Figure 9-12**).

1347 We agree with Referee #4, that MAITs might be important and thus included quantification of  
1348 MAITs in our newly performed scRNA-seq and CYTOF analyses of patient-derived hepatic  
1349 lymphocytes. In these analyses, no change of relative abundance of MAITs was observed  
1350 when comparing control vs. NAFLD/NASH patients. Moreover, we would like to draw attention  
1351 to the co-submitted manuscript Dudek et al., which analyzed - together with us - CD-HFD-fed





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1352 Ja18<sup>-/-</sup> and CD1d<sup>-/-</sup> mice. The latter did not display significant changes in pathology compared  
1353 to CD-HFD-fed control mice at time points of established NASH.

1354 We agree with Referee #4, that  $\gamma\delta$  T-cells may be important, however in our mouse model  
1355 upon NASH establishment, we detected no difference in hepatic abundance of  $\gamma\delta$  T-cells  
1356 between chow or CD-HFD-fed control mice (**Extended Data 3**). Furthermore, data presented  
1357 in **Figures 1 and 4** and **Extended Data 3** argue against a major direct contribution of  $\gamma\delta$  T-  
1358 cells in the preclinical model at time points of 6 or 12 months of diet-feeding. We agree that  $\gamma\delta$   
1359 T-cells might be important in the pathogenesis of NASH and NASH to HCC transition, however,  
1360 e.g. rather in collaboration with CD8<sup>+</sup> T cells, also in the context of PD1-related  
1361 immunotherapy. In humans, our data is not conclusive in all experiments, e.g. our data indicate  
1362 for  $\gamma\delta$  T-cells, if we compare: bulk RNA-seq indicates a reduced expression in severe NASH  
1363 pathology of EOMES, TRDC, and TRGC1 (**Extended Data 28** and **Rebuttal Figure 32**),  
1364 however, both flow cytometry cohorts and the scRNA-seq cohort indicate no change of either  
1365  $\gamma\delta^+$  T-cells or  $\gamma\delta^+$  Eomes<sup>+</sup> T-cells comparing control vs NAFLD/NASH patients (**Extended**  
1366 **Data 25, 27** and **Rebuttal Figure 10 and 12**).

1367 Corroborating the human flow cytometry data in our mouse model upon NASH establishment,  
1368 we detected no difference in hepatic abundance of  $\gamma\delta$  T-cells between chow- or CD-HFD-fed  
1369 control mice. Furthermore, data presented in **Figures 1** and **Extended Data 3** argues against  
1370 the major contribution of  $\gamma\delta$ T-cells in the mouse model of NASH. We did not observe significant  
1371 differences in the “other leukocytes” subset. In the revised manuscript, we analyzed  $\gamma\delta$ -T-cells  
1372 separately to strengthen the point, that these cells are not significantly changed (**Extended**  
1373 **Data 3, 20-23** and **Rebuttal Figure 8** and **18-21**).

1374 Figure 1e: What are the p values on the right referencing? The difference in the PD1<sup>+</sup> population does  
1376 not appear to be significant. How valid is the PD1<sup>+</sup> subset as a subcluster and also what are the critical  
1377 significant differences apart from elevated PD1 expression – some justification for this early on would  
1378 be helpful. Often PD1 expression is more of a gradient (even within PD1<sup>+</sup> cells) so a binary distinction  
1379 needs a bit more justification. Does this group of cells have distinct TCRs from the non-PD1 (or lower  
1380 PD1) subset or are they the same population with distinct expression? Some data on this would address  
1381 the question about specificity – although this would be better addressed by defining actual TCR-specific  
1382 (or independent) functionality.

1383 We thank Referee #4 for raising important points about **Figure 1**. We have now improved our  
1384 manuscript by clarifying, that the p-values on the right-side reference to abundance in CD-  
1385 HFD-fed mice compared to chow-fed control mice. We agree with Referee 4, that the CD8<sup>+</sup>PD-  
1386 1<sup>+</sup> subpopulation was (initially) not significantly changed (p= 0.09). Upon adding novel data,  
1387 and re-analysis according to the comment of Referee #4, by highlighting NKT cells, CD8<sup>+</sup>PD1<sup>+</sup>  
1388 (p= 0.03) are significantly changed. Furthermore, by using AI-based analysis of various  
1389 parameters displaying our used CD-HFD-fed cohorts as a total, we observed that pathology



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1390 severity correlated with the hepatic abundance of CD8+ T-cells and PD1 polarization of these  
1391 cells (**Figure 1 and 4, Extended Data 4 and 24 and Rebuttal Figure 6, 7c-e, 16 and 26**).  
1392 These analyses indicate, that besides changes e.g. in myeloid subsets, CD8+PD1+ cells are  
1393 a key subset in NASH-diseased mice as well as in human patients (**Figure 5 and Rebuttal**  
1394 **Figure 9**). To underline the importance of a CD8+PD-1+ subset -expressing  
1395 effector/exhaustion markers correlating with disease progression- we have connected the data  
1396 of **Figure 1** more closely to single-cell RNA-seq data presented in **Figure 1** (e.g. unique  
1397 transcriptional activity in NASH-derived CD8+ T-cells (**Figure 1 and Rebuttal Figure 26**) and  
1398 improved cross-referencing to the data co-submitted manuscript Dudek et al. in the discussion.  
1399 Furthermore, we have included in the revised manuscript, that we did not observe for CD8+ T-  
1400 cells a sufficient/non-binary gradient of PD-1 expression, allowing dissection into PD-  
1401  $1^{\text{negative}}$ /PD-1 $^{\text{intermediate}}$ /PD-1 $^{\text{high}}$  subsets upon 12 months CD-HFD-feeding, (**Extended Data 3**).  
1402 Moreover, we functionally show that CD8+ T-cell are indeed the drivers of anti-PD1-related  
1403 therapy induced liver cancer.

1404 We thank Referee #4 for pointing out the question about TCR dependency and thus would like  
1405 to draw the attention to the co-submitted manuscript Dudek et al., which describes TCR-  
1406 independent mechanisms on a cellular and molecular level driving CD8+ T cell-mediated  
1407 hepatocyte cell death. NASH-diet feeding experiments using mice with impaired TCR-  
1408 dependent effector function have been performed in collaboration with Dudek et al. 12-months  
1409 CD-HFD-fed perforin $^{-/-}$  mice developed NASH (including systemic obesity, fibrosis, ALT) and  
1410 NASH-induced hepatocarcinogenesis similar to WT control animals. We have now addressed  
1411 the question on TCR-specificity by improved cross-referencing to the co-submitted manuscript  
1412 Dudek et al.. In fact, it turns out that the effect of CD8+ T-cells is TCR-effector function  
1413 independent. Furthermore, we have performed combination therapy of 1) anti-TNF with/without  
1414 PD-1 targeted immunotherapy; 2) anti-CD4 with/without PD-1 targeted immunotherapy; 3) anti-  
1415 CD8 with PD-1 targeted immunotherapy and 4) PD-L1 targeted immunotherapy, to strengthen  
1416 hypotheses about TCR-independent mechanisms (**Figure 4, Extended Data 20-23 and**  
1417 **Rebuttal Figure 16 and 18-21**).

1418  
1419 Figure 1f: The stains are both single stains. It should be possible to show a double staining CD8+PD1+  
1420 population and enumerate them as this seems like the critical part of the study.

1421 We thank Referee #4 for pointing that out. We performed an additional double staining  
1422 corroborating our flow cytometry data in **Figure 1**. In line, we have now included histological  
1423 double staining in a revised manuscript (**Figure 1, Extended Data 3, 12, and Rebuttal Figure**  
1424 **8, 26 and 35**). These data indicated that PD1+ expression is indeed associated with CD8+  
1425 staining.

1426



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1427 Figure 1j: One of the most upregulated genes in the PD1+ subset is IL-10. Do the authors have any data  
1428 on whether this is secreted by this subset. Although the subset is labelled as “PD1+” it is not the top  
1429 upregulated gene here (as above). A side-by-side broader functional study would add a bit of resolution  
1430 here and if they do secrete IL-10 this may impact on the overall interpretation. The interpretations about  
1431 function are all via the screening approaches so some further specific back up by FACS/ELISA would  
1432 be helpful in confirming functionality, especially in the context of an “exhausted” phenotype – this would  
1433 clarify the statement on line 199 about “potential effector function”. Such an experiment would also be  
1434 valuable in the anti-PD1 treated mice in later parts of the manuscript.

1435 We fully agree and thank Referee #4 for raising this important point of IL-10 expression, which  
1436 was also raised in a recent study (Breuer et al., 2020). We analyzed IL-10+ CD8+PD-1+ T-  
1437 cells in our revised manuscript (**Extended Data 19** and **Rebuttal Figure 30**).

1438 However, we did not see any changes in IL10+ CD8+PD1+ in comparison to CDHFD-fed and  
1439 control mice. Moreover, IL10 levels measured by ELISA did neither drop upon CD8-depletion  
1440 (**Extended Data 10** and **Rebuttal Figure 29**) nor increase significantly upon anti-PD1  
1441 treatment (**Extended Data 13** and **Rebuttal Figure 28**). Thus, an increased anti-inflammatory  
1442 role by IL-10 expressing CD8+ T-cells upon PD1-targeted immunotherapy could not be  
1443 corroborated (**Extended Data 19** and **Rebuttal Figure 30k**) (Breuer et al., 2020). Of note, in  
1444 this publication diet-based NAFLD induction was achieved by feeding either WD or CD-HFD  
1445 for 8-10 weeks. This is in strong contrast to our experimental regime of applying diet for 3, 6,  
1446 or 12 months as we show, that the preclinical model presents different stages of NASH  
1447 pathology severity including hepatocarcinogenesis (**Figure 1** and **Rebuttal Figure 26**). Thus,  
1448 in our opinion, CD8+PD1+ cells are the main effector population driving liver inflammation and  
1449 liver cancer – most likely independent of IL10 being one of the most upregulated genes in this  
1450 subset. In line with our mouse data scRNA-seq of CD8+PD1+ cells derived from control vs  
1451 NAFLD/NASH patients did not reveal increased IL10 expression. Besides in bulk RNA-seq of  
1452 human liver tissue, we observed a variable expression pattern depending on NASH pathology  
1453 severity (**Figure 5**, **Extended Data 28** and **Rebuttal Figure 9, 28**).

1454  
1455 Figure 2: It was not that clear why depleting CD8s had no impact on ALT, suggesting they are not playing  
1456 a role in vivo, while blocking PD1 had some impact (AST is not shown for the anti-CD8 treatment).

1457  
1458 We thank Referee #4 for highlighting that CD8+-T cell depletion in the context of NASH-HCC  
1459 transition had no or only minor impact on ALT reduction, an effect that has also come to our  
1460 attention and has puzzled us. On the other hand, we would like to note that in the context of  
1461 anti-PD1-related immunotherapy triggered liver damage CD8+ T cell depletion did lead to a  
1462 significant reduction in liver damage and NAFLD activity score. Thus, we believe that the anti-  
1463 PD1 therapy-related damage in NASH and NASH to HCC transition is mainly triggered by  
1464 CD8+ T cells. In contrast, in the context of NASH development without anti-PD1 antibody  
1465 treatment, other cells than CD8+ T-cell also contribute to liver damage – and that progressive



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1466 NASH is characterized by multi-faceted, collateral damage through myeloid cells, adaptive  
1467 cells, and cell death.

1468 We think that CD8+ T-cells have an important *in vivo* role driving NASH to HCC transition, as  
1469 we strongly decreased or eliminated HCC by CD8+ T-cell depletion (both in NASH or NASH  
1470 with anti-PD1 treatment). In line, the co-submitted manuscript by Dudek et al., described  
1471 hepatocyte death by a CD8-dependent mechanism.

1472 Notably, ALT can be elevated as a result of the chronic metabolic environment and/or as a  
1473 result of the still ongoing hepatic inflammation independent of CD8+ or NK1.1+ cells  
1474 (**Extended Data 9** and **Rebuttal Figure 17**).

1475 Further, it can be that actually at late time points of co-existence of tumors and NASH – the  
1476 collateral damage might be mainly triggered by non-CD8+ T-cells. We have confirmed the  
1477 efficient depletion of the CD8 T-cells in our models, excluding that this might be a reason. AST  
1478 levels are included in our AI-based analysis (**Figure 1 and 4, Extended Data 4 and 24** and  
1479 **Rebuttal Figure 6, 7c-e, 16** and **26**), indicating no change upon CD8 depletion as well.

1480  
1481 Line 202 – lack of impact of anti-PD1. Is there a control for this experiment? The implication is that this  
1482 lack of impact is etiology-specific but it may also be that the intervention does not work well in other  
1483 HCC models.

1484  
1485 We thank Referee #4 for highlighting the etiology-dependent potential outcome of PD-1-  
1486 targeted immunotherapy against HCC. We agree with Referee #4, that there might be  
1487 bivalence in other HCC models and, more importantly, only a subset of HCC patient react to  
1488 PD-1 targeted immunotherapy (El-Khoueiry et al., 2017; Hage et al., 2019). Thus, we have  
1489 also performed anti-PD-L1 targeted immunotherapy in CDHFD-fed mice with and without  
1490 established liver cancer (**Extended Data 7** and **Rebuttal Figure 13**).

1491 The data of our study indicate that similar to anti-PD1 - anti-PDL1-treatment does not induce  
1492 an anti-liver cancer effect for NASH-induced HCC but rather induces similar to anti-PD1  
1493 treatment a pro-inflammatory and pro-carcinogenic effect. These data further suggest that in  
1494 the preclinical NASH models used, both PD1- or PDL1-targeted immunotherapy induces  
1495 adverse effects. This is corroborated by our increased, retrospective cohort HCC-patients of  
1496 different etiologies under PD(L)1-targeted immunotherapy, in which multivariate analysis  
1497 results in NAFLD/NASH being an independent negative factor for overall survival.  
1498 Furthermore, we corroborated our hypothesis of non-viral (NASH-related) HCC being less  
1499 responsive to immunotherapy by a meta-analysis including 1656 patients of the three most  
1500 important clinical trials, identifying immunotherapy vs control for viral HCC as favorable  
1501 treatment (HR(viral)= 0.64), in contrast, non-viral-HCC showed less benefit (HR(non-viral)=  
1502 0.92) for immunotherapy (**Figure 6, Extended Data 30-32, Supplementary Table 9** and  
1503 **Rebuttal Figure 1-4**)).

1504



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1505 Figure 5b and the text are presented in a slightly confusing way. It would be easier to understand the  
1506 disease associations of %CD8 (of CD3), and % PD1+ (or MFI) of CD3+CD8+ first. The association of  
1507 CD103 with tissue residency in the liver is not as good as other tissues, so a broader look at the  
1508 CD8+PD1+ population by flow would be better as well as some caution in interpretation.

1509  
1510 We agree with this comment and thank Referee #4 for highlighting this problem. Inline, we  
1511 have now improved our manuscript as suggested by Referee#4 (included in **Extended Data**  
1512 **25 and 27** and **Rebuttal Figure 10 and 12**). Moreover, we corroborated the association of  
1513 NASH patients and CD103 in a second patient cohort using CYTOF (included in **Figure 5** and  
1514 **Rebuttal Figure 9**).

1515  
1516 Figure 5e could include some study of CD4s as well for reference. That subset has been linked to NASH  
1517 pathogenesis as well. As above, it should be possible to perform some dual CD8 and PD1 staining to  
1518 map the subset of interest.

1519 We thank Referee #4 for highlighting this point, that CD4 T-cells and their expression of PD-1  
1520 might play a crucial role in the observed phenotype and thus included an in detail analysis of  
1521 CD4 T-cells to the majority of our experiments (e.g. **Extended Data 3** and **Rebuttal Figure 8**).  
1522 However, in the preclinical model the magnitude of effects observed in CD4+ T-cells is minor  
1523 when compared to CD8+ T-cells (e.g. **Extended Data 11** mean (CD8+CD62L-CD44+CD69+)  
1524 ~12% (%of CD45+) vs mean(CD4+CD62L-CD44+CD69+) ~4% (%of CD45+) upon PD-1  
1525 targeted immunotherapy).

1526 Data obtained from CD4 depletion with/without PD1-targeted immunotherapy indicate, that the  
1527 increased hepatocarcinogenesis in the context of anti-PD1 related immunotherapy is  
1528 independent of hepatic abundance of CD4+ T-cells in the preclinical NASH/HCC model  
1529 (included in **Figure 4**, **Extended Data 22 and 23** and **Rebuttal Figure 16, 20 and 21**).  
1530 However, CD4+ T-cells might have a diverse set of effector functions (e.g. interpreting tumor  
1531 incidence in anti-CD8/anti-PD1 treated animals: in the absence of CD8+ T-cells but  
1532 immunotherapy, thus CD4+ T-cells might be responsible for baseline tumor incidence; or the  
1533 trends of increased tumor incidence upon anti-CD4/anti-PD1 co-treatment in **Figure 4** and  
1534 **Rebuttal Figure 16n**). To allow a wider interpretation of a potential effect of CD4+ T-cells in  
1535 our preclinical model, we integrated and correlated the variety and potential changes upon 12  
1536 months of diet-feeding in the AI-based analyses correlating disease parameters with cellular  
1537 abundance and polarization (**Figure 1**, **Extended Data 4 and 24** and **Rebuttal Figure 6, 7c-**  
1538 **e and 26**). These data further strengthens that CD4+ T-cells play a minor role, as we see no  
1539 significant correlation of CD4-depleted animals with histological, or serological markers.

1540 Of note, CD4+ T-cells are also significantly changed in the human situation by classical flow  
1541 cytometry, but in the light of the results obtained in the preclinical model, we decided to not  
1542 investigate this result extensively (**Extended Data 25** and **Rebuttal Figure 10**). Of note, CD4+  
1543 T-cells are also significantly changed in the human situation and have also analyzed human



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1544 CD4+ cells a by scRNASeq (**Extended Data 27** and **Rebuttal Figure 12**). In addition, we have  
1545 performed a velocity analyses of the scRNA Seq data of mouse and human CD4 T cells (see  
1546 **Rebuttal Figure 31**). In mouse, no significant velocity flow was detected in 12 months CD-  
1547 HFD-fed mice, indicating, that CD4 cells are not transcriptionally activated and driven by  
1548 NASH-conditions or PD-1-targeted immunotherapy in NASH. However, we want to point out,  
1549 that in the mouse NASH model CD8 T-cells increase statistically significant and thus CD4 are  
1550 relatively fewer cells compared to CD8. Therefore, the velocity analysis of mouse CD4 T-cells  
1551 need to be taken with caution, because we included 300-500 cells only per described subset.  
1552 As consequence, we included the negative CD4 T-cell data not in the manuscript but in the  
1553 Rebuttal letter. Velocity analyses on human CD4 lead to comparable problems like seen in  
1554 mouse. As a consequence, we included the negative CD4 T-cell data not in the manuscript but  
1555 in the Rebuttal letter as **Rebuttal Figure 31**.

1556 However, we discuss the potential role of CD4+ T-cells in greater detail in the main text.

1557  
1558 Figure 5f is not really that convincing of a relationship with TNF – the r-squared value would be better  
1559 to illustrate and would be very low. If the authors think TNF secretion is critical it would be possible to  
1560 explore this further in the mouse model.

1561  
1562 We thank Referee #4 for highlighting this point. Although TNF is correlated significantly with  
1563 PD1 abundance, the correlation is weak as indicated by the r-value and therefore moved the  
1564 data to the Extended Data. Moreover, we fully agree with this Referee that further experiments  
1565 were needed to underline the role of TNF in NASH/HCC transition in the context of anti-PD1  
1566 related immunotherapy.

1567 Thus, we have performed an anti-TNF treatment with or without PD-1- targeted  
1568 immunotherapy in the context of NASH/HCC. Anti-TNF treatment without PD1-targeted  
1569 immunotherapy led to liver cancer formation comparable to control-treated CD-HFD-fed mice.  
1570 However, anti-TNF treatment in the context of PD1-targeted immunotherapy leads to a  
1571 significant reduction of tumor incidence compared to anti-PD1 treated CD-HFD-fed mice,  
1572 indicating that TNF exerts key functions of the observed adverse effects triggered by PD1-  
1573 targeted immunotherapy, namely the increased NAS, liver damage, and hepatocarcinogenesis  
1574 (**Figure 4, Extended Data 20 and 21** and **Rebuttal Figure 16, 18** and **19**).

1575 Moreover, the combination of anti-PD1 therapy with anti-CD8 – also ablating the adverse and  
1576 pro-carcinogenic effects of CD8+ T-cells emphasize that CD8+ T-cells are a major cell  
1577 population mediating increased hepatocarcinogenesis in a TNF-dependent mechanism upon  
1578 PD1-targeted immunotherapy (included in **Figure 4, Extended Data 20 and 21** and **Rebuttal**  
1579 **Figure 16, 18** and **19**).

1580 Importantly, by comparing classical flow cytometry, CYTOF, and on scRNA-seq level of  
1581 mouse-human of CD8+ T-cells isolated from liver tissue of NASH mice or patients, we



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1582 identified similar populations and transcriptional activation of CD8+ PD1+ in a total of three  
1583 independent center patient cohorts (**Figure 5, Extended Data 25 and 27 and Rebuttal Figure**  
1584 **9, 10 and 12**). These data indicate that results obtained and hypotheses built from the  
1585 preclinical NASH model and are in line with published results, where TNF blockade uncouples  
1586 mediated toxicity in dual CTLA-4 and PD-1 immunotherapy (Perez-Ruiz et al., 2019).

1587  
1588 For Figure 5G some disease controls would be valuable.

1589 We thank Referee #4 for his/her comment for pointing out the lack of appropriate control groups  
1590 (e.g. NASH-HCC vs different etiology-induced HCC under Sorafenib/different multi-kinase  
1591 inhibitors as a second/third-line therapy). Although of extreme interest for public health and  
1592 public knowledge, we described this important issue in our discussion and to the best of our  
1593 knowledge there are no NASH-HCC treated cohorts available (apart from, possibly, inside of  
1594 the big pharma-industry), which would allow an adequate control arm. Thus, we evaluated  
1595 potential disease controls in the manuscript by performing a meta-analysis including 1656  
1596 patients of the three major clinical trials (Imbrave 150; Checkmate 459; Keynote 240). Here  
1597 we could identify immunotherapy vs control for viral HCC as favorable treatment ( $HR(viral)=$   
1598  $0.64$ ), in contrast non-viral-HCC showed less benefit ( $HR(non-viral)= 0.92$ ) for immunotherapy  
1599 (**Figure 6, Extended Data 30-32, Supplementary Table 9 and Rebuttal Figure 1-4**)).

1600 Furthermore, we toned down the conclusions of our retrospective cohort in the manuscript and  
1601 would like to point out, that bigger cohorts and prospective clinical trials are of utmost  
1602 importance for the scientific community.

1603  
1604 Line 493+: This sentence is perhaps overstating the data, which were not significant in all those  
1605 parameters. It is likely quite hard to make the firmest comparisons, especially in such a retrospective  
1606 analysis, where the heterogeneous group of patients with eg viral aetiologies will be on effective  
1607 therapies - the actual aetiologies were not obvious in the supplementary data. This interpretation could  
1608 be a bit more cautious throughout (eg. it is in the abstract).

1609 We would like to thank Referee #4 for the important comment and agree. Thus, we toned down  
1610 the wording and interpretation of our data. As described previously, we recruited additional  
1611 patients to increase the number of patients in our initial clinical cohort from 65 to 130 HCC  
1612 patients under anti-PD(L)1-targeted immunotherapy, which we validated in a second cohort  
1613 (**Figure 6 and Rebuttal Figure 1f,g**).

1614 We agree with Referee #4, that the presented retrospective PD-(L)1 targeted immunotherapy  
1615 treated NAFLD/NASH-associated HCC cohort - although unique for Europe and treatment not  
1616 officially licensed and thus reimbursement - is still small, although we would like to point out,  
1617 that prominent trends or effects can be seen in small retrospective cohorts as well. Thus, our  
1618 analyses of BCLC-C NAFLD/NASH-HCC vs other-etiological-HCC patients indicated, that  
1619 NAFLD/NASH-HCC has significantly reduced overall survival compared to other-etiological-



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1620 HCC in this small retrospective cohort. Of note, multivariate analyses identified NAFLD/NASH  
1621 as an independent factor for treatment response and thus identifying NAFLD/NASH as a  
1622 negative predictor for HCC immunotherapy (**Supplementary Table 9**).

1623 Like previously mentioned, we corroborated our hypothesis of non-viral (NASH-related) HCC  
1624 being less responsive to immunotherapy by a meta-analysis including 1656 patients of the  
1625 three most important clinical trials (IMbrave 150; Checkmate 459; Keynote 240), identifying  
1626 immunotherapy vs control for viral HCC as favorable treatment ( $HR(viral)= 0.64$ ), in contrast,  
1627 non-viral-HCC showed less benefit ( $HR(non-viral)= 0.92$ ) for immunotherapy (**Figure 6,**  
1628 **Extended Data 30-32, Supplementary Table 7 and Rebuttal Figure 1-4**)).

1629 Thus, we toned down the conclusions of our retrospective cohort in the manuscript and again  
1630 would like to point out, that bigger cohorts and prospective clinical trials are of utmost  
1631 importance for the scientific community.

1632





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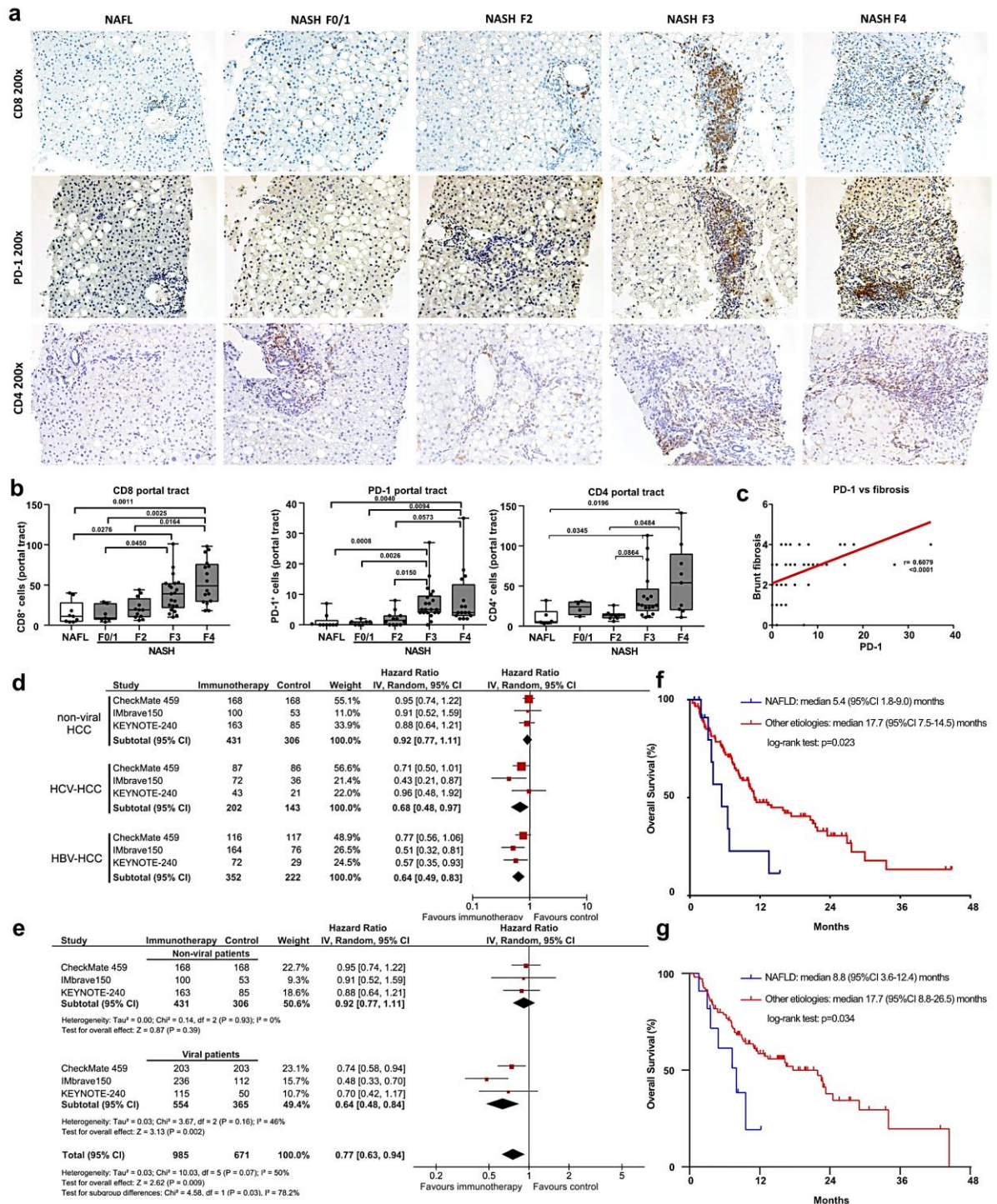
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1739 **Rebuttal Figures**

**Rebuttal Figure 1: PD-1 and PD-L1 targeted immunotherapy in advanced HCC has a distinct effect depending on disease etiology**

(a) Immunohistochemical staining and (b) quantification of hepatic PD-1, CD8 and CD4 expressing cells of NAFLD and NASH patients in **Supplementary Table 3** with varying stages of fibrosis (NAFLD n= 9 patients; NASH F1/0 n= 7 patients; NASH F2 n= 12 patients; NASH F3 n= 21 patients; NASH F4 n= 16 patients; CD4: NAFL n= 6 patients; NASH F1/0 n= 4 patients; NASH F2 n= 8 patients; NASH F3 n= 17 patients; NASH F4 n= 9 patients). (c) Correlation analysis of PD-1 against fibrosis scoring according to Brunt by immunohistochemical staining by RNA-sequencing (NAFLD/NASH n= 65 patients). (d) A total of 1656 patients were included in all three randomized trials, and 985 patients received a checkpoint inhibitor (**Supplementary Table 7**). Separate meta-analyses were performed for each of the three etiologies: non-viral (including mostly NASH and alcohol intake), HCV and HBV. (e) HCV and HBV were pooled into a separate category, termed "viral", and a subsequent meta-analysis comparing viral (n=919) and non-viral, including mostly NASH and alcohol intake (n=737) was performed. Hazard ratios for each trial are represented by squares, the size of the square represents the weight of the trial in the meta-analysis.

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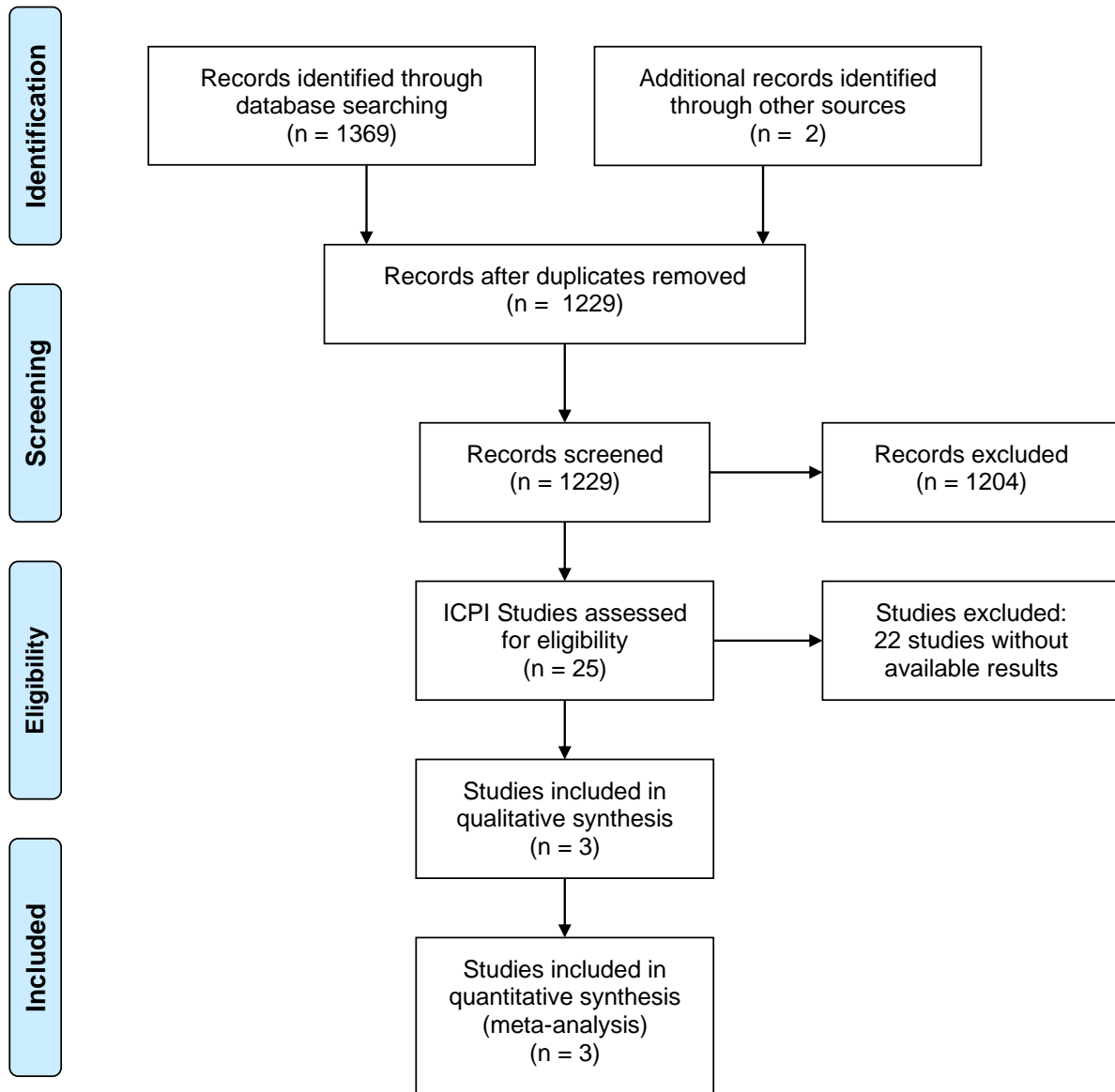


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1754 The horizontal line crossing the square represents the 95% confidence interval (CI). The diamonds represent the  
1755 estimated overall effect based on the meta-analysis random effect of all trials. Inverse variance and random effects  
1756 methods were used to calculate HRs, 95% CIs, P values, and the test for overall effect; these calculations were  
1757 two-sided. The Cochran's Q-test and  $I^2$  were used to calculate heterogeneity. Random = random effects method,  
1758 IV = Inverse variance. **(f)** Nonalcoholic fatty liver disease (NAFLD) is associated with a worse outcome in patients  
1759 with hepatocellular carcinoma (HCC) treated with PD-(L)1-targeted immunotherapy. A total of 130 patients with  
1760 advanced HCC received PD-(L)1-targeted immunotherapy (**Supplementary Table 8**). Kaplan-Meier curve display  
1761 overall survival of patients with NAFLD vs. those with any other etiology; all 130 patients were included in these  
1762 survival analyses (NAFLD n=13, any other etiology n=117). **(g)** Validation cohort of patients with HCC treated with  
1763 PD-(L)1-targeted immunotherapy. A total of 118 patients with advanced HCC received PD-(L)1-targeted  
1764 immunotherapy (**Supplementary Table 10**). Kaplan-Meier curve display overall survival of patients with NAFLD vs.  
1765 those with any other etiology; all 118 patients were included in these survival analyses (NAFLD n=11, any other  
1766 etiology n=107).



## PRISMA 2009 Flow Diagram



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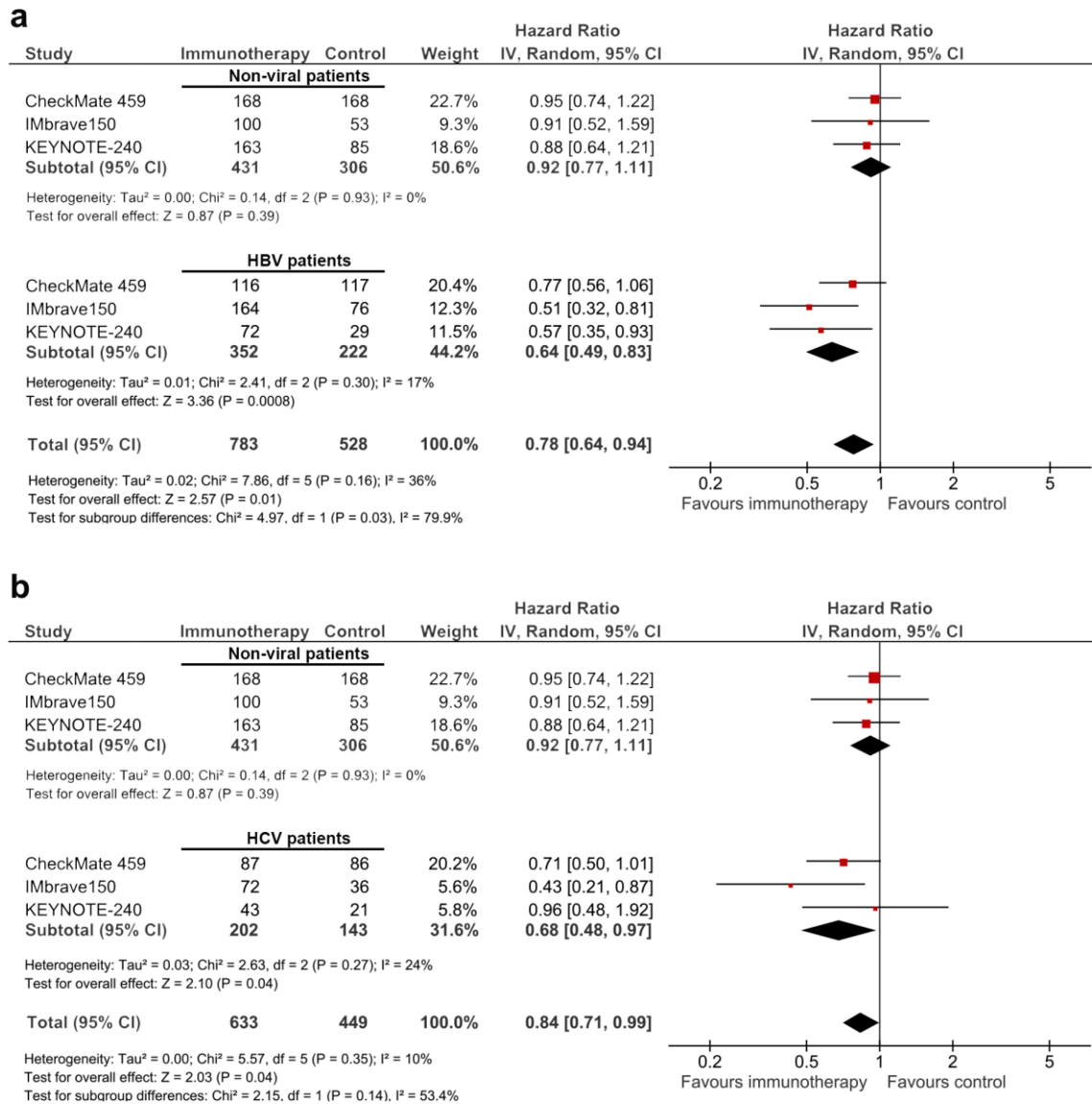
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**Rebuttal Figure 2: PRISMA Flow chart of the systematic review of targeted immunotherapy in HCC.**

Selection of articles assessing the clinical outcome of immune checkpoint inhibitors in advanced HCC for inclusion in the systematic review and meta-analysis. ICPI: Immune checkpoint inhibitor.

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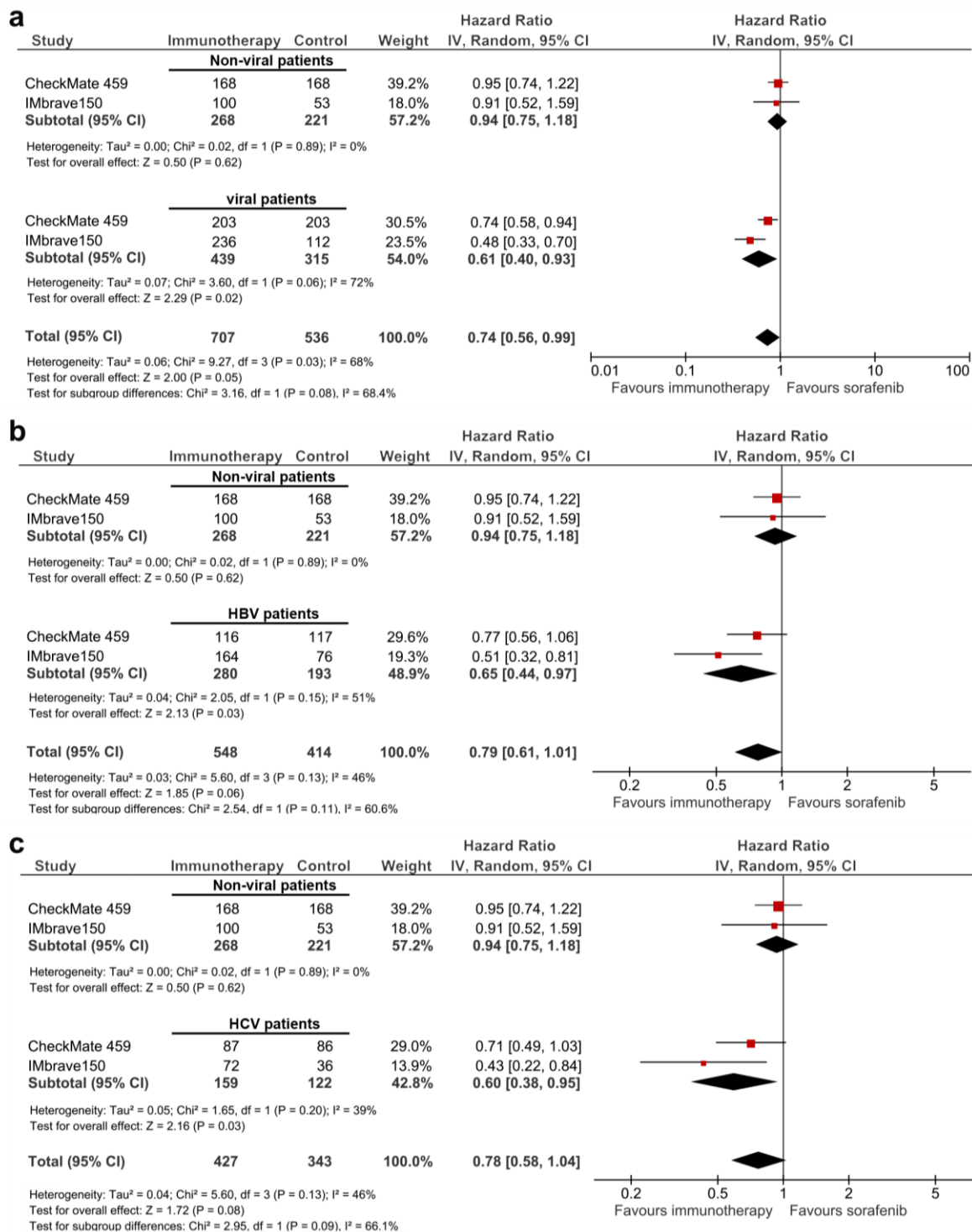


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**Rebuttal Figure 3: PD-1 and PD-L1 targeted immunotherapy in advanced HCC has a distinct effect depending on disease etiology**

A total of 1656 patients were included in all three randomized trials, and 985 patients received a checkpoint inhibitor. Subgroup analysis was performed to study the specific effects of immunotherapy comparing non-viral etiologies (n=737) with (a) HBV (n=574) or (b) HCV (n=345). Hazard ratios for each trial are represented by squares, the size of the square represents the weight of the trial in the meta-analysis. The horizontal line crossing the square represents the 95% confidence interval (CI). The diamonds represent the estimated overall effect based on the meta-analysis random effect of all trials.

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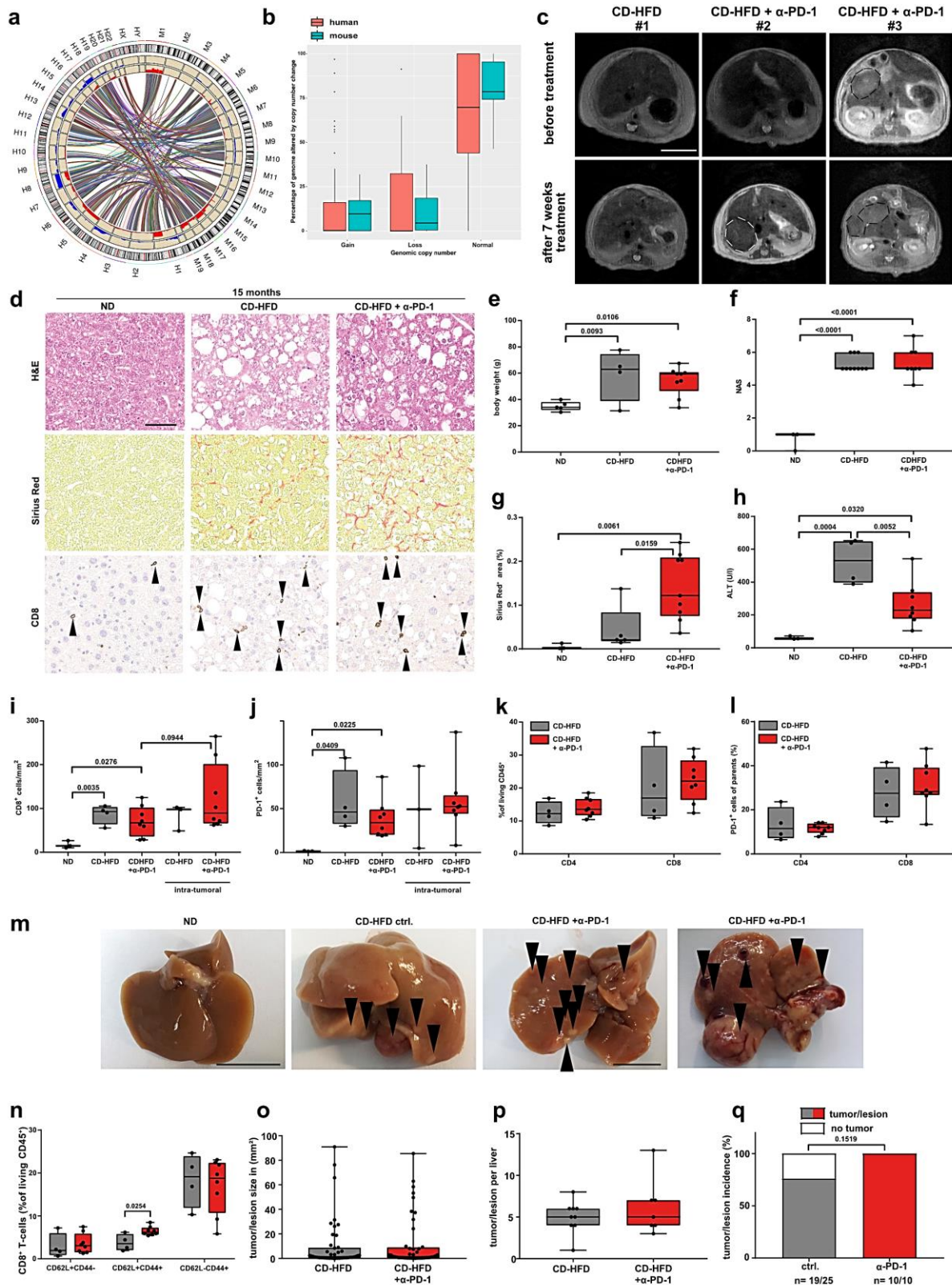


**Rebuttal Figure 4: Subgroup analysis of PD-1 and PD-L1 targeted immunotherapy in first-line trials of advanced HCC**

A total of 1243 patients were included in two first-line trials comparing PD-1 or PD-L1 targeted immunotherapy to sorafenib. 707 patients received an immune checkpoint inhibitor (either PD-1 or anti-PD-1). (a) HCV and HBV were pooled into a separate category, termed "viral", and a subsequent meta-analysis comparing viral (n=754) and non-viral (n=489), mostly NASH and alcohol intake, was performed. A subgroup analysis studying the specific effects of non-viral etiologies (n=489) on the magnitude of effect of immunotherapy are presented, when compared to (b) HBV (n=473) or (c) HCV (n=281). Hazard ratios for each trial are represented by squares, the size of the square represents the weight of the trial in the meta-analysis. The horizontal line crossing the square represents the 95% confidence interval (CI). The diamonds represent the estimated overall effect based on the meta-analysis random effect of all trials.

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**Rebuttal Figure 5:  $\alpha$ -PD-1 treatment does not achieve anti-tumor effects in NASH-induced tumors**

(a) Synteny analysis of mouse-HCC and (b) quantification of genomic aberrations by array-based Comparative Genomic Hybridization (aCGH) after 12 months on CD-HFD (n= 19) and human NALFD/NASH-HCC (n= 78). (c) MRI pictures of liver of mice after 13- months CD-HFD-fed mice followed by 7 weeks treatment of CD-HFD or CD-HFD + 7 weeks by  $\alpha$ -PD-1 antibodies (CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice). Lines indicate tumor nodule. Scale bar: 10 mm. (d) Histological staining of hepatic tissue by H&E, Sirius Red and CD8 of 15 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (H&E: ND n= 3 mice; CD-HFD n= 10 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice; Sirius Red: ND n= 3 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 9 mice; CD8: ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 3 mice). Scale bar: 50  $\mu$ m. Arrowheads indicate CD8<sup>+</sup> cells. (e) Body weight of 15 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1

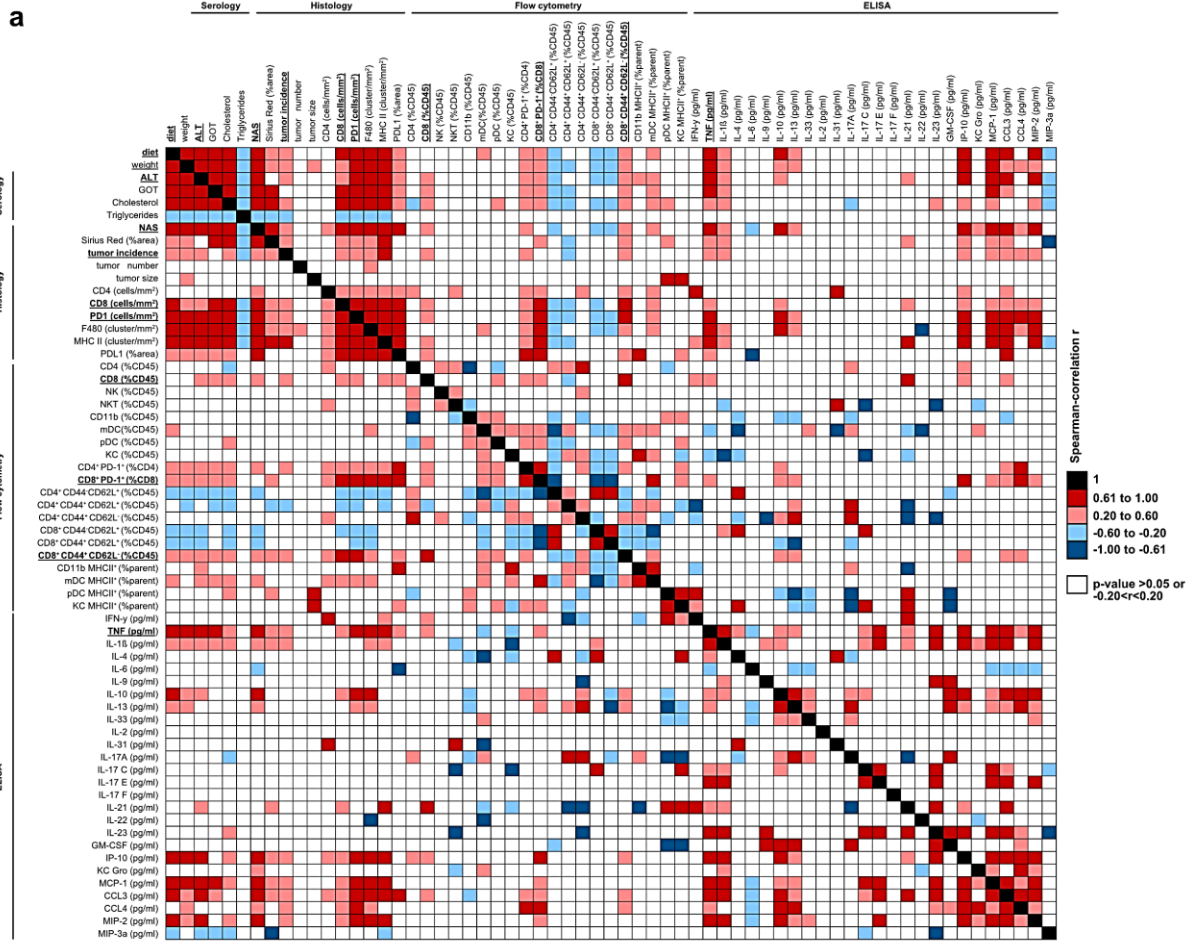
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1802 antibodies (ND n= 5 mice; CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 9 mice). **(f)** NAS evaluation by H&E of 15  
1803 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 3 mice; CD-HFD n=  
1804 10 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). **(g)** Fibrosis quantification (Sirius Red) of 15 months ND, CD-HFD or CD-  
1805 HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 3 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1  
1806 n= 9 mice). **(h)** ALT levels of 15 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1  
1807 antibodies (ND n= 3 mice; CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). **(i)** Quantification of CD8<sup>+</sup> and **(j)** PD-  
1808 1<sup>+</sup> cells in hepatic tissue by immunohistochemistry of 15 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks  
1809 treatment by  $\alpha$ -PD-1 antibodies (ND n= 3 mice; CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice; intra-tumoral  
1810 staining: CD-HFD n= 3 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). **(k)** Quantification and **(l)** expression of PD-1 of hepatic  
1811 CD4<sup>+</sup> and CD8<sup>+</sup> T-cells by flow cytometry of 15 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -  
1812 PD-1 antibodies (CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). **(m)** Macroscopy of liver of 15 months ND, CD-  
1813 HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies. Arrowheads indicate tumor/lesions. Scale  
1814 bar: 10 mm. **(n)** Quantification of CD8<sup>+</sup> T-cells by flow cytometry of 15 months CD-HFD or CD-HFD-fed mice + 8  
1815 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 3 mice; CD-HFD n= 4 mice; CD-HFD +  $\alpha$ -PD-1 n= 8 mice). **(o)**  
1816 Quantification of tumor/lesion size, **(p)** tumor load and **(q)** tumor incidence of 15 months CD-HFD or CD-HFD-fed  
1817 mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (tumor/lesion size and tumor load: CD-HFD n= 9 mice; CD-HFD +  
1818  $\alpha$ -PD-1 n= 7 mice; tumor incidence: CD-HFD n= 17 tumors/lesions in 22 mice; CD-HFD +  $\alpha$ -PD-1 n= 10  
1819 tumors/lesions in 10 mice).  
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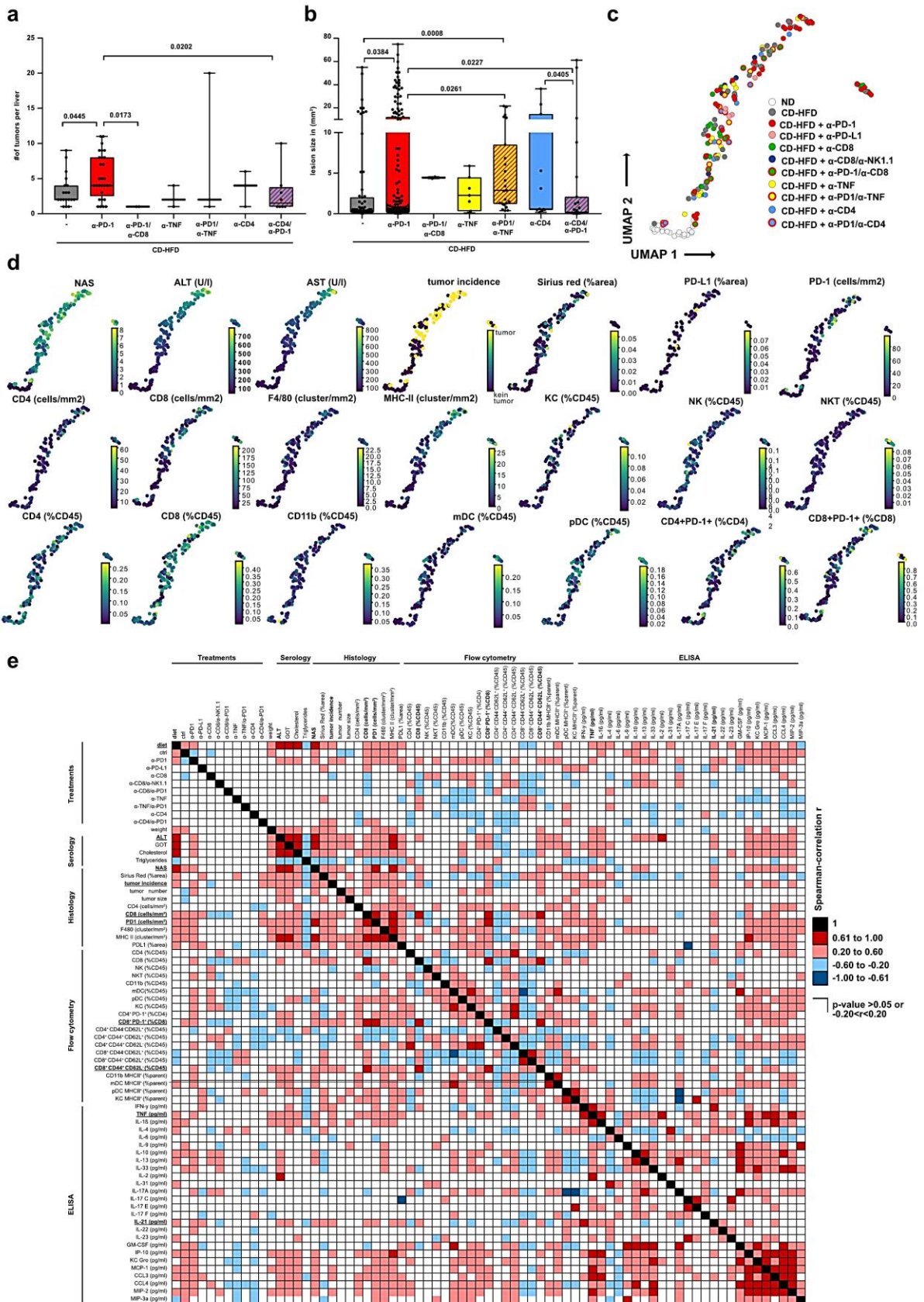


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**Rebuttal Figure 6: Hepatic immune cell environment, including CD8<sup>+</sup> T-cells abundance and effector phenotype correlate with NASH pathology and liver cancer incidence**

(a) Data gathered from hepatic tissue analyses was binary correlated with each other of 6- or 12-months ND or CD-HFD-fed mice (ND n= 47 mice; CD-HFD n= 72 mice). NAS correlated with diet, weight, ALT, GOT, cholesterol, Sirius red, CD8 cells/mm<sup>2</sup>, PD-1 cells/mm<sup>2</sup>, F4/80 cluster/mm<sup>2</sup>, MHCII cluster/mm<sup>2</sup>, PD-L1 (%area), CD8 (%CD45), CD8<sup>+</sup>CD44<sup>+</sup>CD62L<sup>-</sup> (%CD45), mDC MHC II<sup>+</sup> (%parent), TNF (pg/ml), IL-1β (pg/ml), IL-10 (pg/ml), IL-13 (pg/ml), IP-10 (pg/ml), MCP-1 (pg/ml), CCL3 (pg/ml), CCL4 (pg/ml), MIP-2 (pg/ml). Tumor incidence correlated with diet, weight, ALT, cholesterol, NAS, Sirius red, CD8 cells/mm<sup>2</sup>, PD-1 cells/mm<sup>2</sup>, F4/80 cluster/mm<sup>2</sup>, MHCII cluster/mm<sup>2</sup>, CD8 (%CD45), CD8<sup>+</sup>CD44<sup>+</sup>CD62L<sup>-</sup> (%CD45), TNF (pg/ml), IL-1β (pg/ml), IP-10 (pg/ml), MCP-1 (pg/ml), CCL3 (pg/ml), CCL4 (pg/ml), MIP-2 (pg/ml).

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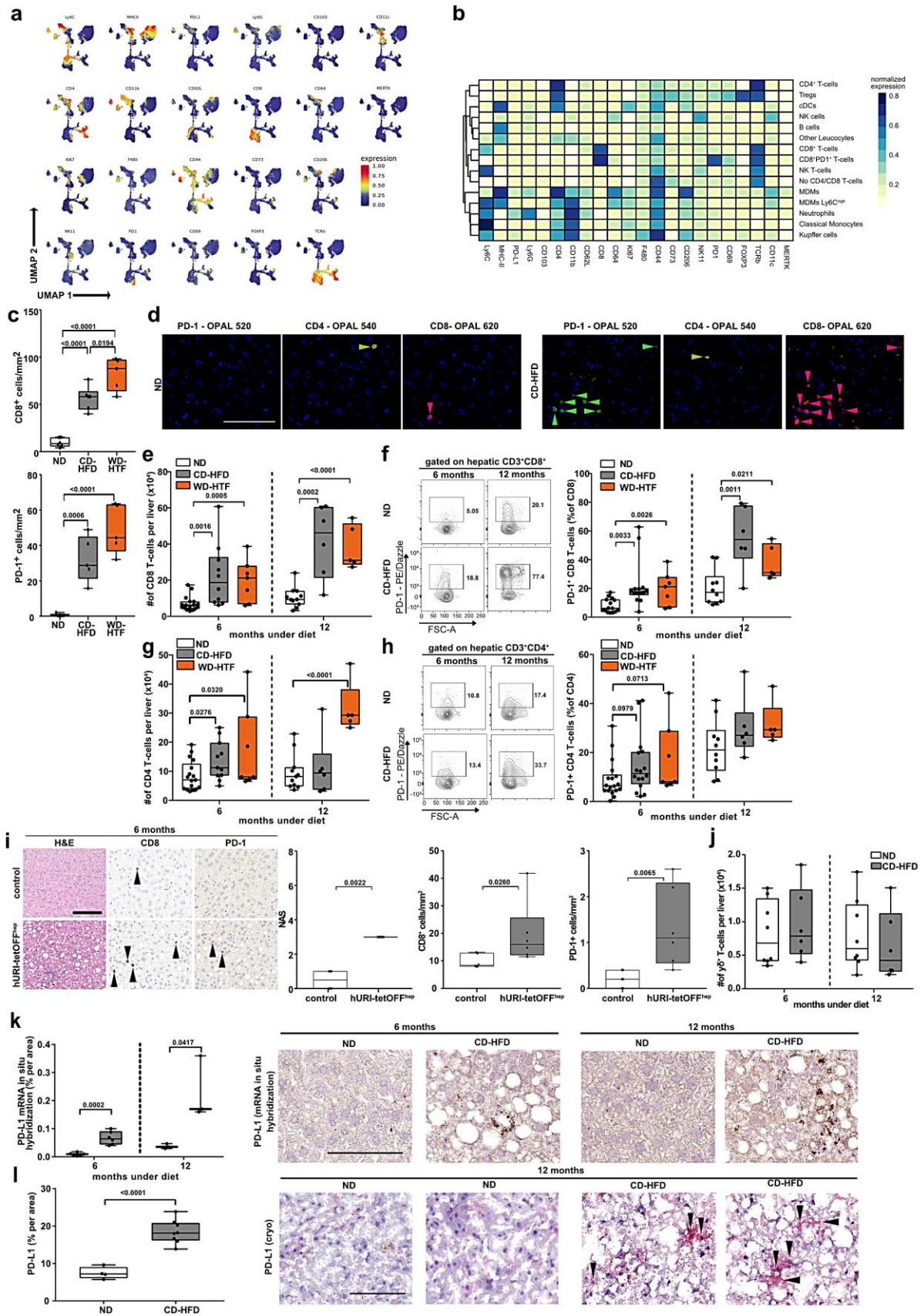
**Rebuttal Figure 7: PD-1-targeted immunotherapy induces hepatic inflammation, which drives hepatocarcinogenesis in a CD8<sup>+</sup> T-cell dependent manner**  
**(a)** Tumor/lesion load and **(b)** tumor/lesion size of 12-months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-PD-1/α-CD8, α-TNF, α-PD-1/α-TNF, α-CD4, or α-PD-1/α-CD4 antibodies (CD-HFD n= 19 mice; CD-HFD + α-PD-1 n= 29 mice; CD-HFD + α-PD-1/α-CD8 n= 2 mice; CD-HFD + α-TNF n= 3 mice; CD-HFD + α-PD-1/α-TNF n= 3 mice; CD-HFD + α-CD4 n= 3 mice; CD-HFD + α-PD-1/α-CD4 n= 8 mice). **(c)** UMAP representation



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1839 of 63 parameters (serology, flow cytometry, histology) and **(d)** selected display of analyzed parameters indicating  
1840 NASH pathology severity measured of 12 months ND, CD-HFD or CD-HFD-fed mice+ 8 weeks treatment by  $\alpha$ -  
1841 CD8,  $\alpha$ -CD8/ $\alpha$ -NK1.1;  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  $\alpha$ -CD4, or  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (ND n=  
1842 22 mice; CD-HFD n= 31 mice; CD-HFD +  $\alpha$ -PD-1 n= 41 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; CD-HFD +  $\alpha$ -CD8 n=  
1843 24 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice;  
1844 CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice). **(e)** Data  
1845 gathered from hepatic tissue analyses was binary correlated with each other of 6- or 12-months ND, CD-HFD or  
1846 CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8,  $\alpha$ -CD8/ $\alpha$ -NK1.1;  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -TNF,  
1847  $\alpha$ -CD4, or  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (ND n= 47 mice; CD-HFD n= 72 mice; CD-HFD +  $\alpha$ -PD-1 n= 41 mice; CD-HFD  
1848 +  $\alpha$ -PD-L1 n= 6 mice; CD-HFD +  $\alpha$ -CD8 n= 29 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 6 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8  
1849 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-  
1850 HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice).

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**Rebuttal Figure 8: T-cell activation and hepatic abundance correlate with NASH pathology**

(a) Umap showing the expression intensity of the indicated marker of scholastically selected CD45+ cells define distinct marker expression of 12 months ND or CD-HFD-fed mice (ND n= 4 mice; CD-HFD n= 8 mice). (b) Heatmap showing the median marker expression of the defined CD45+ subsets displayed in (a) by flow cytometry of 12 months ND or CD-HFD-fed mice (ND n= 4 mice; CD-HFD n= 8 mice). (c) Quantification of hepatic CD8+ cells and PD-1+ expressing cells by immunohistochemistry of 12 months ND, CD-HFD or WD-HTF-fed mice (PD-1: n= 5

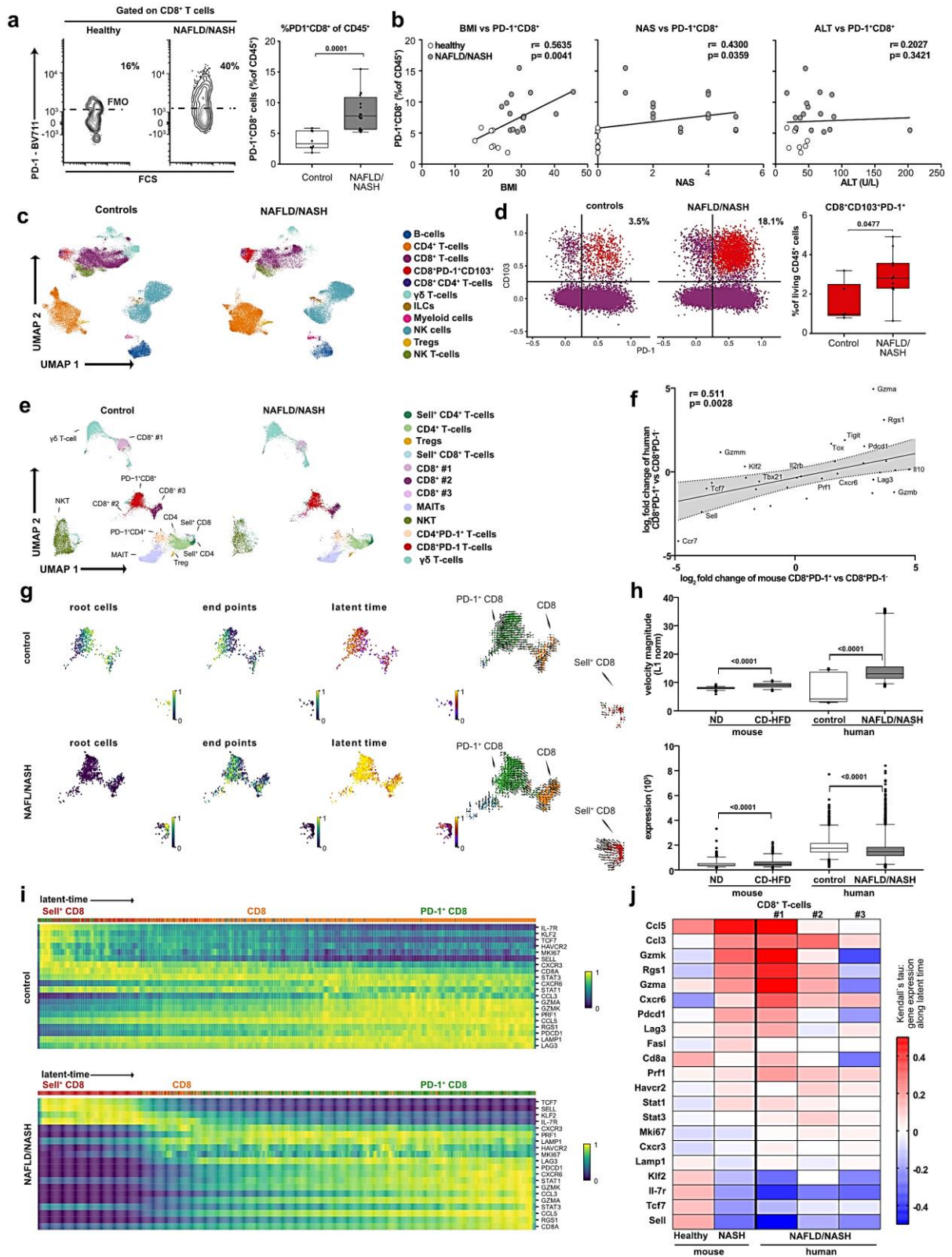
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1858 mice/group; CD8: ND n= 6 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice). **(d)** Immunofluorescence staining of  
1859 single channel-staining PD-1, CD8 and CD4 of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). Arrowheads  
1860 indicate CD8<sup>+</sup> (red), PD-1<sup>+</sup> (green) or CD4<sup>+</sup> (ocher) cells. Scale bar: 100  $\mu$ m. **(e)** Quantification of abundance, **(f)**  
1861 PD-1 expression and flow cytometry plots of hepatic CD8<sup>+</sup> T-cells by flow cytometry of 6 or 12 months ND or CD-  
1862 HFD-fed mice (abundance of CD8: 6 months: ND n= 17 mice; CD-HFD n= 10 mice; WD-HTF n= 7 mice; 12 months:  
1863 ND n= 11 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice; PD-1 expression in CD8<sup>+</sup> T-cells: 6 months: ND n= 15  
1864 mice; CD-HFD n= 14 mice; WD-HTF n= 7 mice; 12 months: ND n= 10 mice; CD-HFD n= 6 mice; WD-HTF n= 5  
1865 mice). **(g)** Quantification of abundance, **(h)** PD-1 expression and flow cytometry plots of hepatic CD4<sup>+</sup> T-cells by  
1866 flow cytometry of 6 or 12 months ND or CD-HFD-fed mice (abundance of CD4: 6 months: ND n= 17 mice; CD-HFD  
1867 n= 10 mice; WD-HTF n= 7 mice; 12 months: ND n= 11 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice; PD-1  
1868 expression in CD4<sup>+</sup> T-cells: 6 months: ND n= 15 mice; CD-HFD n= 14 mice; WD-HTF n= 7 mice; 12 months: ND  
1869 n= 10 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice). **(i)** H&E, CD8 and PD-1 staining, evaluation by NAS and  
1870 quantification of CD8<sup>+</sup> cells and PD-1<sup>+</sup> expressing cells by immunohistochemistry of 32-weeks old hURI-tetOFFhep  
1871 and non-transgenic litter control mice (n=6 mice/group). Arrowheads indicate specific staining positive cells. Scale  
1872 bar: 100  $\mu$ m. **(j)** Hepatic abundance of TCR $\gamma\delta$  T-cells of 6 or 12 months ND or CD-HFD-fed mice (6 months ND n=  
1873 8 mice; CD-HFD n= 6 mice; 12 months ND n= 8 mice; CD-HFD n= 6 mice). **(k)** Quantification of hepatic PD-L1<sup>+</sup>  
1874 expression by mRNA *in situ* hybridization of 6- or 12-months ND or CD-HFD-fed mice (6 months: ND n= 13 mice;  
1875 CD-HFD n= 11 mice; 12 months: ND n= 7 mice; CD-HFD n= 7 mice). Scale bar: 100  $\mu$ m. **(l)** Quantification of hepatic  
1876 PD-L1<sup>+</sup> expression by immunohistochemistry of 12 months ND or CD-HFD-fed mice (6 months: ND n= 4 mice; CD-  
1877 HFD n= 8 mice). Scale bar: 100  $\mu$ m.

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**Rebuttal Figure 9: Hepatic resident-like CD8<sup>+</sup>PD-1<sup>+</sup> T-cells are increased in livers of non-alcoholic fatty liver disease (NAFLD) patients**

(a) Flow cytometry plots, quantification of patient-liver-derived PD-1<sup>+</sup>CD8<sup>+</sup> T-cells, and (b) correlation of PD-1<sup>+</sup>CD8<sup>+</sup> T-cells with BMI, NAS and ALT of healthy or NAFLD/NASH patients (**Supplementary Table 1**: healthy n= 8 patients; NAFLD/NASH n= 16 patients). Fluorescence-minus-one (FMO). (c) UMAP representation showing the FlowSOM-guided clustering of CD45<sup>+</sup> cells and (d) flow cytometry plots and quantification of CD8<sup>+</sup>PD-1<sup>+</sup>CD103<sup>+</sup> derived from hepatic biopsies of control, or NAFLD/NASH patients (**Supplementary Table 2**: control n= 6 patients; NAFLD/NASH n= 11 patients) Populations: CD8<sup>+</sup> (violet), CD8<sup>+</sup>PD-1<sup>+</sup>CD103<sup>+</sup> (red). (e) UMAP representation of CD3<sup>+</sup> cells and analyses of differential gene expression by scRNA-seq of control, or NAFLD/NASH patients (control

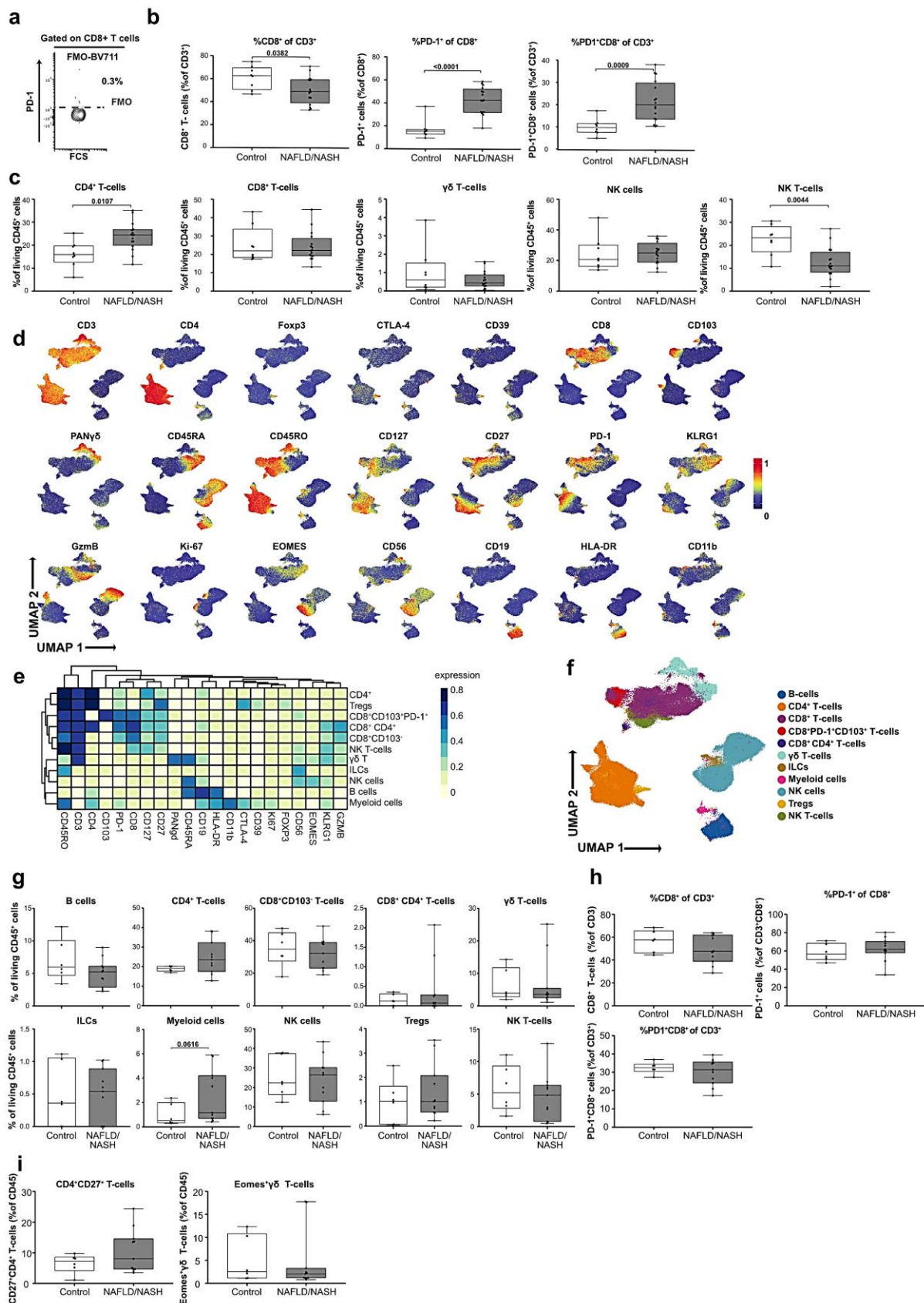


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1888 n= 4 patients; NAFLD/NASH n= 7 patients). **(f)** Correlation of significant differentially expressed genes in liver-  
1889 derived CD8<sup>+</sup>PD-1<sup>+</sup> compared to CD8<sup>+</sup>PD-1<sup>-</sup> T-cells subsets of 12 months CD-HFD-fed mice and NAFLD/NASH  
1890 patients (mouse: n= 3 mice; human: n= 3 patients). **(g)** Velocity analyses of scRNA-seq data showing **(h)**  
1891 expression, transcriptional activity, **(i)** gene expression and **(j)** correlation of expression along the latent-time of  
1892 selected genes along the latent-time of patient-liver-derived CD8<sup>+</sup> T-cells of control, or NAFLD/NASH patients in  
1893 comparison to mouse-liver-derived CD8<sup>+</sup> T-cells (patients: NAFLD/NASH n= 3 patients; mouse: n= 3 mice/group).  
1894 Root cells: yellow cells indicate root cells, blue cells indicate cells farthest away from the root by RNA velocity. End  
1895 points: yellow cells indicate end point cells, blue cells indicate cells farthest away from defined end point cells by  
1896 RNA velocity. Latent time: pseudo-time by RNA velocity, dark color indicate start of RNA velocity, yellow color  
1897 indicate end point of latent time. RNA velocity flow: Blue cluster defined as start point, orange cluster as  
1898 intermediate, green cluster as end point. Arrows indicate the trajectory of cells.



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**Rebuttal Figure 10: An inflammatory cellular polarization of T-cells can be found in liver biopsies of NAFLD/NASH patients**  
**(a)** Flow cytometry plot of FMO control, **(b)** quantification of patient-liver-derived PD-1<sup>+</sup>CD8<sup>+</sup> T-cells, and **(c)** quantification of CD4, CD8, γδ, NK and NKT cells healthy or NAFLD/NASH patients (**Supplementary Table 1**: healthy n= 8 patients; NAFLD/NASH n= 16 patients). **(d)** Umap showing the expression intensity of the indicated marker on stochastically selected CD45<sup>+</sup> cells and **(e)** Heatmap showing the median marker expression of the defined CD45<sup>+</sup> subsets of figure 5c by flow cytometry derived from hepatic biopsies of control and NAFLD/NASH

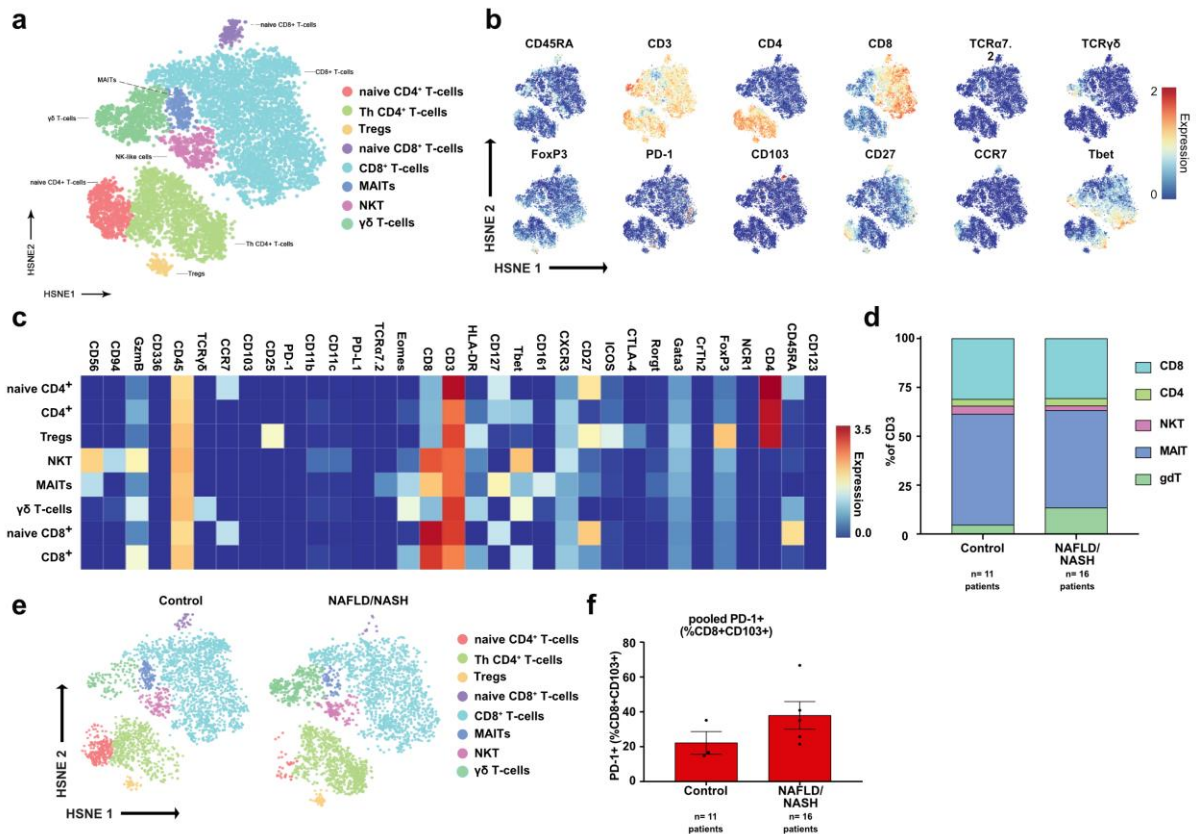
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1906 patients to define distinct marker expression (**Supplementary Table 2**: control n= 6 patients; NAFLD/NASH n= 11  
1907 patients). **(f)** Definition of cellular subsets, **(g)** relative quantification of defined cellular subsets of randomly chosen  
1908 CD45<sup>+</sup> cells, **(h)** polarization of CD8<sup>+</sup> T-cells and **(i)** quantification of CD4<sup>+</sup>CD27<sup>+</sup>, or  $\gamma\delta$  TCR<sup>+</sup>Eomes<sup>+</sup> T-cells by  
1909 flow cytometry derived from hepatic biopsies of healthy and NAFLD/NASH patients (**Supplementary Table 2**:  
1910 control n= 6 patients; NAFLD/NASH n= 11 patients).

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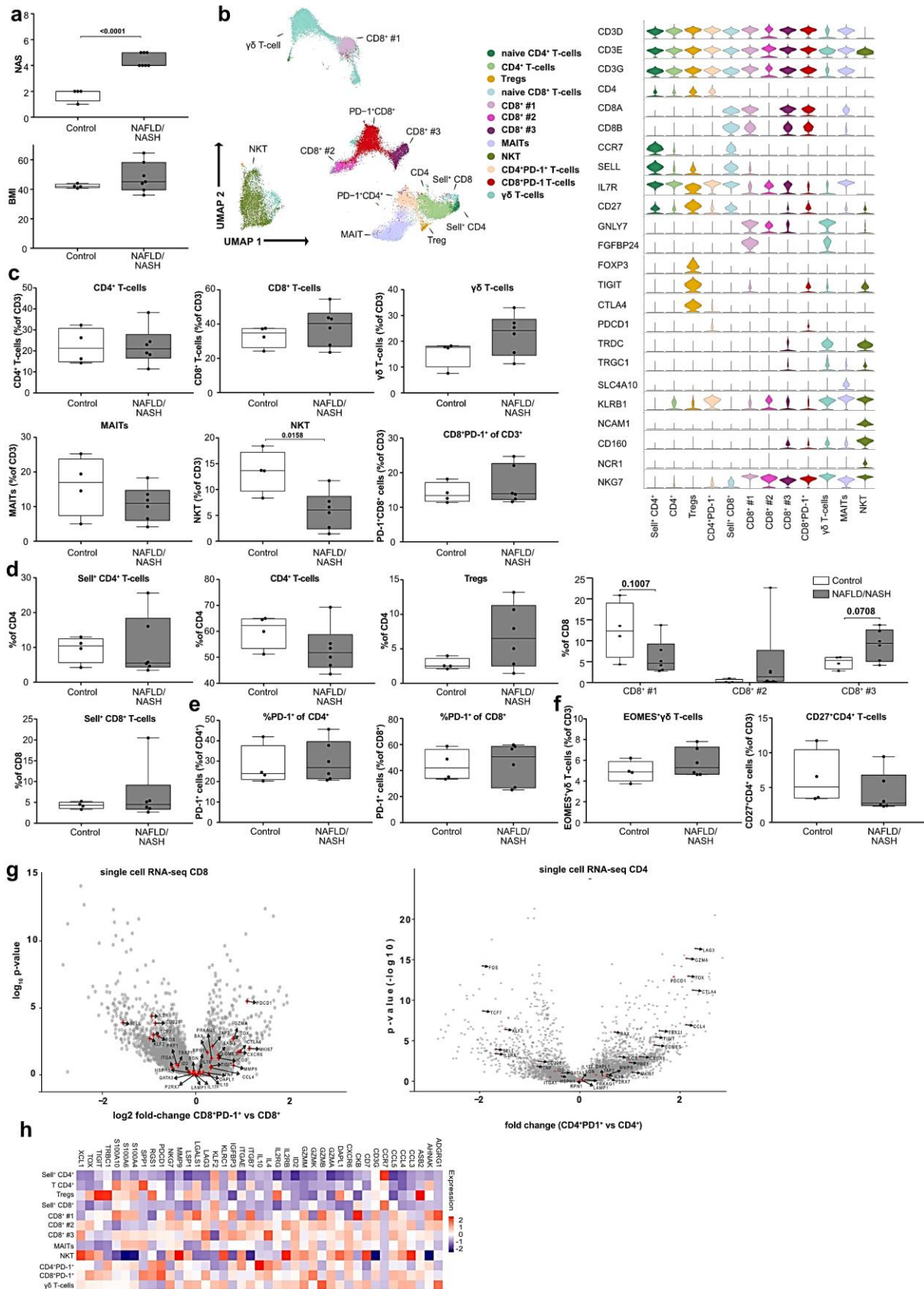


**Rebuttal Figure 11: CyTOF analyses of T-cells from liver biopsies of NAFLD/NASH patients reveals co-expression of PD-1 and CD103 in CD8<sup>+</sup> T-cells**

(a) tSNE representation, (b) marker expression, (c) average marker expression of defined T-cell subsets of patient-liver-derived T-cells analyzed by CyTOF of control and NAFLD/NASH patients (control n= 11 patients pooled in 3 analyses; NAFLD/NASH n= 16 patients pooled in 5 analyses). (d) Composition, (e) HSNE representation of defined T-cell subsets and (f) quantification of CD8<sup>+</sup>CD103<sup>+</sup>PD-1<sup>+</sup> cells of patient-liver-derived T-cells analyzed by CyTOF of control and NAFLD/NASH patients (control n= 11 patients pooled in 3 analyses; NAFLD/NASH n= 16 patients pooled in 5 analyses).

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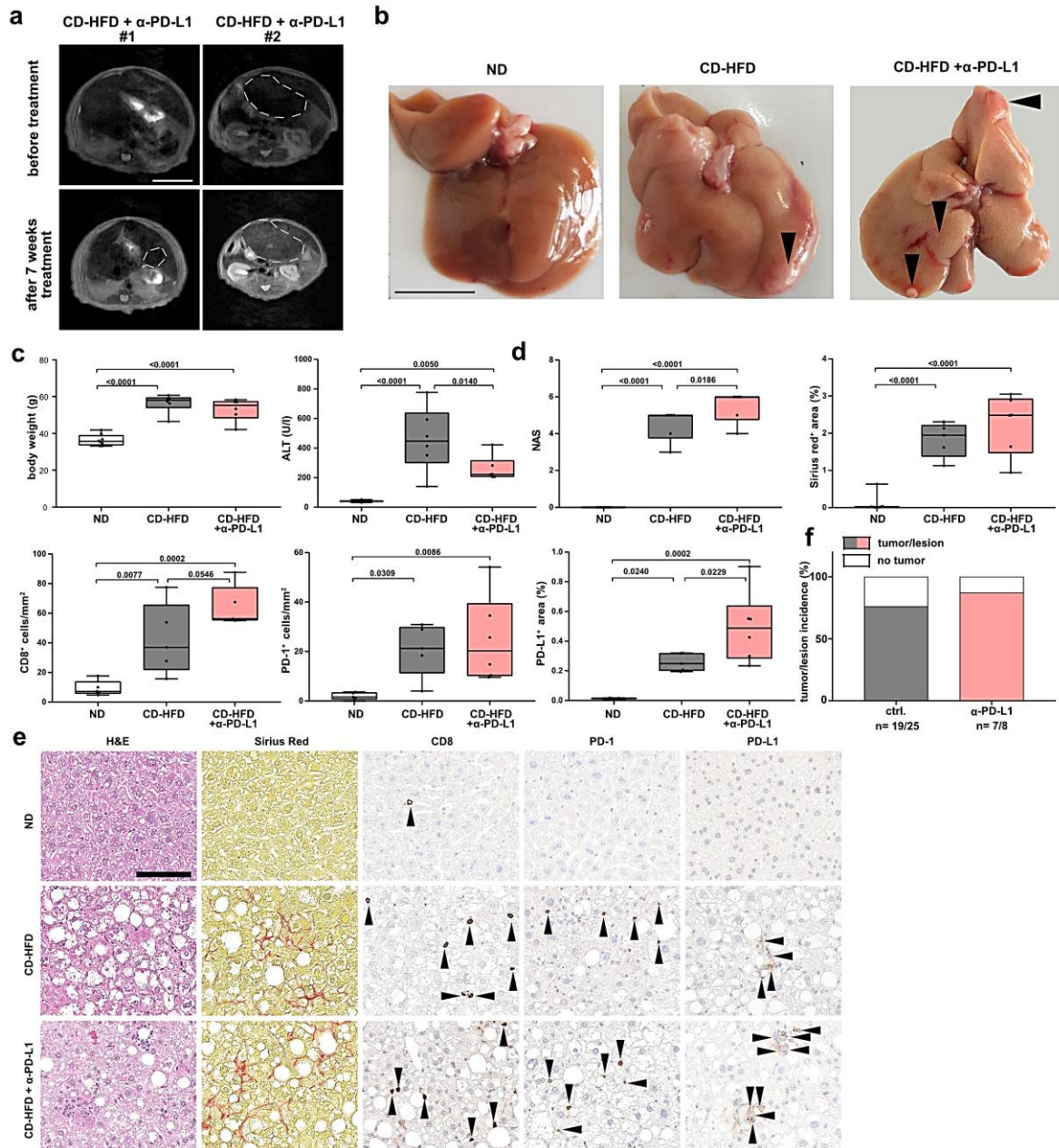
**Rebuttal Figure 12: Single cell RNA-sequencing of T-cells found in patient liver biopsies of NAFLD/NASH corroborate mouse gene expression inflammatory**

(a) NAS and BMI of patients used for scRNA-seq analyses of patient-liver-derived T-cells of control and NAFLD/NASH patients (control n = 4 patients; NAFLD/NASH n = 7 patients). (b) UMAP representation, marker expression, (c) relative quantification and (d), (e), (f) polarization of defined T-cell subsets of defined T-cell subsets of patient-liver-derived T-cells by scRNA-seq of control and NAFLD/NASH patients (control n = 4 patients;



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1927 NAFLD/NASH n= 7 patients). **(g)** Differential gene expression of CD4+PD-1+ vs CD4+ T-cells and **(h)** selected  
1928 average marker expression in CD4+ and CD8+ T-cell subsets of by scRNA-seq of control and NAFLD/NASH patients  
1929 (control n= 4 patients; NAFLD/NASH n= 7 patients).

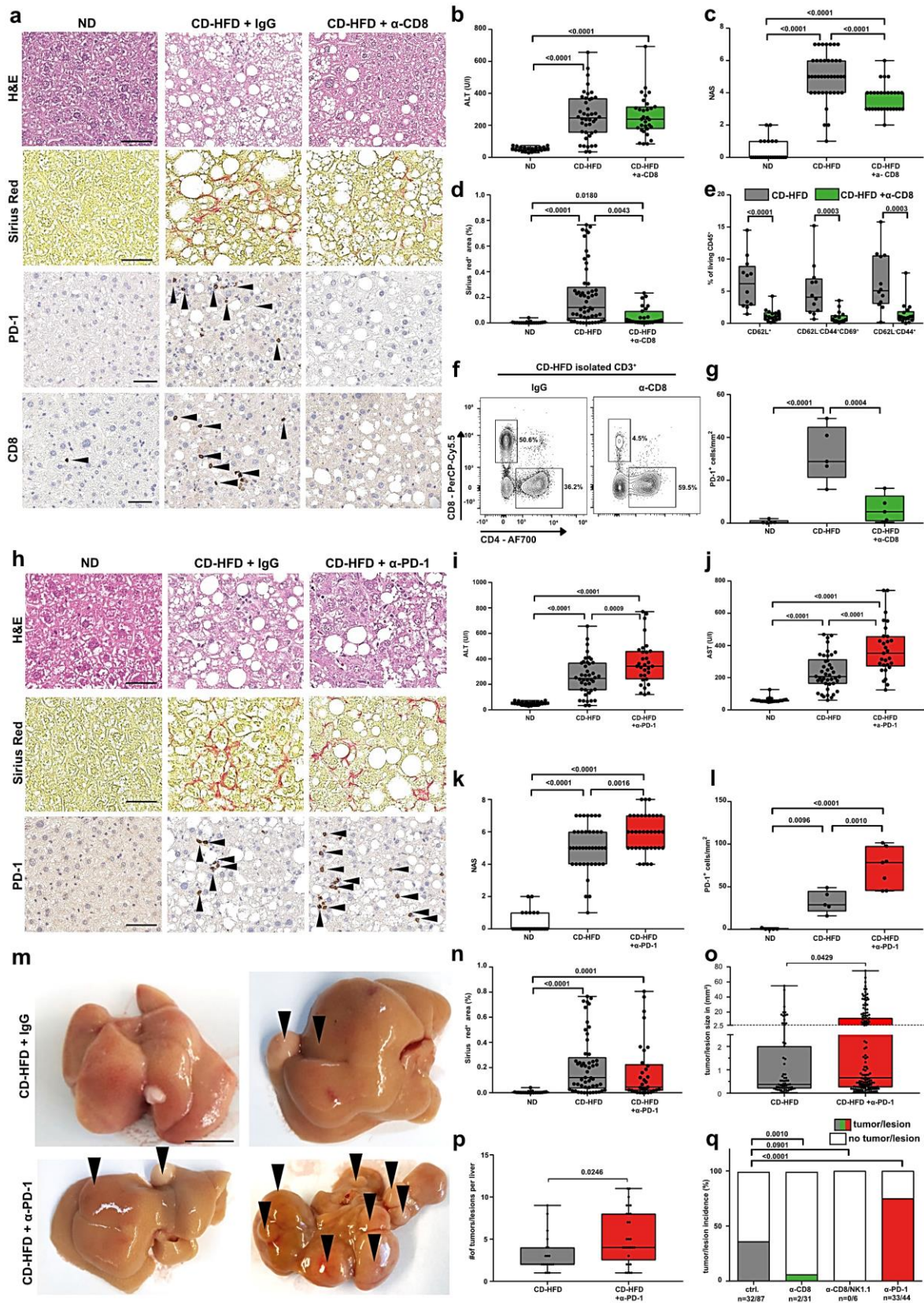


**Rebuttal Figure 13:  $\alpha$ -PD-L1 treatment does not achieve anti-tumor effects in NASH-induced tumors**

(a) MRI pictures of liver of mice after 13 months CD-HFD followed by 7 weeks treatment to CD-HFD or CD-HFD-fed mice + 7 weeks by  $\alpha$ -PD-L1 antibodies (CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-L1 n= 8 mice). Lines indicate tumor nodule. Scale bar: 10 mm. (b) Macroscopy of liver of ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-L1 antibodies. Arrowheads indicate tumor/lesions. Scale bar: 10 mm. (c) Body weight, ALT levels ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-L1 antibodies (Body weight, ALT, : ND n= 8 mice; CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice) (d) and (e) NAS evaluation by H&E, fibrosis quantification (Sirius Red), quantification of CD8, PD-1 and PD-L1 staining of hepatic tissue by immunohistochemistry of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-L1 antibodies (NAS: ND n= 7 mice; CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; Sirius Red: ND n= 7 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice; CD8, : ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 5 mice; PD-1, PD-L1: ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-L1 n= 6 mice). Scale bar: 100  $\mu$ m. (f) Tumor/Lesion incidence in CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-L1 antibodies (CD-HFD n= 19 tumors/lesions in 25 mice; CD-HFD +  $\alpha$ -PD-L1 n= 7 tumors/lesions in 8 mice). Arrowheads indicate specific staining positive cells.

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**Rebuttal Figure 14: Figure 2: Anti-PD-1 treatment drives hepatocarcinogenesis in a CD8-dependent manner in NASH**  
**(a)** Histological staining of hepatic tissue by H&E, Sirius Red, PD-1 and CD8 of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (H&E: ND n= 24 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -CD8 n= 29 mice; Sirius Red: ND n= 19 mice; CD-HFD n= 31 mice; CD-HFD +  $\alpha$ -CD8 n= 24 mice; PD-1: n= 5 mice/group; CD8: ND n= 6 mice; CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -CD8 n= 5 mice). Arrowheads indicate CD8<sup>+</sup> or PD-1<sup>+</sup> cells. Scale bar: 50  $\mu$ m. **(b)** ALT levels of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (ND n= 22 mice; CD-HFD n= 42 mice; CD-HFD +  $\alpha$ -CD8 n= 31 mice). **(c)** NAS evaluation by H&E of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (ND n= 24 mice;

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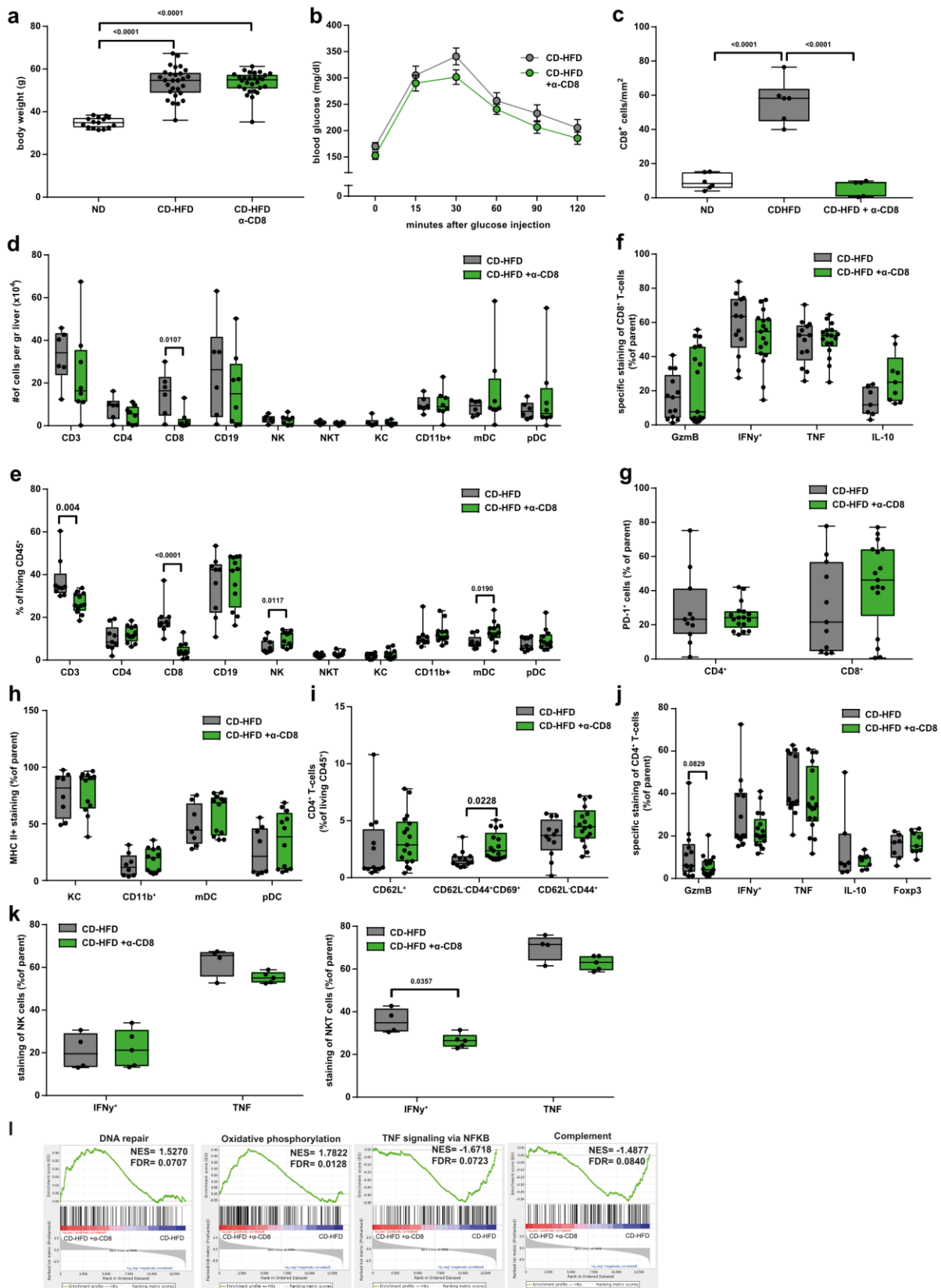


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1954 CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -CD8 n= 29 mice). **(d)** Fibrosis quantification (Sirius Red) of 12 months ND, CD-  
 1955 HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (ND n= 19 mice; CD-HFD n= 53 mice; CD-  
 1956 HFD +  $\alpha$ -CD8 n= 27 mice) **(e)** Flow cytometry analysis for polarization of hepatic CD8<sup>+</sup> T-cells of 12 months CD-  
 1957 HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -CD8 n=  
 1958 17 mice). **(f)** Flow cytometry plots of 12 months ND, CD-HFD or CD-HFD + 8 weeks treatment by  $\alpha$ -CD8 antibodies.  
 1959 **(g)** Quantification of PD-1<sup>+</sup> cells of hepatic tissue by immunohistochemistry of 12 months ND, CD-HFD or CD-HFD  
 1960 + 8 weeks treatment by  $\alpha$ -CD8 antibodies (n= 5 mice/group). **(h)** Histological staining of hepatic tissue by H&E,  
 1961 Sirius Red, PD-1 and CD8 of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1  
 1962 antibodies (H&E: ND n= 24 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 36 mice; Sirius Red: ND n= 19 mice;  
 1963 CD-HFD n= 31 mice; CD-HFD +  $\alpha$ -PD-1 n= 27 mice; PD-1: ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1  
 1964 n= 7 mice). Arrowheads indicate PD-1<sup>+</sup> cells. Scale bar: 50  $\mu$ m. **(i)** ALT and **(j)** AST levels of 12 months ND, CD-  
 1965 HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ALT: ND n= 22 mice; CD-HFD n= 42 mice;  
 1966 CD-HFD +  $\alpha$ -PD-1 n= 30 mice). **(k)** NAS evaluation by H&E of 12 months ND, CD-HFD or CD-HFD + 8 weeks  
 1967 treatment by  $\alpha$ -PD-1 antibodies (ND n= 24 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 36 mice). **(l)**  
 1968 Quantification of PD-1<sup>+</sup> cells of hepatic tissue by immunohistochemistry of 12 months ND, CD-HFD or CD-HFD-fed  
 1969 mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 5 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 7 mice).  
 1970 **(m)** Macroscopy of liver of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies.  
 1971 Arrowheads indicate tumor/lesions. Scale bar: 10 mm. **(n)** Fibrosis quantification (Sirius Red) of 12 months ND,  
 1972 CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 19 mice; CD-HFD n= 53 mice;  
 1973 CD-HFD +  $\alpha$ -PD-1 n= 33 mice). **(o)** Quantification of tumor/lesion size and **(p)** tumor load of 12 months CD-HFD or  
 1974 CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (tumor/lesion size, tumor load: CD-HFD n= 19 mice;  
 1975 CD-HFD +  $\alpha$ -PD-1 n= 29 mice). **(q)** Quantification of tumor incidence of 12 months CD-HFD or CD-HFD-fed mice  
 1976 + 8 weeks treatment by  $\alpha$ -CD8, co-depletion of  $\alpha$ -CD8/NK1, or  $\alpha$ -PD-1 antibodies (tumor incidence: CD-HFD n= 32  
 1977 tumors/lesions in 87 mice; CD-HFD +  $\alpha$ -CD8 n= 2 tumors/lesions in 31 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n= 0  
 1978 tumors/lesions in 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 33 tumors/lesions in 44 mice).



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**Rebuttal Figure 15: CD8 T-cell depletion in NASH does not induce compensatory immunological reactions**

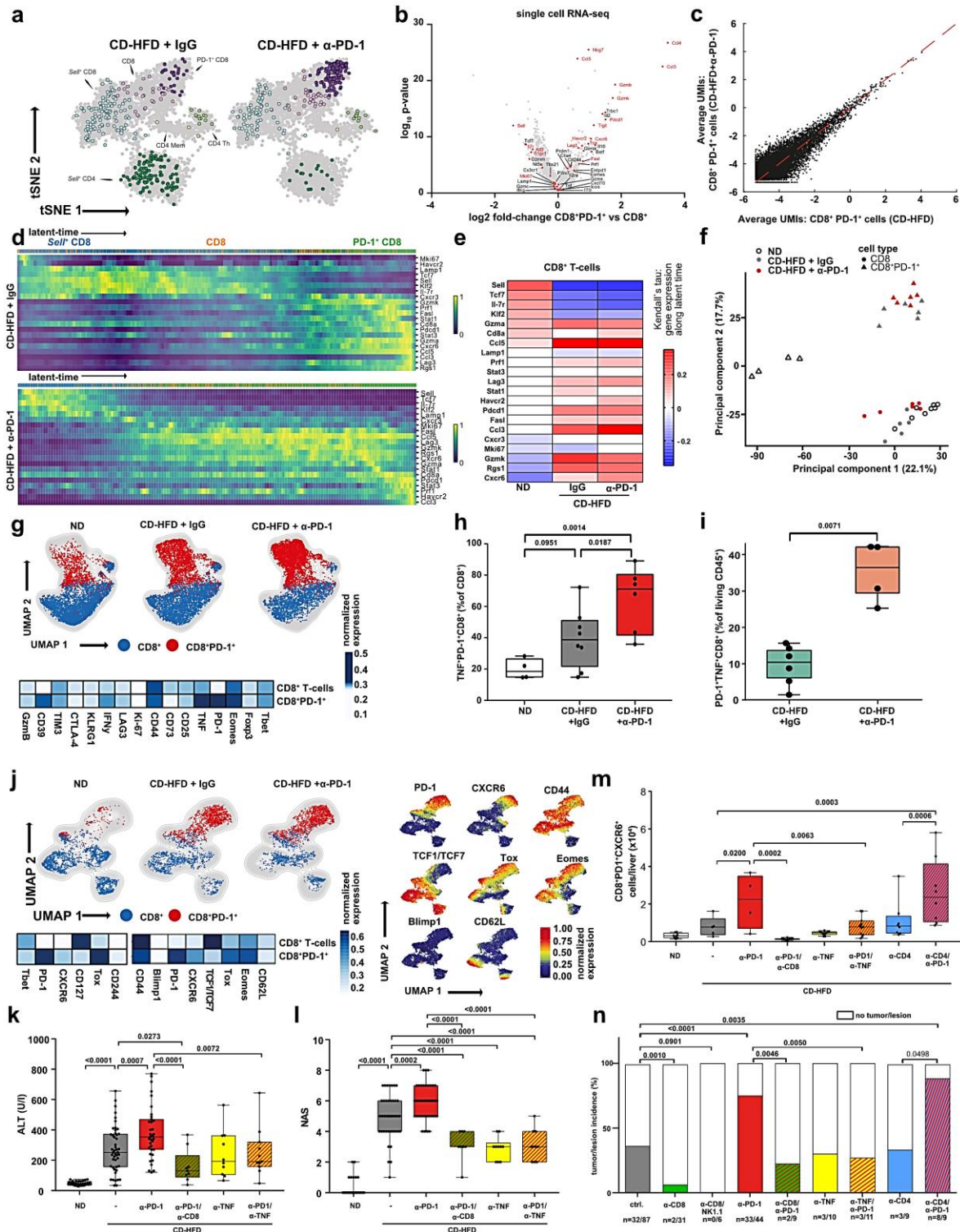
(a) Body weight of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (ND n= 15 mice; CD-HFD n= 28 mice; CD-HFD +  $\alpha$ -CD8 n= 28 mice). (b) Assessment of metabolic tolerance by intra peritoneal glucose tolerance test of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 8 mice; CD-HFD +  $\alpha$ -CD8 n= 10 mice). (c) Quantification of CD8 staining of hepatic tissue by immunohistochemistry of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (ND n= 6 mice; CD-HFD n= 6 mice; CD-HFD +  $\alpha$ -CD8 n= 5 mice). (d) Absolute and (e) relative quantification of hepatic leukocytes of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8



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1988 antibodies (CD-HFD n= 9 mice; CD-HFD +  $\alpha$ -CD8 n= 12 mice). **(f)** Analyses of cytokine expression for polarization  
 1989 of hepatic CD8<sup>+</sup> T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies  
 1990 (GzmB, IFN $\gamma$ , TNF: CD-HFD n= 13 mice;  $\alpha$ -CD8 + CD-HFD n= 17 mice; IL-10: CD-HFD n= 7 mice;  $\alpha$ -CD8 + CD-  
 1991 HFD n= 9 mice). **(g)** Expression of PD-1 of hepatic CD4<sup>+</sup> and CD8<sup>+</sup> T-cells by flow cytometry of 12 months CD-HFD  
 1992 or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 11 mice;  $\alpha$ -CD8 + CD-HFD n= 17  
 1993 mice). **(h)** Flow cytometry analysis for polarization of hepatic myeloid cells of 12 months CD-HFD or CD-HFD-fed  
 1994 mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 8 mice;  $\alpha$ -CD8 + CD-HFD n= 12 mice). **(i)** Flow  
 1995 cytometric analysis for polarization of hepatic CD4<sup>+</sup> T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks  
 1996 treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 12 mice;  $\alpha$ -CD8 + CD-HFD n= 17 mice). **(j)** Cytokine expression of  
 1997 hepatic CD4<sup>+</sup> T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (GzmB,  
 1998 IFN $\gamma$ , TNF: CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -CD8 n= 17 mice; IL-10, Foxp3: CD-HFD n= 7 mice; CD-HFD +  $\alpha$ -  
 1999 CD8 n= 9 mice). **(k)** Cytokine expression for polarization of hepatic NK and NKT-cells of 12 months CD-HFD or  
 2000 CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (CD-HFD n= 4 mice;  $\alpha$ -CD8 + CD-HFD n= 5 mice). **(l)**  
 2001 Gene set enrichment analysis of RNA sequencing data of hepatic tissue comparing CD-HFD with CD-HFD-fed mice  
 2002 +  $\alpha$ -CD8 of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (n= 5  
 2003 mice/group).

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**Rebuttal Figure 16: Resident-like CD8<sup>+</sup>PD-1<sup>+</sup> T-cells drive hepatocarcinogenesis in a TNF-dependent manner upon anti-PD-1 treatment in NASH**

(a) ScRNA-seq analysis of hepatic TCR $\beta$ <sup>+</sup> cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 or  $\alpha$ -CD8 antibodies (n = 3 mice/group). (b) Selected marker expression in hepatic CD8<sup>+</sup> T-cells by scRNA-seq comparing CD8<sup>+</sup> with CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n = 3 mice/group). (c) Average UMI comparison of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n = 3 mice/group). (d) RNA velocity analyses of scRNA-seq data showing expression and (e) correlation of expression along the latent-time of selected genes along the latent-time (n = 3 mice/group). Root cells: yellow cells indicate root cells, blue cells indicate cells farthest away from root by RNA velocity. End points: yellow cells indicate end point cells, blue cells indicate cells farthest away from defined end point cells by RNA velocity. Latent time: pseudo-time by RNA velocity, dark color indicate start of RNA velocity, yellow color indicate end point of latent time. RNA velocity flow: Blue cluster defined as start point, orange cluster as intermediate, green cluster as end point. Arrows indicate trajectory of cells.

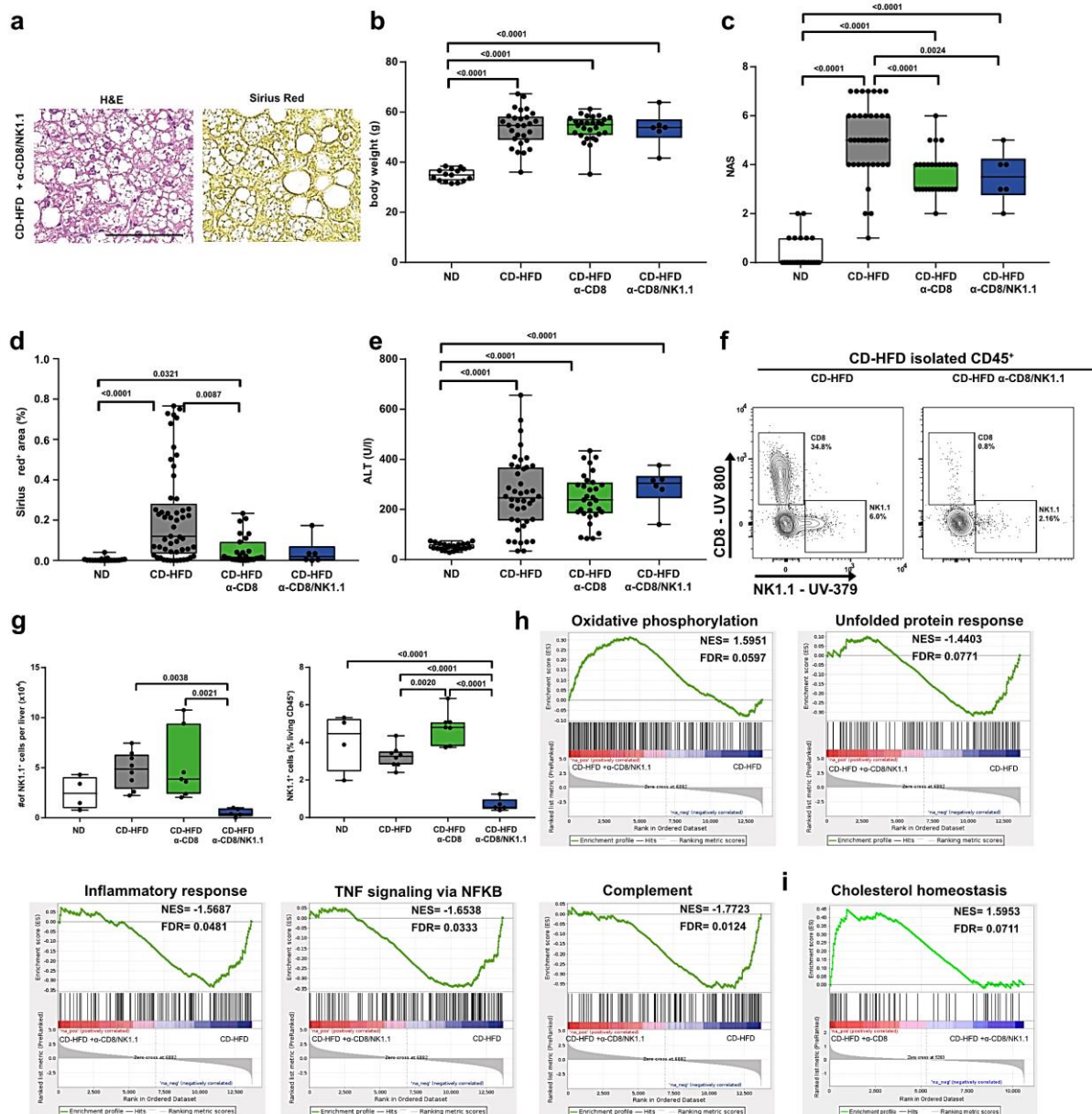
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2018 **(f)** PCA plot of hepatic CD8<sup>+</sup> or CD8<sup>+</sup>PD-1<sup>+</sup> T-cells sorted TCRβ<sup>+</sup> cells by mass spectrometry of 12 months ND, CD-  
 2019 HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (CD8<sup>+</sup>: ND n= 6 mice, CD-HFD + IgG n= 5  
 2020 mice; CD-HFD + α-PD-1 n= 6 mice; CD8<sup>+</sup>PD-1<sup>+</sup>: ND n= 4 mice, CD-HFD + IgG n= 6 mice; CD-HFD + α-PD-1 n= 6  
 2021 mice). **(g)** UMAP representation showing the FlowSOM-guided clustering, heatmap showing the median marker  
 2022 expression, and **(h)** quantification of hepatic CD8<sup>+</sup> T-cells of 12 months ND, CD-HFD + IgG or CD-HFD-fed mice +  
 2023 8 weeks treatment by α-PD-1 antibodies (ND n= 4 mice; CD-HFD + IgG n= 8 mice; CD-HFD + α-PD-1 n= 6 mice).  
 2024 **(i)** Quantification of CellCNN analyzed flow cytometry data of hepatic CD8<sup>+</sup> T-cells of 12 months CD-HFD + IgG or  
 2025 CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (CD-HFD + IgG n= 6 mice; CD-HFD + α-PD-1 n= 4  
 2026 mice). **(j)** UMAP representation showing the FlowSOM-guided clustering, the expression intensity of the indicated  
 2027 marker and heatmap showing the median marker expression of flow cytometry data of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells  
 2028 of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (ND n= 6 mice; CD-  
 2029 HFD n= 5 mice; CD-HFD + α-PD-1 n= 6 mice). **(k)** ALT and **(l)** NAS evaluation of 12 months ND, CD-HFD, CD-  
 2030 HFD-fed mice + 8 weeks treatment by α-PD-1, α-PD-1/α-CD8, α-TNF, or α-PD-1/α-TNF antibodies (ND n= 30 mice;  
 2031 CD-HFD n= 47 mice; CD-HFD + α-PD-1 n= 35 mice; CD-HFD + α-PD-1/α-CD8 n= 9 mice; CD-HFD + α-TNF n= 10  
 2032 mice; CD-HFD + α-PD-1/α-TNF n= 11 mice). **(m)** Quantification of hepatic CD8<sup>+</sup>PD-1<sup>+</sup>CXCR6<sup>+</sup> T-cells ND, CD-  
 2033 HFD, CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-PD-1/α-CD8, α-TNF, α-PD-1/α-TNF, α-CD4, or α-PD-  
 2034 1/α-CD4 antibodies (ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD + α-PD-1 n= 35 mice; CD-HFD + α-PD-1/α-  
 2035 CD8 n= 9 mice; CD-HFD + α-TNF n= 10 mice; CD-HFD + α-PD-1/α-TNF n= 11 mice); CD-HFD + α-CD4 n= 8 mice;  
 2036 CD-HFD + α-PD-1/α-CD4 n= 8 mice). **(n)** Quantification of tumor incidence of 12 months CD-HFD or CD-HFD-fed  
 2037 mice + 8 weeks treatment by α-CD8, α-CD8/NK1.1, α-PD-1, α-PD-1/α-CD8, α-TNF, α-PD-1/α-TNF, α-CD4, or α-  
 2038 PD-1/α-CD4 antibodies (tumor incidence: CD-HFD n= 32 tumors/lesions in 87 mice; CD-HFD + α-CD8 n= 2  
 2039 tumors/lesions in 31 mice; CD-HFD + α-CD8/NK1.1 n= 0 tumors/lesions in 6 mice; CD-HFD + α-PD-1 n= 33  
 2040 tumors/lesions in 44 mice; CD-HFD + α-PD-1/α-CD8 n= 2 tumors/lesions in 9 mice; CD-HFD + α-TNF n= 3  
 2041 tumors/lesions in 10 mice; CD-HFD + α-PD-1/α-TNF n= 3 tumors/lesions in 11 mice); CD-HFD + α-CD4 n= 3  
 2042 tumors/lesions in 9 mice; CD-HFD + α-PD-1/α-CD4 n= 8 tumors/lesions in 9 mice).

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**Rebuttal Figure 17:  $\alpha$ -CD8/NK1.1 co-depletion does not further ameliorate NASH pathology compared to CD8 T-cell depletion alone**

(a) H&E and Sirius Red staining, (b) body weight, (c) NASH evaluation by H&E, (d) fibrosis quantification (Sirius Red) and (e) ALT levels of 12 months ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 or CD-HFD-fed mice + 8 weeks co-depletion of  $\alpha$ -CD8/NK1.1 antibodies (body weight: ND n = 15 mice; CD-HFD n = 28 mice; CD-HFD +  $\alpha$ -CD8 n = 28 mice; fibrosis ND n = 19 mice; CD-HFD n = 53 mice; CD-HFD +  $\alpha$ -CD8 n = 27 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n = 6 mice; NASH: ND n = 24 mice; CD-HFD n = 40 mice; CD-HFD +  $\alpha$ -CD8 n = 29 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n = 6); ALT: ND n = 22 mice; CD-HFD n = 42 mice; CD-HFD +  $\alpha$ -CD8 n = 31 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n = 6). Scale bar: 100  $\mu$ m. (f) Flow cytometry plots and (g) quantification of hepatic NK1.1 abundance of 12 months ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 or CD-HFD-fed mice + 8 weeks co-depletion of  $\alpha$ -CD8/NK1.1 antibodies (ND n = 4 mice; CD-HFD n = 8 mice; CD-HFD +  $\alpha$ -CD8 n = 7 mice; CD-HFD +  $\alpha$ -CD8/NK1.1 n = 6 mice). (h) Gene set enrichment analysis of RNA sequencing data of hepatic tissue comparing CD-HFD with CD-HFD-fed mice + co-depletion of  $\alpha$ -CD8/NK1.1 of 12 months ND, CD-HFD or CD-HFD-fed mice + co-depletion of  $\alpha$ -CD8/NK1.1 antibodies (n = 5 mice/group). (i) Gene set enrichment analysis of RNA sequencing data of hepatic tissue comparing or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 with CD-HFD-fed mice + co-depletion of  $\alpha$ -CD8/NK1.1 of 12 months ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 or CD-HFD-fed mice + co-depletion of  $\alpha$ -CD8/NK1.1 antibodies (n = 5 mice/group).



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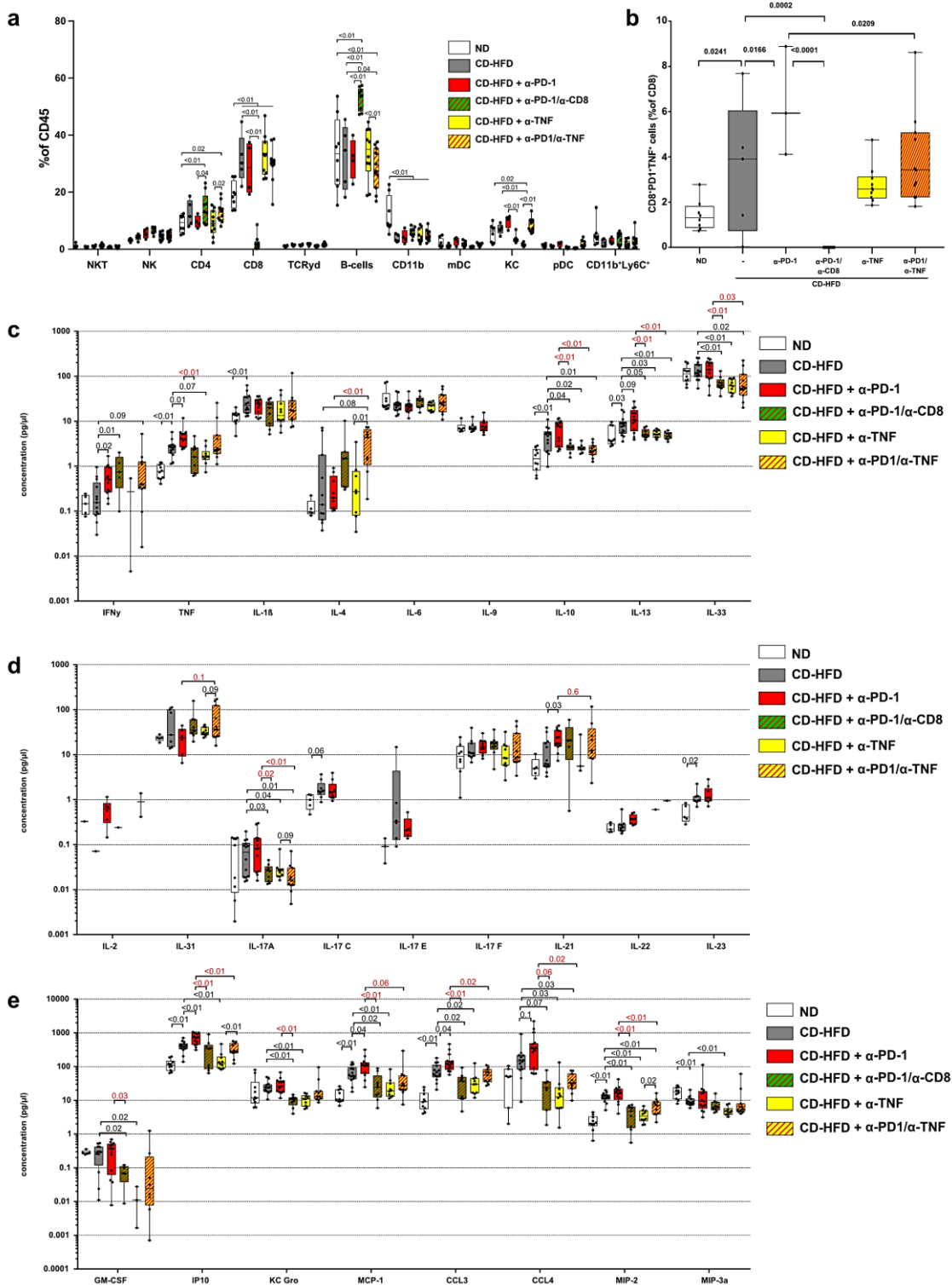
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2062 **Rebuttal Figure 18: CD8<sup>+</sup> T-cells drive hepatic inflammation and subsequent liver cancer in a TNF-dependent manner upon**  
2063 **PD-1-targeted immunotherapy**  
2064 **(a)** Body weight, AST, and histological evaluation by **(b)** Sirius red, CD4, CD8, PD-1, PD-L1, F4/80, MHC-II and **(c)**  
2065 staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -PD-1/ $\alpha$ -CD8,  $\alpha$ -TNF,  $\alpha$ -PD-1/ $\alpha$ -  
2066 TNF antibodies (body weight: ND n= 16 mice; CD-HFD n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -  
2067 PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; AST: body weight:  
2068 ND n= 30 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-  
2069 HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; Sirius red: ND n= 11 mice; CD-HFD n= 12 mice;



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2070 CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -  
 2071 PD-1/ $\alpha$ -TNF n= 11 mice; CD4: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -  
 2072 PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; CD8: ND n= 10 mice;  
 2073 CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n=  
 2074 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; PD-1: ND n= 12 mice; CD-HFD n= 12  
 2075 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 8 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD  
 2076 +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 10 mice; PD-L1: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD  
 2077 +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; F4/80: ND n= 11  
 2078 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -  
 2079 CD8 n= 9 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice; MHC-II: ND n= 11 mice; CD-  
 2080 HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD8 n= 9  
 2081 mice; CD-HFD +  $\alpha$ -TNF n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -TNF n= 11 mice). Scale bar: 100  $\mu$ m.

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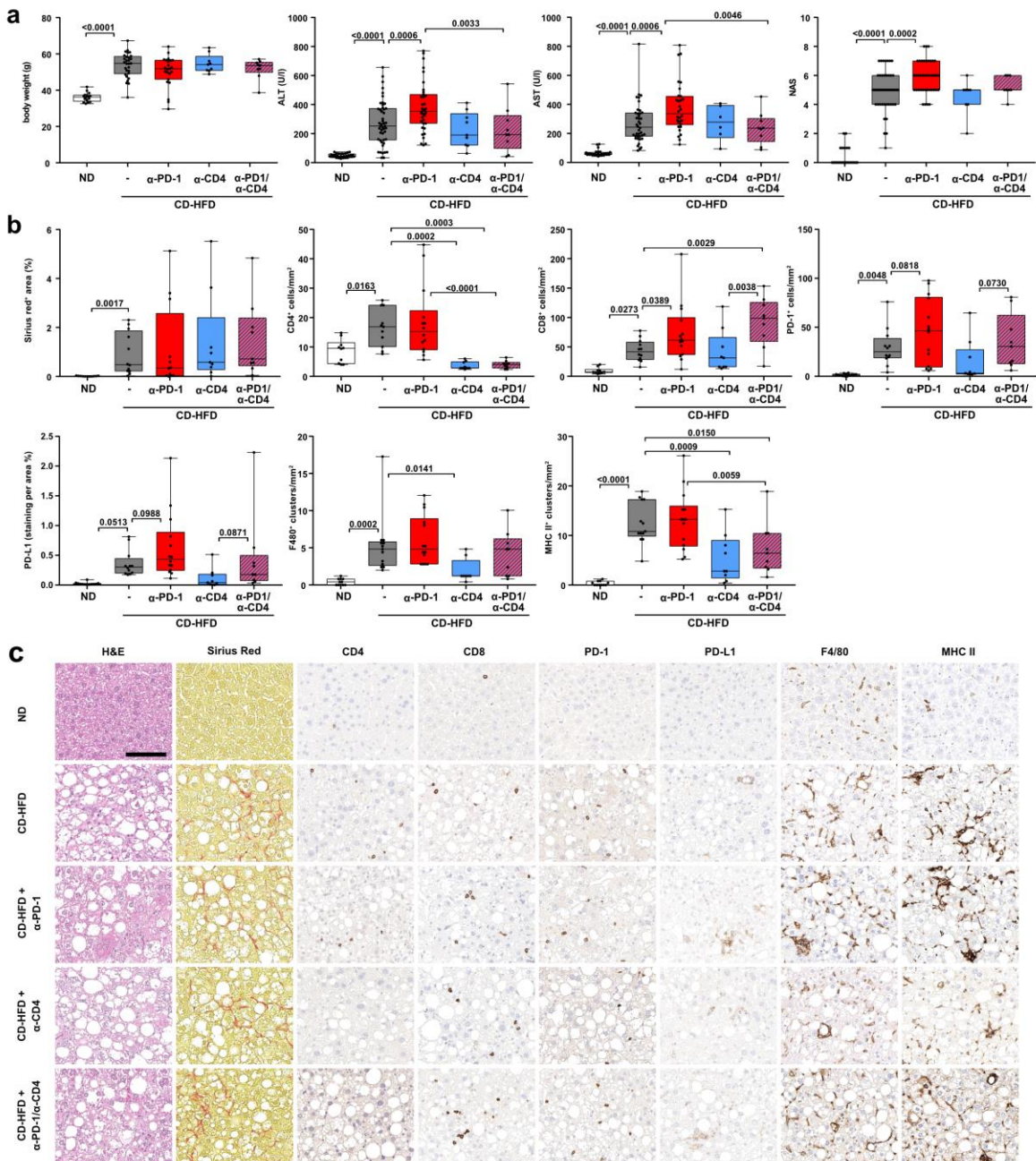
**Rebuttal Figure 19: Inflammation associated hepatic cytokine and chemokine environment in CD8<sup>+</sup> T-cells driven hepatic inflammation upon PD-1-targeted immunotherapy**

(a) Quantification of hepatic immune cell composition and (b) CD8<sup>+</sup>PD-1<sup>+</sup>TNF<sup>+</sup> T-cells by flow cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-PD-1/α-CD8, α-TNF, α-PD-1/α-TNF antibodies (Hepatic immune cell composition: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD + α-PD-1 n= 4 mice; CD-HFD + α-PD-1/α-CD8 n= 9 mice; CD-HFD + α-TNF n= 10 mice; CD-HFD + α-PD-1/α-TNF n= 11 mice; CD8<sup>+</sup>PD-1<sup>+</sup>TNF<sup>+</sup>: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD + α-PD-1 n= 3 mice; CD-HFD + α-PD-1/α-CD8 n= 9 mice; CD-HFD + α-TNF n= 10 mice; CD-HFD + α-PD-1/α-TNF n= 11 mice). (c) and (d) multiplex ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-PD-1/α-CD8, α-TNF, α-PD-1/α-TNF antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD + α-PD-1 n= 13 mice; CD-HFD + α-PD-1/α-CD8 n= 9 mice; CD-HFD + α-TNF n= 10 mice; CD-HFD + α-PD-1/α-TNF n= 11 mice).

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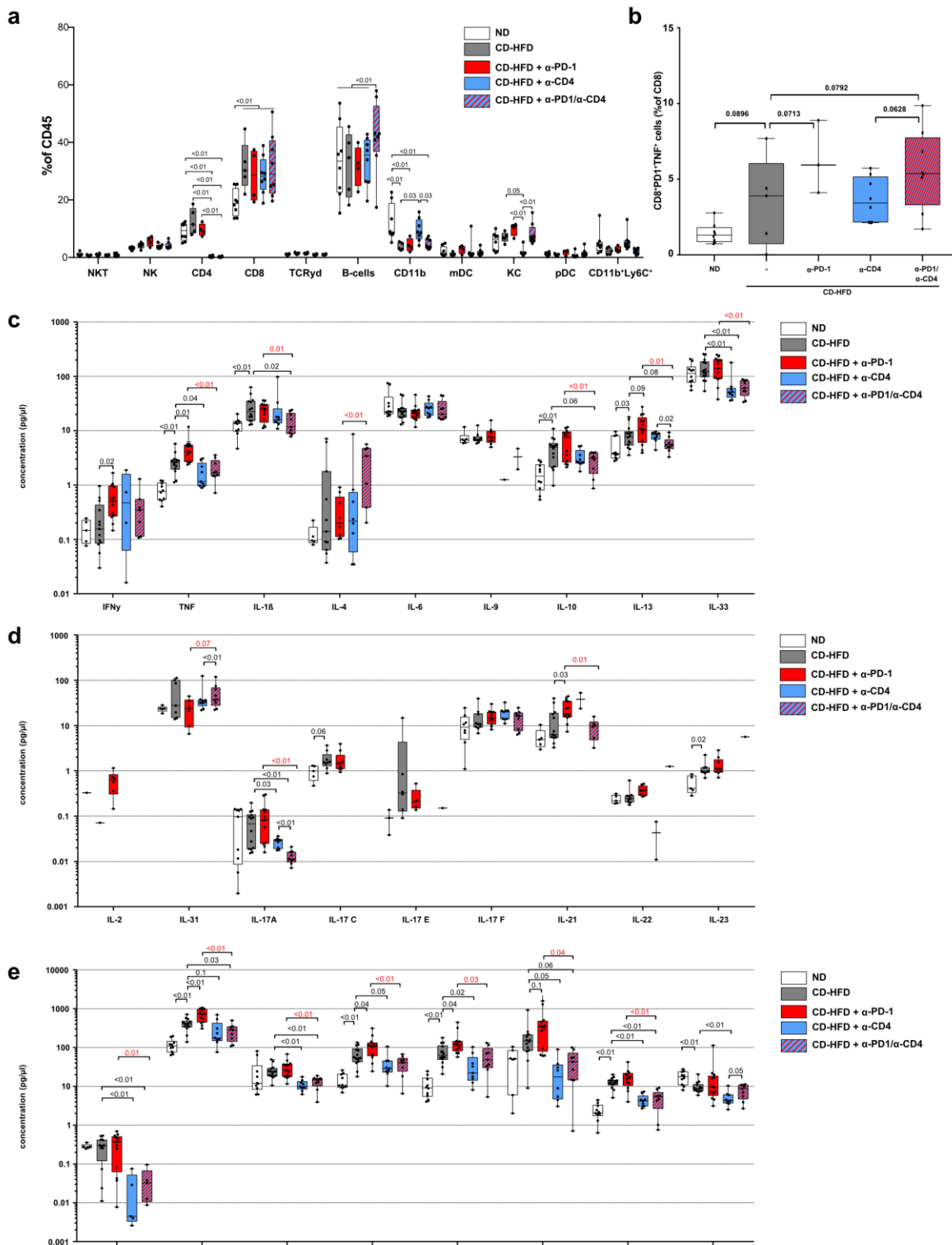


**Rebuttal Figure 20: Depletion of CD4<sup>+</sup> T-cells does not impair hepatic inflammation in NASH upon PD-1-targeted immunotherapy**

**(a)** Body weight, ALT, AST, NAS, and histological evaluation by **(b)** Sirius Red, CD4, CD8, PD-1, PD-L1, F4/80, MHC-II and **(c)** staining of ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1,  $\alpha$ -CD4,  $\alpha$ -PD-1/ $\alpha$ -CD4 antibodies (body weight: ND n= 16 mice; CD-HFD n= 29 mice; CD-HFD +  $\alpha$ -PD-1 n= 23 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; ALT ND n= 30 mice; CD-HFD n= 47 mice; CD-HFD +  $\alpha$ -PD-1 n= 35 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; AST: ND n= 30 mice; CD-HFD n= 40 mice; CD-HFD +  $\alpha$ -PD-1 n= 30 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; NAS: ND n= 31 mice; CD-HFD n= 46 mice; CD-HFD +  $\alpha$ -PD-1 n= 40 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 8 mice; Sirius red: ND n= 11 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 12 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; CD4: ND n= 10 mice; CD-HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 10 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 11 mice; CD8: ND n= 10 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-1: ND n= 13 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; PD-L1: ND n= 12 mice; CD-HFD n= 12 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; F4/80: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 8 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice; MHC-II: ND n= 11 mice; CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD-HFD +  $\alpha$ -CD4 n= 9 mice; CD-HFD +  $\alpha$ -PD-1/ $\alpha$ -CD4 n= 9 mice). Scale bar: 100  $\mu$ m.

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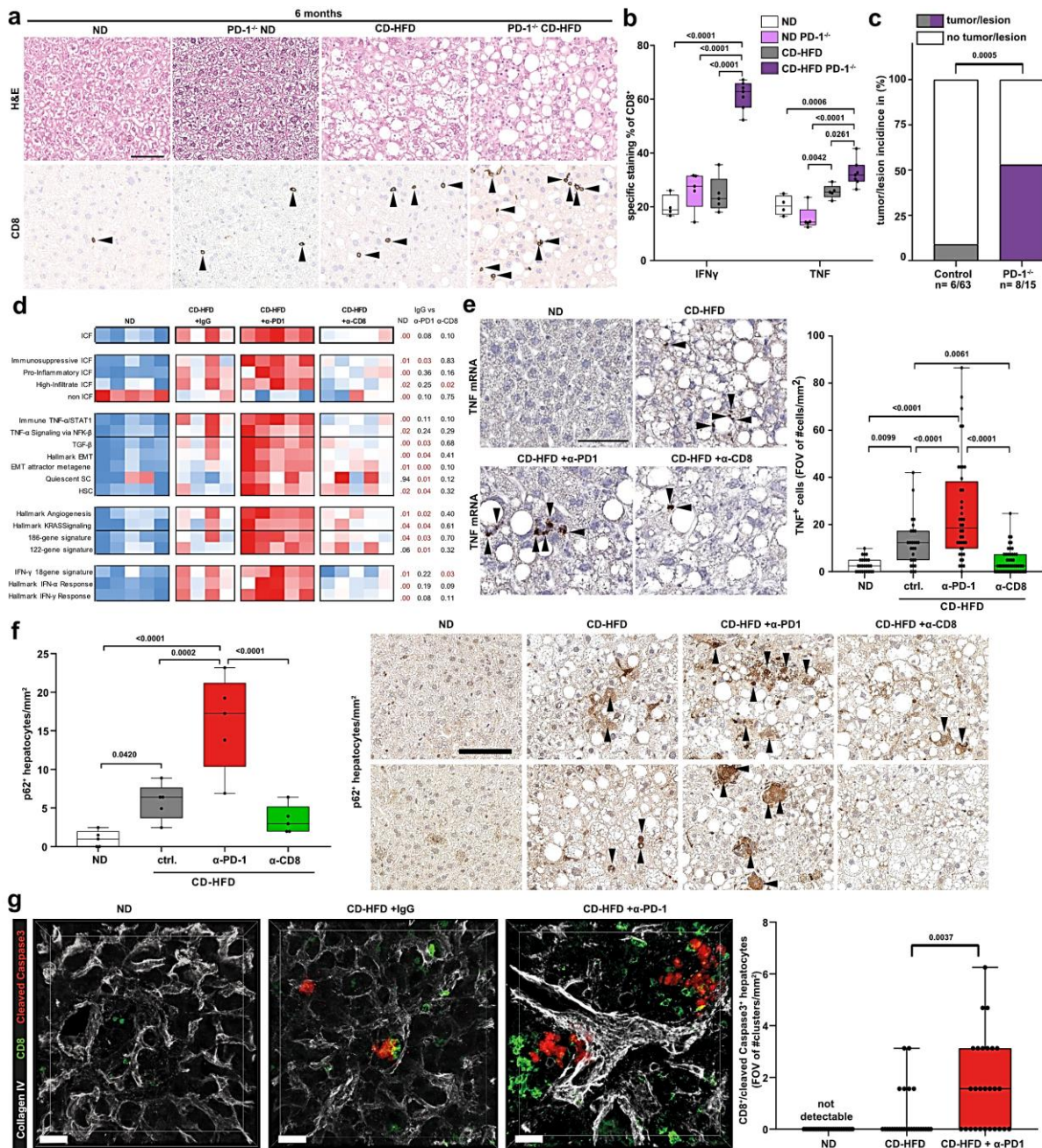
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**Rebuttal Figure 21: Inflammation associated hepatic cytokine and chemokine environment in CD4-depleted animals with or without PD-1-targeted immunotherapy**

(a) Quantification of hepatic immune cell composition and (b) CD8<sup>+</sup>PD1<sup>+</sup>TNF<sup>+</sup> T-cells by flow cytometry of 12 months ND, CD-HFD, or CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-CD4, α-PD-1/α-CD4 antibodies (Hepatic immune cell composition: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD + α-PD-1 n= 4 mice; CD-HFD + α-CD4 n= 8 mice; CD-HFD + α-PD-1/α-CD4 n= 8 mice; CD8<sup>+</sup>PD1<sup>+</sup>TNF<sup>+</sup>: ND n= 8 mice; CD-HFD n= 5 mice; CD-HFD + α-PD-1 n= 3 mice; CD-HFD + α-CD4 n= 8 mice; CD-HFD + α-PD-1/α-CD4 n= 8 mice). (c) and (d) multiplex ELISA of hepatic inflammation associated cytokines and (e) chemokines of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1, α-CD4, α-PD-1/α-CD4 antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD + α-PD-1 n= 13 mice; CD-HFD + α-CD4 n= 9 mice; CD-HFD + α-PD-1/α-CD4 n= 9 mice).

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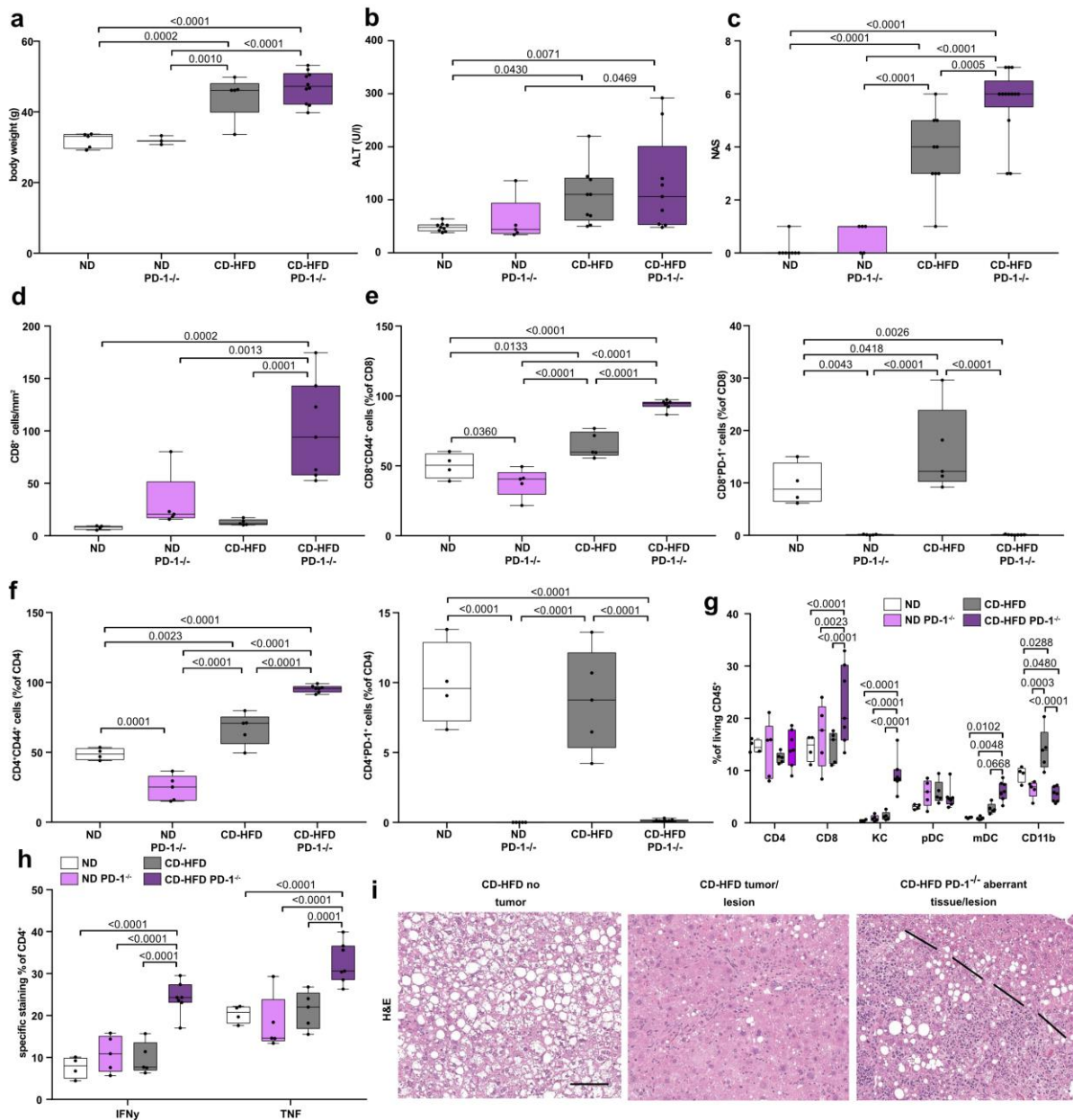


**Rebuttal Figure 22: Anti-PD-1 treatment drives hepatocarcinogenesis by enhancing an inflammatory and pro-tumorigenic liver microenvironment**

(a) Histological staining of hepatic tissue by H&E and CD8 of 6 months ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (H&E: ND n= 8 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 9 mice; PD-1<sup>-/-</sup> CD-HFD n= 13 mice; CD8: ND n= 4 mice; CD-HFD n= 5 mice; PD-1<sup>-/-</sup> CD-HFD n= 7 mice). Arrowheads indicate CD8<sup>+</sup> cells. Scale bar: 50  $\mu$ m. (b) Cytokine expression of hepatic CD8<sup>+</sup> T-cells of 6 months ND, PD-1<sup>-/-</sup> ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (ND n= 4 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 5 mice; PD-1<sup>-/-</sup> CD-HFD n= 6 mice). (c) Tumor/lesion incidence of 6 months CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (tumor incidence: CD-HFD n= 6 tumors/lesions in 63 mice; PD-1<sup>-/-</sup> CD-HFD n= 6 tumors/lesions in 13 mice). (d) Immune cancer field and ICF<sup>38</sup>- patterns of RNA sequencing data of hepatic tissue of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 or  $\alpha$ -CD8 antibodies (ND, CD-HFD +  $\alpha$ -PD-1, CD-HFD +  $\alpha$ -CD8 n= 5 mice/group; CD-HFD n= 4 mice) through single-sample Gene Set Enrichment Analysis (ssGSEA). (e) Quantification of mRNA *in situ* hybridization for hepatic TNF<sup>+</sup> cells of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 or  $\alpha$ -PD-1 antibodies (ND n= 25 fields of view (FOV) in 3 mice; CD-HFD n= 27 FOV in 3 mice; CD-HFD +  $\alpha$ -PD-1 n= 40 FOV in 3 mice; CD-HFD +  $\alpha$ -CD8 n= 55 FOV in 3 mice). Arrowheads indicate TNF<sup>+</sup> cells. Scale bar: 20  $\mu$ m. (f) Histological staining of liver tumor tissue by p62 of 12 months ND, CD-HFD or CD-HFD + 8 weeks treatment by  $\alpha$ -PD-1 antibodies or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (n= 5 mice/group). (g) Immunofluorescence staining for Collagen IV, CD8 and Cleaved Caspase 3 of liver tissue of 12 months ND, CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n= 27 FOV in 3 mice/group). Scale bar: 30  $\mu$ m.

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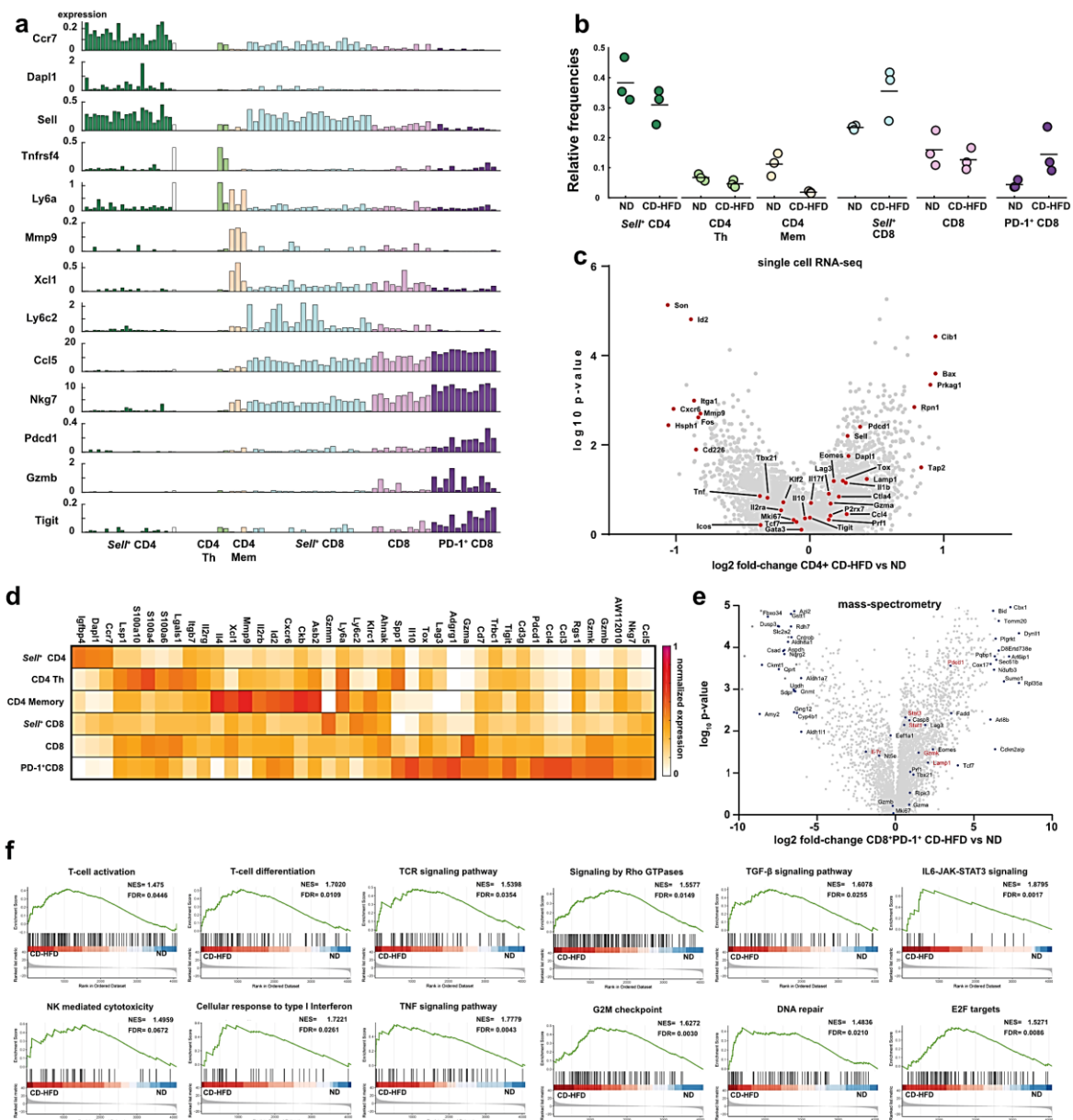
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**Rebuttal Figure 23: PD-1<sup>-/-</sup> mice fed NASH-inducing diet have an increased inflammatory liver environment**

(a) Body weight of 6 months ND, PD-1<sup>-/-</sup> ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (ND n= 5 mice; PD-1<sup>-/-</sup> ND n= 3 mice; CD-HFD n= 5 mice; PD-1<sup>-/-</sup> CD-HFD n= 10 mice). (b) ALT levels of ND, PD-1<sup>-/-</sup> ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (ND n= 9 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 9 mice; PD-1<sup>-/-</sup> CD-HFD n= 10 mice). (c) NASH evaluation by H&E of ND, PD-1<sup>-/-</sup> ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (ND n= 8 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 9 mice; PD-1<sup>-/-</sup> CD-HFD n= 13 mice). (d) Quantification of CD8<sup>+</sup> cells in hepatic tissue by immunohistochemistry of 6 months ND, PD-1<sup>-/-</sup> ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (ND n= 4 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 5 mice; PD-1<sup>-/-</sup> CD-HFD n= 7 mice). (e) – (g) Characterization of hepatic T-cells by flow cytometry of 6 months ND, PD-1<sup>-/-</sup> ND, CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (ND n= 4 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 5 mice; PD-1<sup>-/-</sup> CD-HFD n= 6 mice). (h) Relative quantification of hepatic leukocytes of 6 months CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (ND n= 4 mice; PD-1<sup>-/-</sup> ND n= 5 mice; CD-HFD n= 5 mice; PD-1<sup>-/-</sup> CD-HFD n= 6 mice). (i) Histological staining of hepatic tissue by H&E of CD-HFD or PD-1<sup>-/-</sup> CD-HFD-fed mice (ND n= 8 mice; CD-HFD n= 9 mice; PD-1<sup>-/-</sup> CD-HFD n= 13 mice). Dotted line indicates tumor/lesion border. Scale bar: 100 μm.

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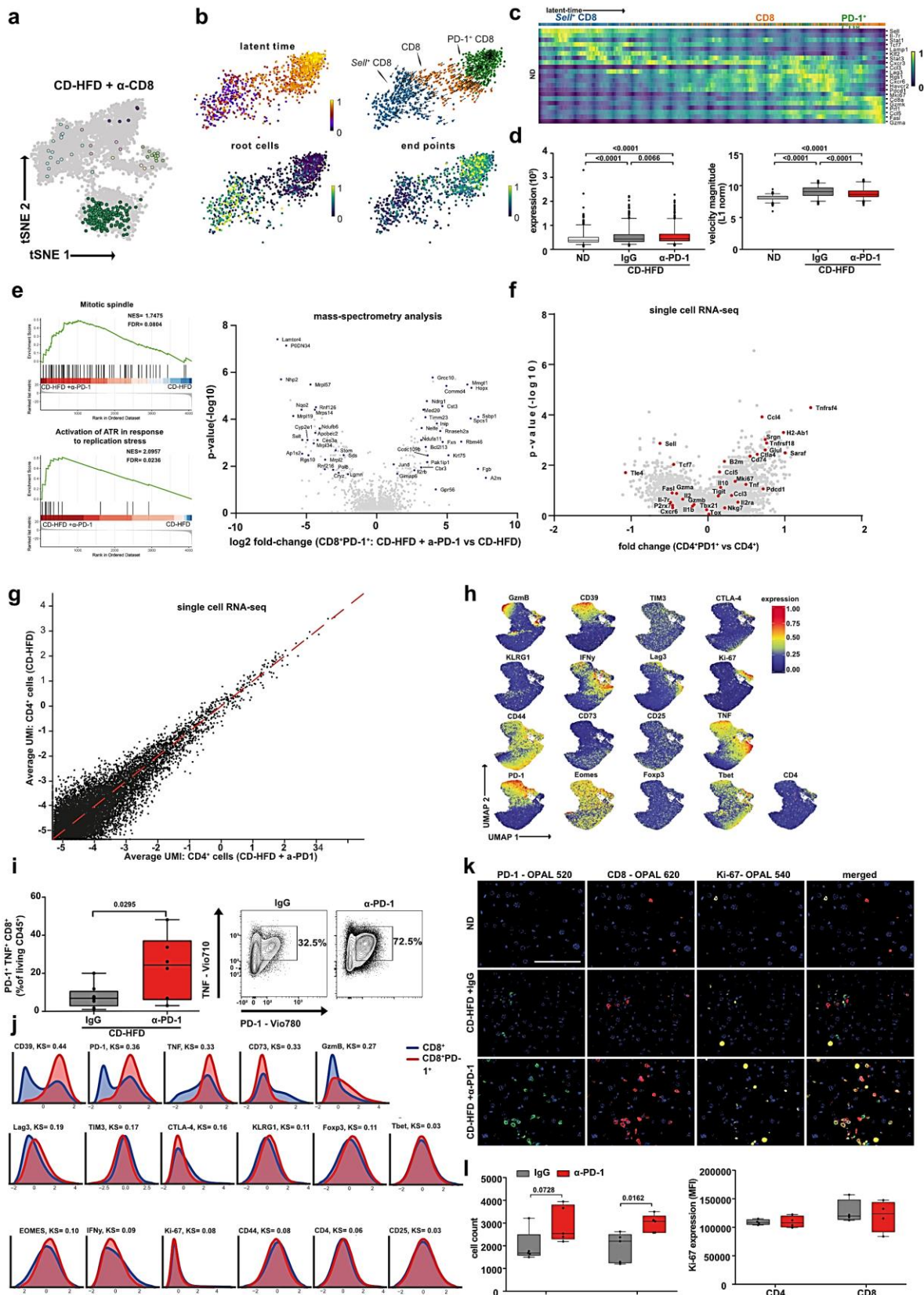


**Rebuttal Figure 24: In depth characterization of hepatic immune cell compartment focusing on T-cells**

(a) Marker expression of CD4<sup>+</sup> and CD8<sup>+</sup> sorted TCRβ<sup>+</sup> cells defining T-cell subsets by single cell RNA-sequencing of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). (b) Relative frequency of CD4<sup>+</sup> and CD8<sup>+</sup> sorted TCRβ<sup>+</sup> cells by single cell RNA-sequencing of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). (c) Selected marker expression in CD4<sup>+</sup> T-cells sorted TCRβ<sup>+</sup> cells by single cell RNA-sequencing of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). (d) Selected average marker expression in T-cell subsets of CD4<sup>+</sup> and CD8<sup>+</sup> sorted TCRβ<sup>+</sup> by scRNA-seq of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). (e) Selected marker expression in hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells by mass spectrometry of 12 months ND or CD-HFD-fed mice (ND n= 4 mice, CD-HFD n= 6 mice). (f) Gene set enrichment analysis of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells sorted TCRβ<sup>+</sup> cells by mass spectrometry of 12 months ND or CD-HFD-fed mice (ND n= 4 mice, CD-HFD n= 6 mice).

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**Rebuttal Figure 25: Cellular drivers of hepatic necroinflammation- and increased hepatocarcinogenesis upon  $\alpha$ -PD-1 treatment in NASH**

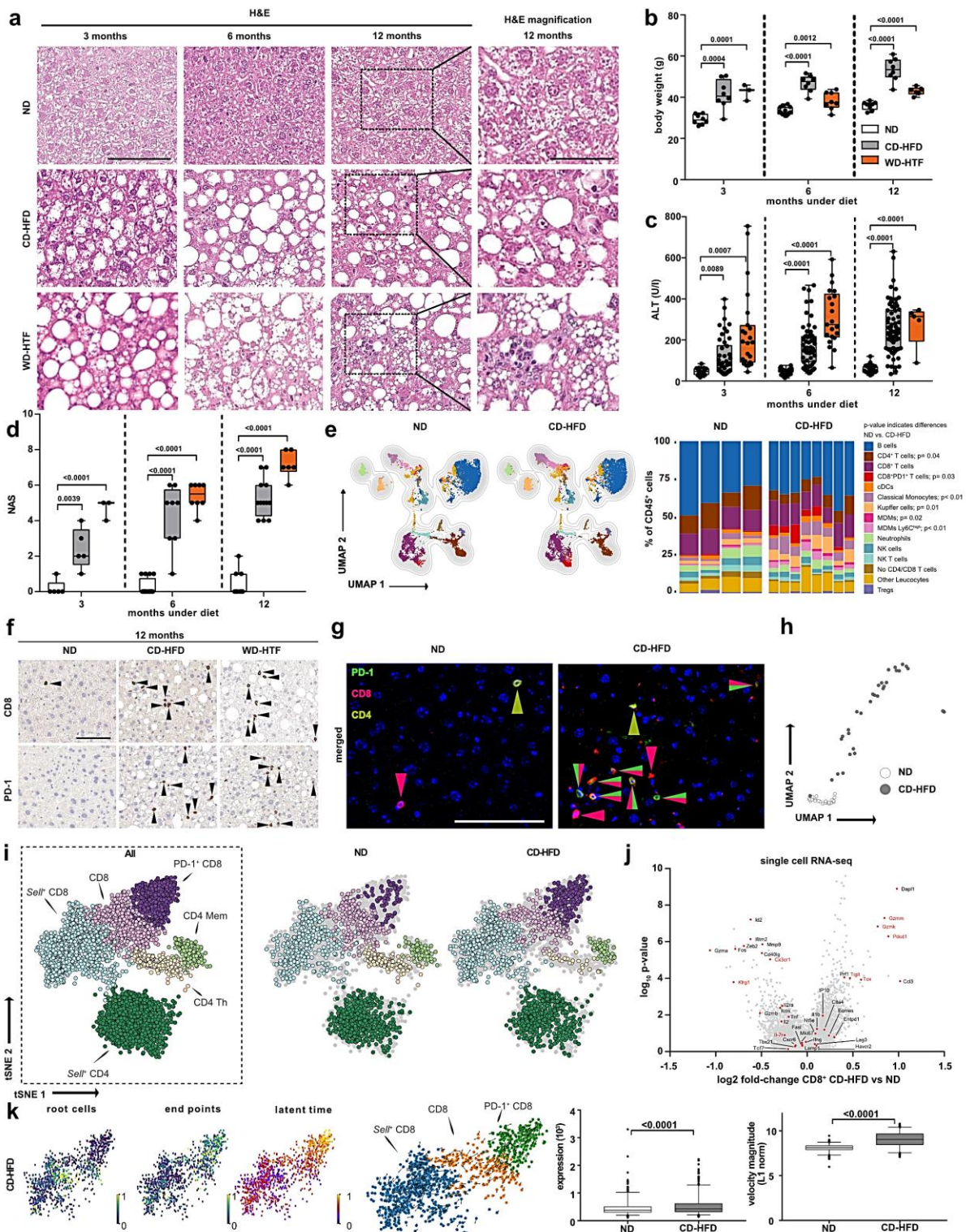
(a) Analysis of hepatic TCR $\beta$ <sup>+</sup> cells by single cell RNA-sequencing of 12 months CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 antibodies (n= 3 mice). (b) Velocity analyses on scRNA-seq data CD8<sup>+</sup> cells of 12 months ND or CD- HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n= 3 mice). (c) Velocity analyses of scRNA-seq data showing correlation of expression along the latent-time of selected genes along the latent-time of ND-fed mice (n= 3 mice). (d) RNA velocity analyses indicating transcriptional activity and gene expression of CD8<sup>+</sup> cells by



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2182 scRNA-seq of 12 months ND, CD-HFD or CD- HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n= 3  
 2183 mice/group). **(e)** Gene set enrichment analysis of mass spectrometry data comparing hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells  
 2184 sorted TCR $\beta$ <sup>+</sup> cells from CD-HFD with CD-HFD-fed mice +  $\alpha$ -PD-1. Selected marker expression in hepatic CD8<sup>+</sup>PD-  
 2185 1<sup>+</sup> T-cells sorted TCR $\beta$ <sup>+</sup> cells by mass spectrometry of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks  
 2186 treatment by  $\alpha$ -PD-1 antibodies (n= 6 mice/group). Candidates developing steady in-/decrease from ND to CD-HFD  
 2187 to CD-HFD + 8 weeks treatment by  $\alpha$ -PD-1 are indicated in red. (n= 6 mice/group). **(f)** Selected marker expression  
 2188 in hepatic CD4<sup>+</sup> T-cells sorted TCR $\beta$ <sup>+</sup> cells by single cell RNA-sequencing comparing CD4<sup>+</sup> with CD4<sup>+</sup>PD-1<sup>+</sup> T-  
 2189 cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 or  $\alpha$ -CD8 antibodies (n= 3  
 2190 mice/group). **(g)** Average UMI comparison of hepatic CD4<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed  
 2191 mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n= 3 mice/group). **(h)** Umap showing the expression intensity of  
 2192 the indicated marker on scholastically selected TCR $\beta$ <sup>+</sup> CD8<sup>+</sup> cells of flow cytometry data to define distinct marker  
 2193 expression of 12 months ND, CD-HFD + IgG, CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n=  
 2194 4 mice; CD-HFD n= 8 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). **(i)** Quantification of manual gating and flow cytometry  
 2195 plots for hepatic CD8<sup>+</sup>PD-1<sup>+</sup> TNF<sup>+</sup> abundance of 12 months CD-HFD + IgG, CD-HFD-fed mice + 8 weeks treatment  
 2196 by  $\alpha$ -PD-1 antibodies (CD-HFD n= 8 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). **(j)** CellCNN analyzed flow cytometry data  
 2197 of hepatic CD8<sup>+</sup> T-cells of 12 months CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies  
 2198 (CD-HFD + IgG n= 6 mice; CD-HFD +  $\alpha$ -PD-1 n= 4 mice). **(k)** Immunofluorescence staining for PD-1, CD8 and Ki-  
 2199 67 of liver tissue of 12 months ND, CD-HFD + IgG or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies  
 2200 (n= 2 mice/group). Scale bar: 100  $\mu$ m. **(l)** In vitro stimulated splenic CD8 T cells from C57Bl/6 mice were treated  
 2201 with  $\alpha$ -PD-1 antibody for 72 hours (cell count: n= 5 experiments/group; Ki-67: n= 4 experiments/group).

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**Rebuttal Figure 26: Progression of NASH pathology is associated with increased, and transcriptionally activated hepatic CD8+PD-1+ T-cells**

**(a)** Histological staining of hepatic tissue by H&E of 3, 6 or 12 months ND, CD-HFD or WD-HTF-fed mice (H&E: 3 months: ND  $n = 5$  mice; CD-HFD  $n = 5$  mice; WD-HTF  $n = 3$  mice; 6 months: ND  $n = 16$  mice; CD-HFD  $n = 8$  mice; WD-HTF  $n = 8$  mice; 12 months: ND  $n = 9$  mice; CD-HFD  $n = 12$  mice; WD-HTF  $n = 6$  mice). Scale bar: 100  $\mu$ m. **(b)** Body weight of 3, 6 or 12 months ND, CD-HFD or WD-HTF-fed mice (3 months: ND  $n = 8$  mice; CD-HFD  $n = 8$  mice; WD-HTF  $n = 3$  mice; 6 months: ND  $n = 14$  mice; CD-HFD  $n = 8$  mice; WD-HTF  $n = 8$  mice; 12 months: ND  $n = 8$  mice; CD-HFD  $n = 8$  mice; WD-HTF  $n = 6$  mice). **(c)** ALT levels of 3, 6 or 12 months ND, CD-HFD or WD-HTF-fed mice (3 months: ND  $n = 15$  mice; CD-HFD  $n = 46$  mice; WD-HTF  $n = 23$  mice; 6 months: ND  $n = 46$  mice; CD-HFD  $n = 59$  mice; WD-HTF  $n = 21$  mice; 12 months: ND  $n = 25$  mice; CD-HFD  $n = 69$  mice; WD-HTF  $n = 5$  mice). **(d)** NASH evaluation by of 3, 6 or 12 months ND, CD-HFD or WD-HTF-fed mice (3 months: ND  $n = 5$  mice; CD-HFD  $n = 5$  mice; WD-HTF  $n = 3$  mice; 6 months: ND  $n = 16$  mice; CD-HFD  $n = 8$  mice; WD-HTF  $n = 8$  mice; 12 months: ND  $n =$

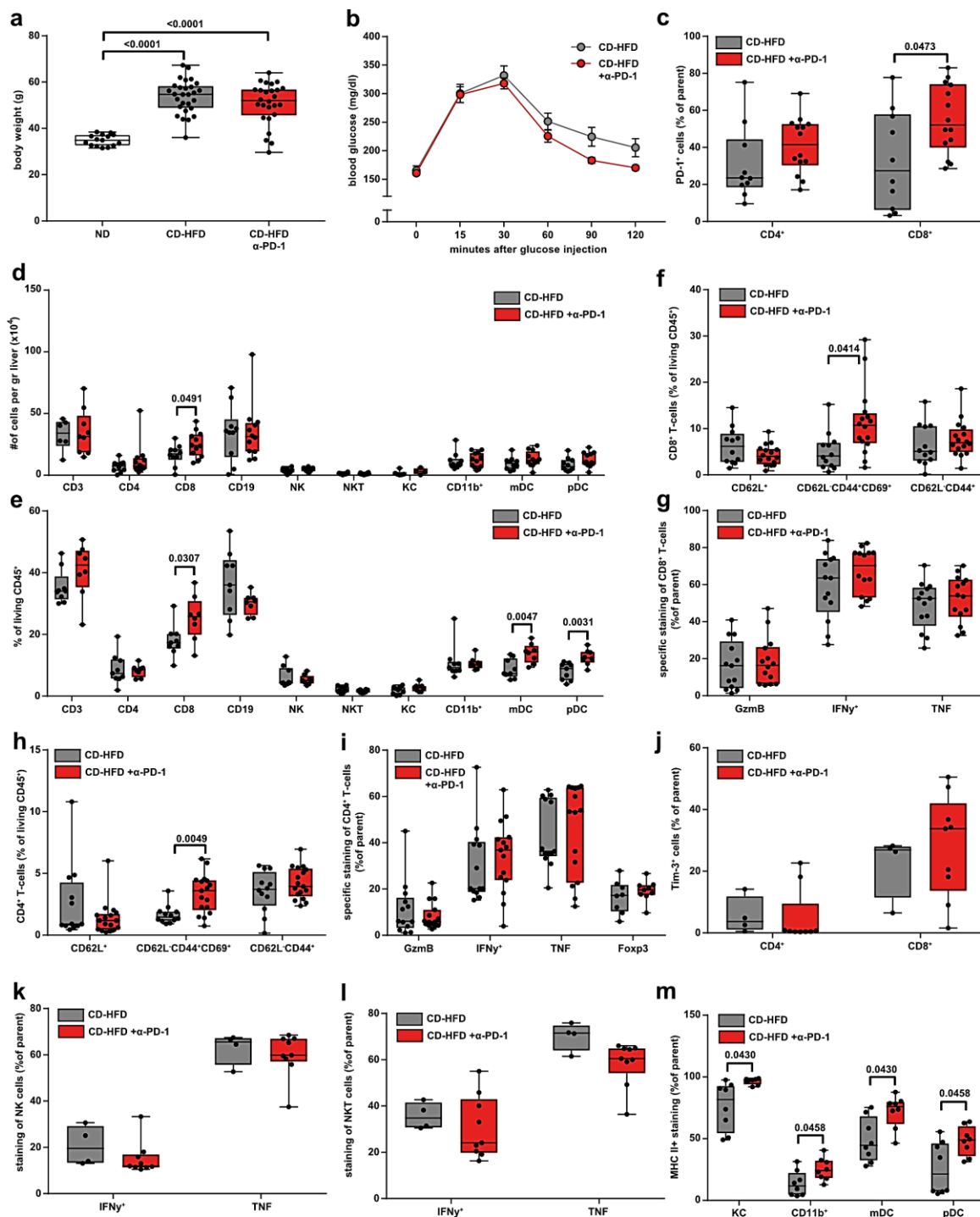




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2215 9 mice; CD-HFD n= 12 mice; WD-HTF n= 6 mice). **(e)** UMAP representation showing the FlowSOM-guided  
 2216 clustering of randomly chosen CD45<sup>+</sup> cells and quantification of hepatic immune cell composition by flow cytometry  
 2217 of 12 months ND or CD-HFD-fed mice (ND n= 4 mice; CD-HFD n= 8 mice). **(f)** CD8 and PD-1 staining of hepatic  
 2218 tissue by immunohistochemistry of 12 months ND, CD-HFD or WD-HTF-fed mice (PD-1: n= 5 mice/group; CD8:  
 2219 ND n= 6 mice; CD-HFD n= 6 mice; WD-HTF n= 5 mice). Scale bar: 100  $\mu$ m. **(g)** Immunofluorescence staining of  
 2220 PD-1, CD8 and CD4 of 12 months ND or CD-HFD-fed mice (n= 3 mice/group). Arrowheads indicate CD8<sup>+</sup> (red),  
 2221 PD-1<sup>+</sup> (green) or CD4<sup>+</sup> (ocher) cells. Scale bar: 100  $\mu$ m. **(h)** UMAP representation of 63 parameters (serology, flow  
 2222 cytometry, histology) indicating NASH pathology severity measured of 12 months ND or CD-HFD-fed mice (ND n=  
 2223 22 mice; CD-HFD n= 31 mice). **(i)** tSNE representation of TCR $\beta$ <sup>+</sup> cells and analyses of **(j)** differential gene  
 2224 expression, **(k)** RNA velocity indicating transcriptional activity, gene expression and the trajectory of CD8<sup>+</sup> cells by  
 2225 scRNA-seq of 12 months ND or CD-HFD-fed mice (n= 3 mice/group)<sup>53</sup>. Root cells: yellow cells indicate root cells,  
 2226 blue cells indicate cells farthest away from root by RNA velocity. End points: yellow cells indicate end point cells,  
 2227 blue cells indicate cells farthest away from defined end point cells by RNA velocity. Latent time: pseudo-time by  
 2228 RNA velocity, dark color indicate start of velocity, yellow color indicate end point of latent time. RNA velocity flow:  
 2229 Blue cluster defined as start point, orange cluster as intermediate, green cluster as end point. Arrows indicate the  
 2230 trajectory of cells.

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### Rebuttal Figure 27: α-PD-1 treatment in NASH does increase intrahepatic CD8 T-cells and PD-1 expression, and only leads to minor changes in other T-cell subsets

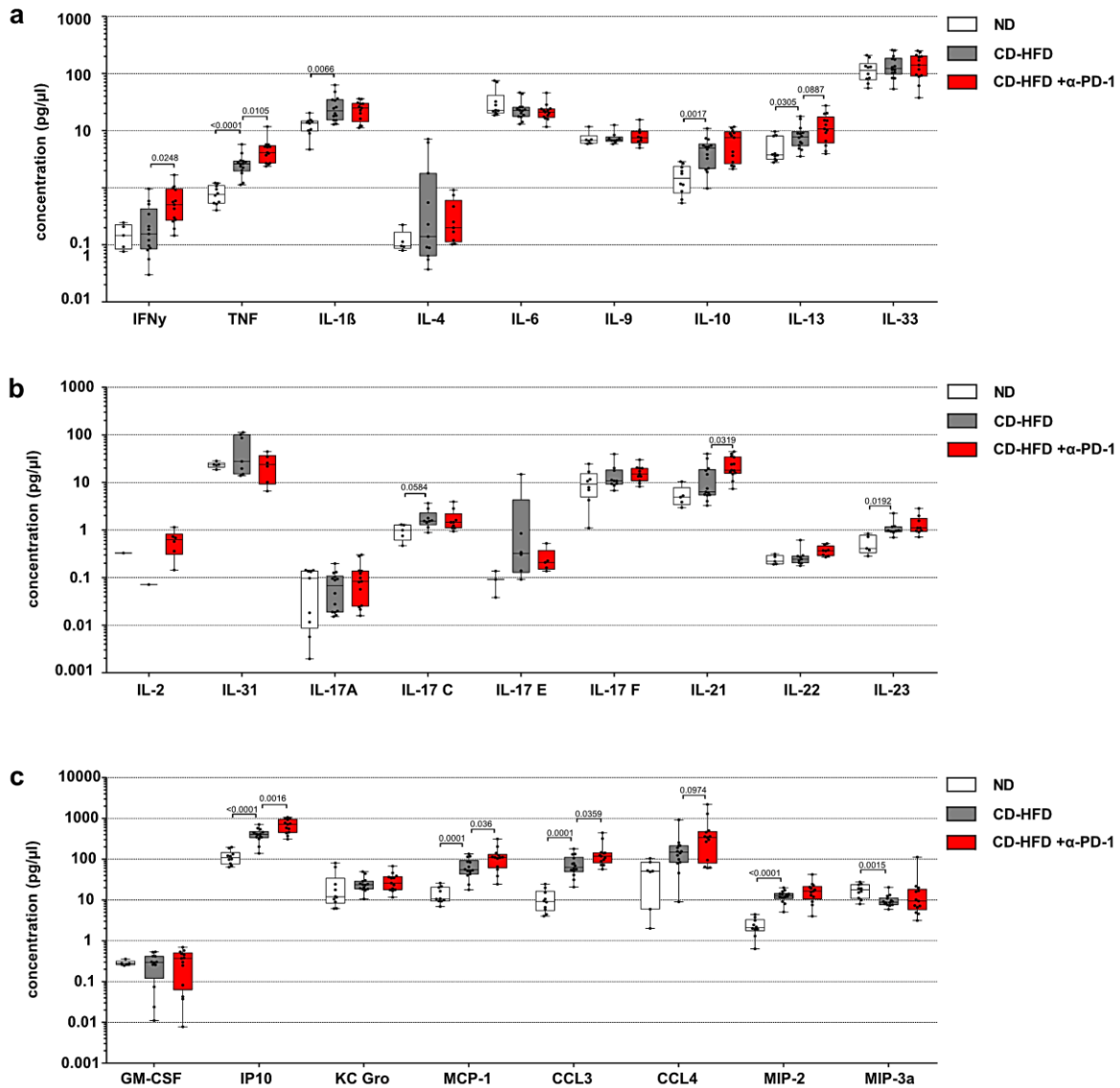
(a) Body weight of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (ND n = 15 mice; CD-HFD n = 28 mice; CD-HFD + α-PD-1 n = 26 mice). (b) Assessment of metabolic tolerance by intra peritoneal glucose tolerance test of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (n = 9 mice/group). (c) Expression of PD-1 of hepatic CD4<sup>+</sup> and PD-1<sup>+</sup> T-cells by flow cytometry of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (CD-HFD n = 10 mice; α-PD-1 + CD-HFD n = 13 mice). (d) Absolute and (e) relative quantification of hepatic leukocytes of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (CD3: CD-HFD n = 6 mice; CD-HFD + α-PD-1 n = 10 mice; CD4, CD8, CD19, NK, NKT, CD11b<sup>+</sup>, mDC, pDC: CD-HFD n = 10 mice; CD-HFD + α-PD-1 n = 12 mice, KC: CD-HFD n = 6 mice; CD-HFD + α-PD-1 n = 4 mice). (f) Flow cytometric analysis for polarization of hepatic CD8<sup>+</sup> T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (CD-HFD n = 10 mice; α-PD-1 + CD-HFD n = 14 mice). (g) Cytokine expression of hepatic CD4<sup>+</sup> T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (CD-HFD n = 13 mice; CD-HFD + α-PD-1 n = 14 mice). (h) Flow cytometry analysis for polarization of hepatic CD4<sup>+</sup> T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by α-PD-1 antibodies (CD-HFD n = 12 mice; α-PD-1 + CD-HFD n = 17 mice). (i) Cytokine expression of

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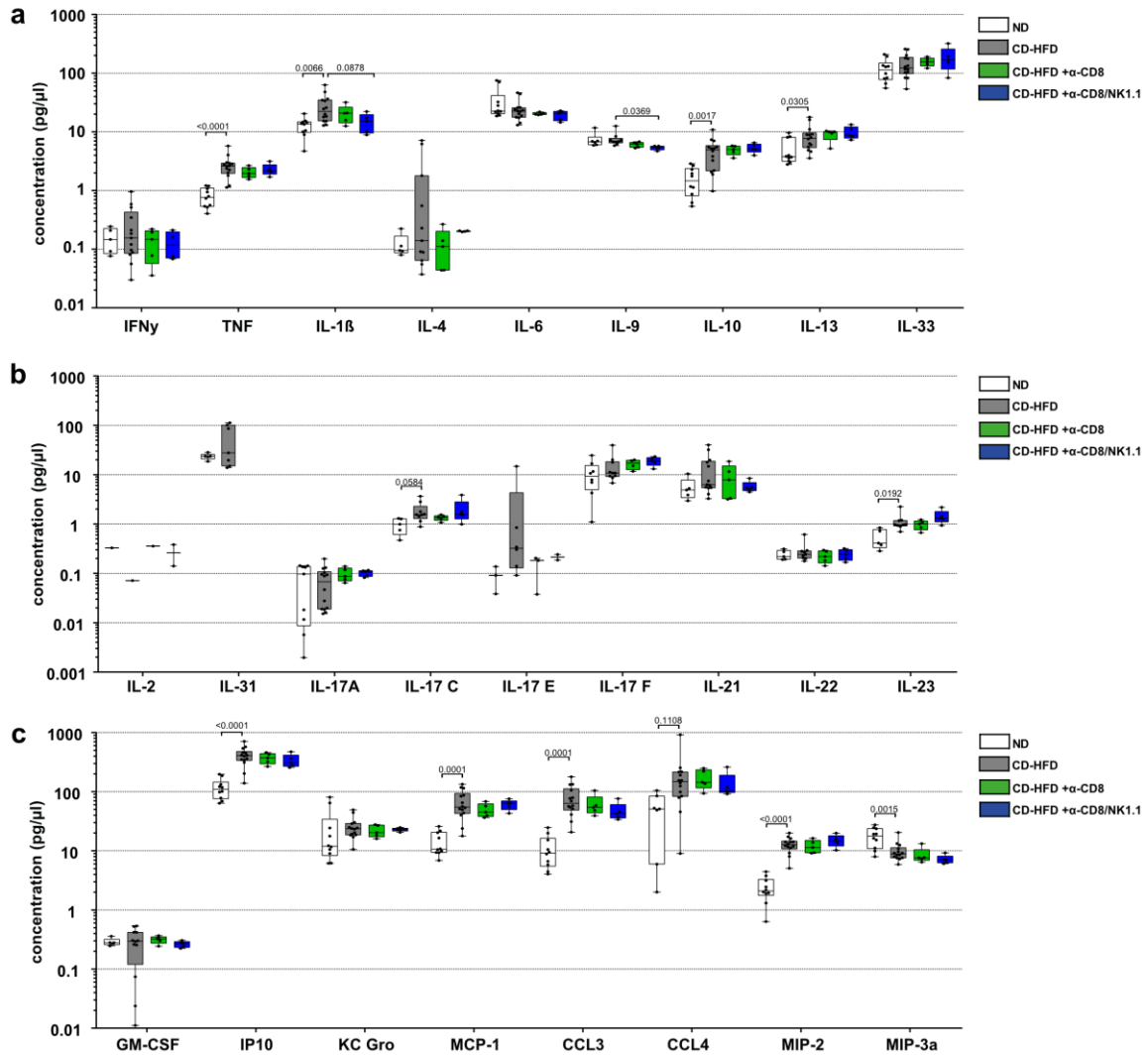
2248 hepatic CD4<sup>+</sup> T-cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (GzmB,  
2249 IFN $\gamma$ , TNF: CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; IL-10, Foxp3: CD-HFD n= 7 mice; CD-HFD +  $\alpha$ -  
2250 PD-1 n= 9 mice). **(j)** Expression of Tim-3 of hepatic CD4<sup>+</sup> and CD8<sup>+</sup> T-cells by flow cytometry of 12 months CD-  
2251 HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD n= 4 mice;  $\alpha$ -PD-1 + CD-HFD n= 9  
2252 mice). **(k)** Cytokine expression for polarization of hepatic NK and **(l)** NKT-cells of 12 months CD-HFD or CD-HFD-  
2253 fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n= 5 mice/group). **(m)** Flow cytometric analysis for polarization  
2254 of hepatic myeloid cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies  
2255 (CD-HFD n= 8 mice;  $\alpha$ -PD-1 + CD-HFD n= 12 mice).



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**Rebuttal Figure 28:  $\alpha$ -PD-1 treatment causes increased inflammation-associated hepatic cytokine and chemokine environment in NASH**

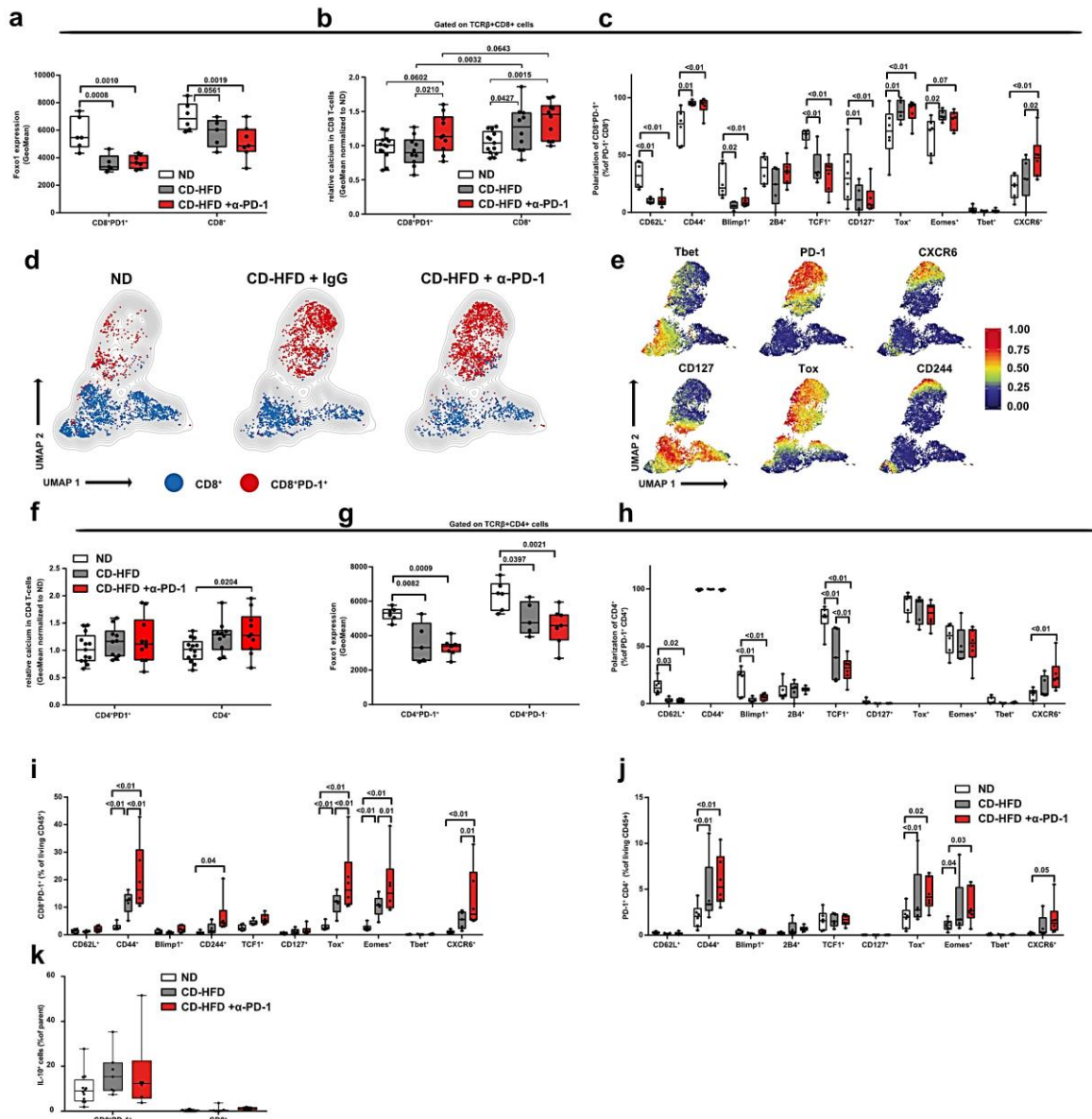
**(a) and (b)** multiplex ELISA concentrations of inflammation-associated hepatic cytokines and **(c)** chemokines of mice submitted to 12 months of ND, CD-HFD, CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD +  $\alpha$ -PD-1 n= 13 mice).



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**Rebuttal Figure 29: Minor changes in inflammation-associated hepatic cytokine and chemokine environment in NASH under CD8 T-cell depletion or CD8/NK1.1 co-depletion treatment**  
**(a) and (b)** multiplex ELISA concentrations of hepatic inflammation-associated cytokines and **(c)** chemokines of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -CD8 or CD-HFD-fed mice + co-depletion of  $\alpha$ -CD8/NK1.1 antibodies (ND n= 10 mice; CD-HFD n= 14 mice; CD-HFD + 8 weeks treatment by  $\alpha$ -CD8 n= 5 mice; CD-HFD + co-depletion of  $\alpha$ -CD8/NK1.1 n= 5 mice).

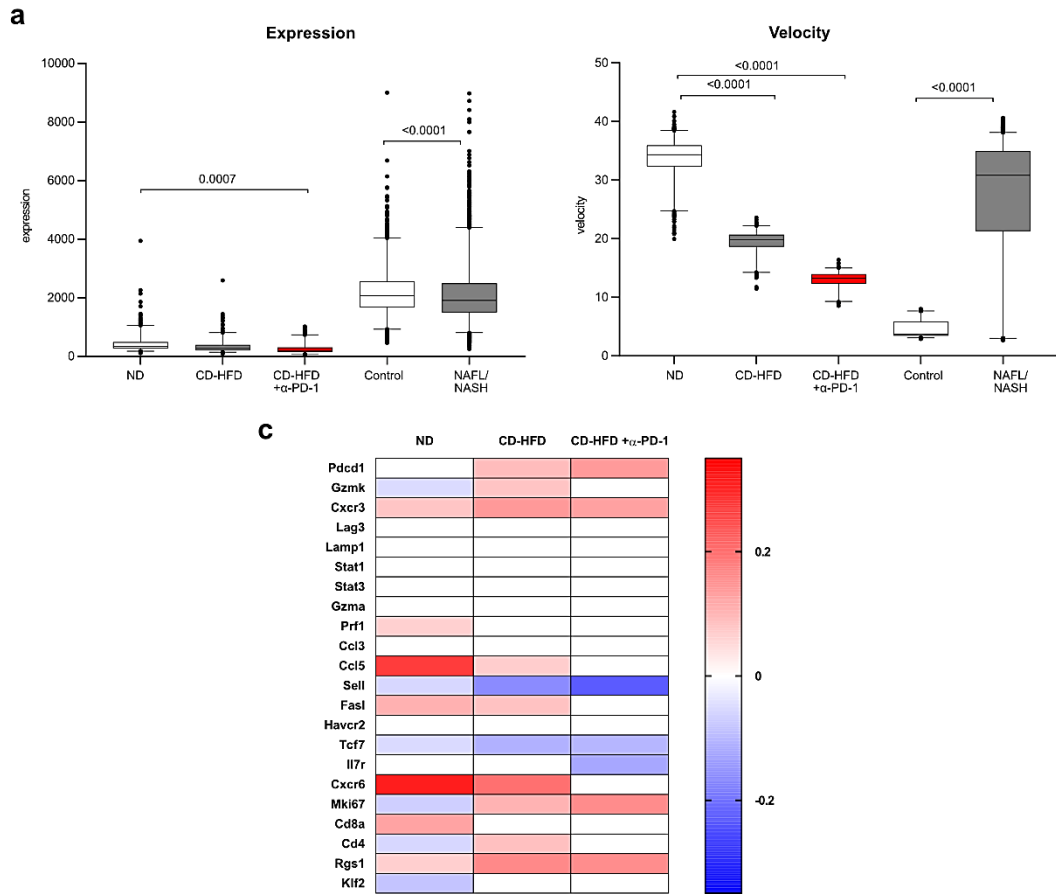
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**Rebuttal Figure 30: CD8<sup>+</sup>PD-1<sup>+</sup> are TOX<sup>high</sup>, have a resident-like character and are enriched upon  $\alpha$ -PD-1 treatment in NASH**  
**(a)** Quantification of intracellular Foxo1 and **(b)** calcium levels in CD8<sup>+</sup> T-cells by flow cytometry of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (Foxo1: ND n= 6 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 7 mice; calcium: ND n= 13 mice; CD-HFD n= 10 mice; CD-HFD +  $\alpha$ -PD-1 n= 10 mice). Polarization of CD8<sup>+</sup>PD-1<sup>+</sup> T-cells **(c)**, as well as Umap showing FlowSOM-guided clustering **(d)** and the expression intensity of the indicated marker **(e)** on stochastically selected hepatic CD8<sup>+</sup> T-cells of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 6 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). **(f)** Quantification of intracellular Calcium and **(g)** Foxo1 levels in CD4<sup>+</sup> T-cells by flow cytometry of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies ((Foxo1: ND n= 6 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 7 mice; calcium: ND n= 13 mice; CD-HFD n= 10 mice; CD-HFD +  $\alpha$ -PD-1 n= 10 mice). **(h)** Polarization analysis by flow cytometry of hepatic CD4<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 6 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). **(i)** Relative quantification of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> and **(j)** CD4<sup>+</sup>PD-1<sup>+</sup> T-cells by flow cytometry of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 6 mice; CD-HFD n= 5 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice). **(k)** Polarization by flowcytometry of hepatic CD8<sup>+</sup>PD-1<sup>+</sup> T-cells of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (ND n= 12 mice; CD-HFD n= 7 mice; CD-HFD +  $\alpha$ -PD-1 n= 6 mice).

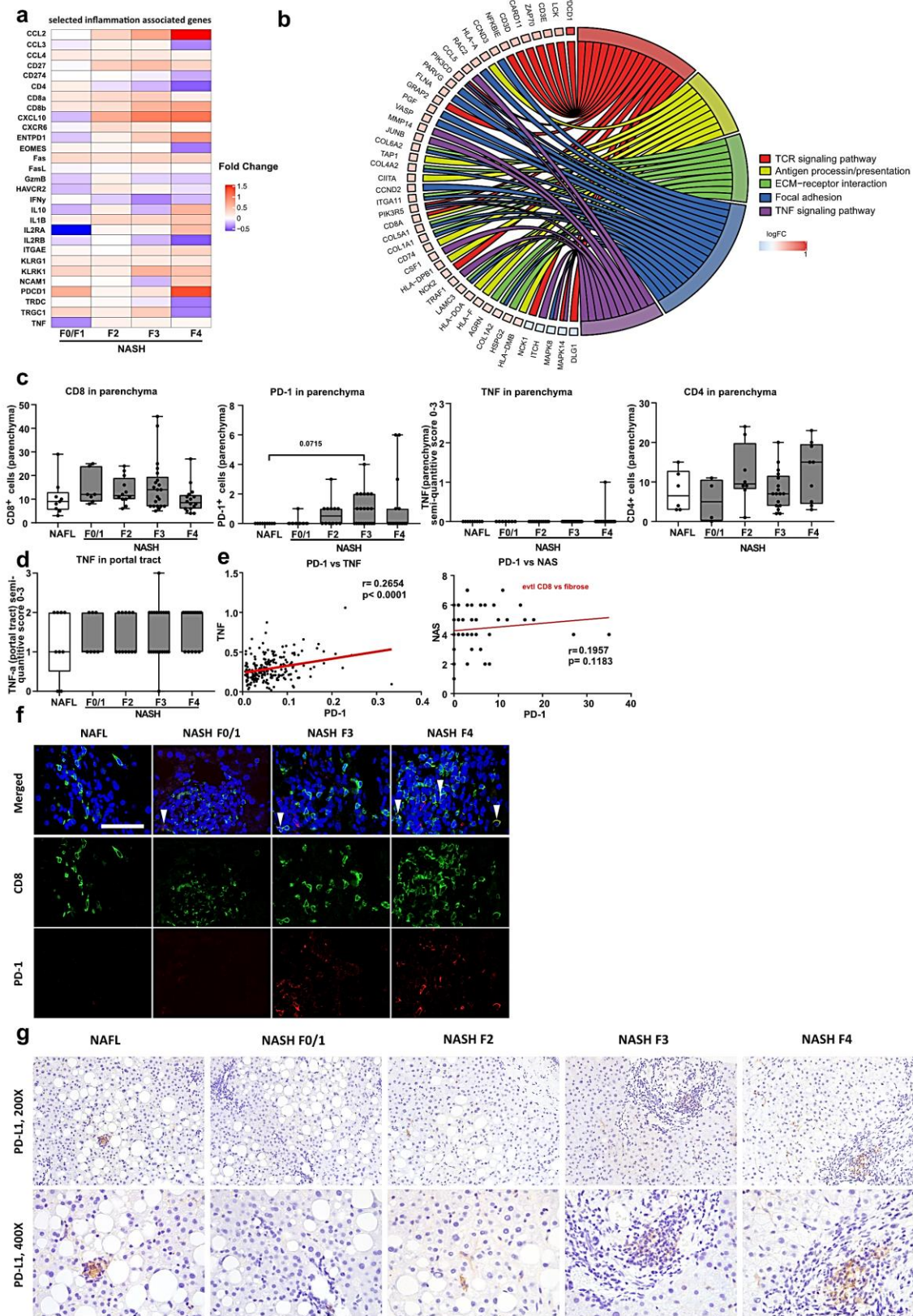
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2289 **Rebuttal Figure 31: RNA velocity analyses on CD4 T-cells in NASH**

2290 (a) RNA Velocity analyses of scRNA-seq data showing expression, and (b) velocity of patient-liver-derived CD4+  
 2291 T-cells of control, or NAFLD/NASH patients in comparison to mouse-liver-derived CD4+ T-cells (patients:  
 2292 NAFLD/NASH n= 3 patients; mouse: n= 3 mice/group). (c) Correlation of expression along the latent-time of  
 2293 selected genes along the latent-time (mouse: n= 3 mice/group).  
 2294



**Rebuttal Figure 32: Severity of NAS is associated with CD8 and PD-1 expression**  
**(a)** RNA-sequencing data comparing NASH with varying fibrosis (F0 – F4 according to Brunt classification) normalized to NAFLD from a total of n= 206 NAFLD/NASH patients corrected for batch, gender and center. **(b)** Single gene PD-1 correlation analysis of RNA-sequencing data from a total of n= 206 NAFLD/NASH patients corrected for batch, gender and center. **(c)** Quantification of hepatic parenchymal PD-1, parenchymal CD8, parenchymal CD4 parenchymal and **(d)** portal tract TNF expressing cells of NAFL and NASH with varying fibrosis patients (NAFL n= 9 patients; NASH F1/0 n= 7 patients; NASH F2 n= 12 patients; NASH F3 n= 21 patients; NASH F4 n= 16 patients; CD4: NAFL n= 6 patients; NASH F1/0 n= 4 patients; NASH F2 n= 8 patients; NASH F3 n= 17

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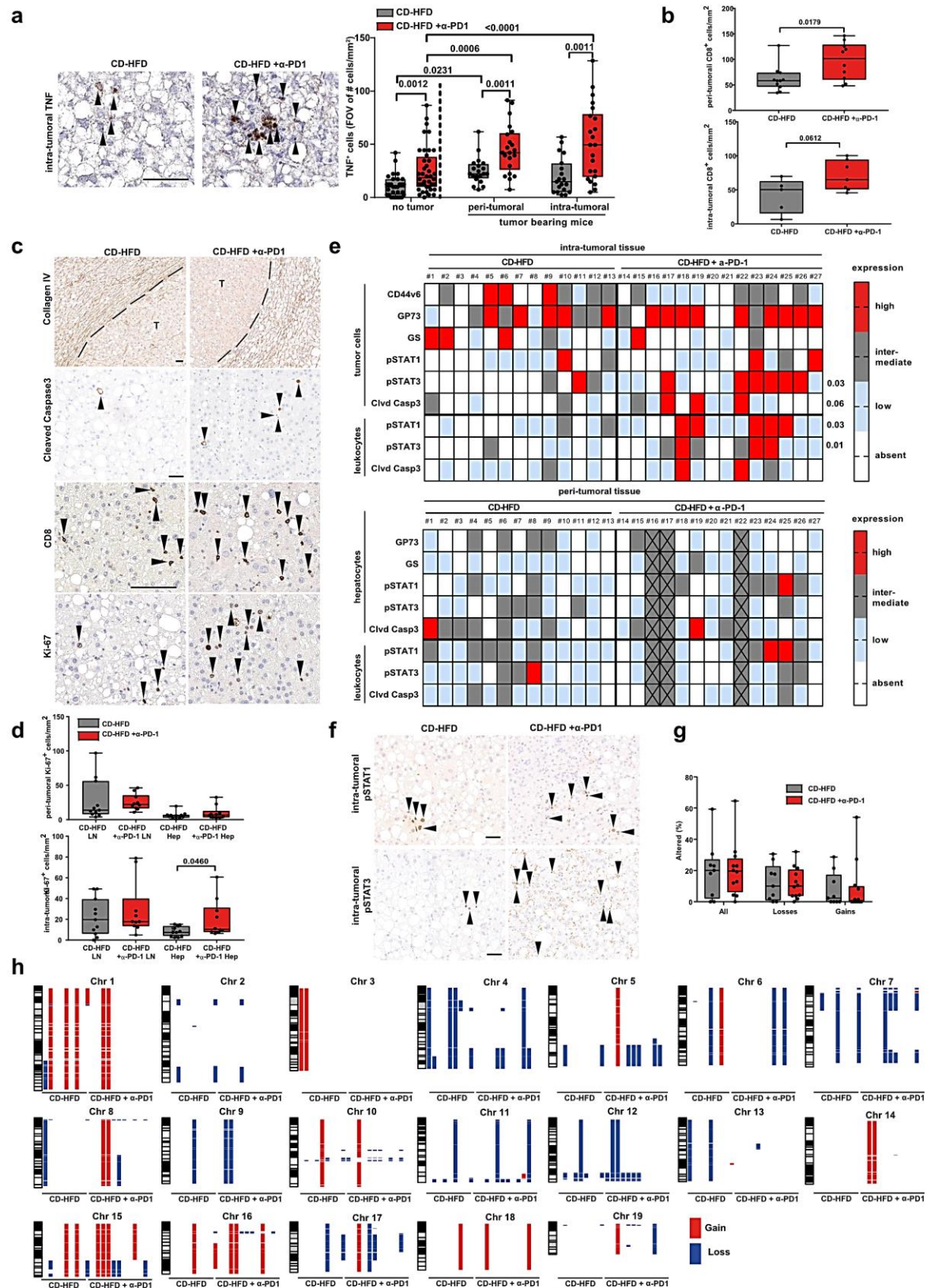




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2304 patients; NASH F4 n= 9 patients). **(e)** (c) Correlation analysis of PD-1 against TNF by RNA-sequencing or NAS by  
2305 immunohistochemical staining (NAFLD/NASH n= 65 patients). **(f)** Immunofluorescence staining of PD-1 and CD8  
2306 of NAFL and NASH with varying fibrosis patients. Arrowheads indicate CD8+PD-1+ cells. Scale bar: 50 µm. (g)  
2307 Immunohistochemical staining of PD-L1 in patient-derived liver samples. Scale bar: 50 µm.

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**Rebuttal Figure 33: CD8<sup>+</sup>PD-1<sup>+</sup> T-cells drive necro-inflammation induced hepatocarcinogenesis in NASH**

(a) Quantification of mRNA *in situ* hybridization for hepatic TNF<sup>+</sup> cells of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies with or without tumor (without tumor: CD-HFD n = 30 field of view (FOV) in 3 mice; CD-HFD +  $\alpha$ -PD-1 n = 40 FOV in 3 mice; peri-tumoral: CD-HFD n = 20 FOV in 3 mice; CD-HFD +  $\alpha$ -PD-1 n = 21 FOV in 3 mice; intra-tumoral: CD-HFD n = 19 FOV in 3 mice; CD-HFD +  $\alpha$ -PD-1 n = 22 FOV in 3 mice). Arrowheads indicate TNF<sup>+</sup> cells. Scale bar: 20  $\mu$ m. (b) Quantification of CD8 staining of peri- and intra-tumoral hepatic tissue by immunohistochemistry of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (peri-tumoral: CD-HFD n = 11 mice; CD-HFD +  $\alpha$ -PD-1 n = 10 mice; intra-tumoral: CD-HFD n = 5 mice;

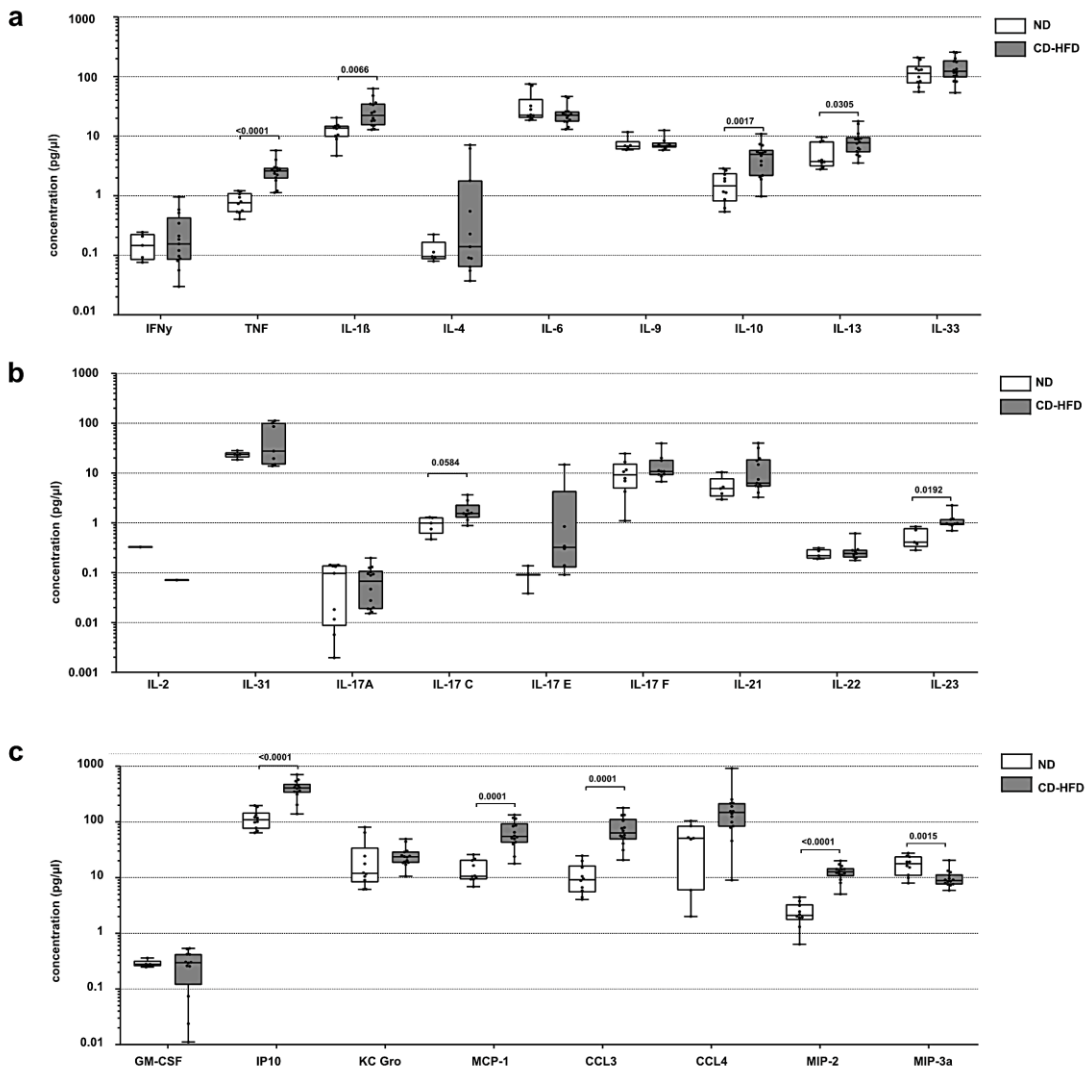
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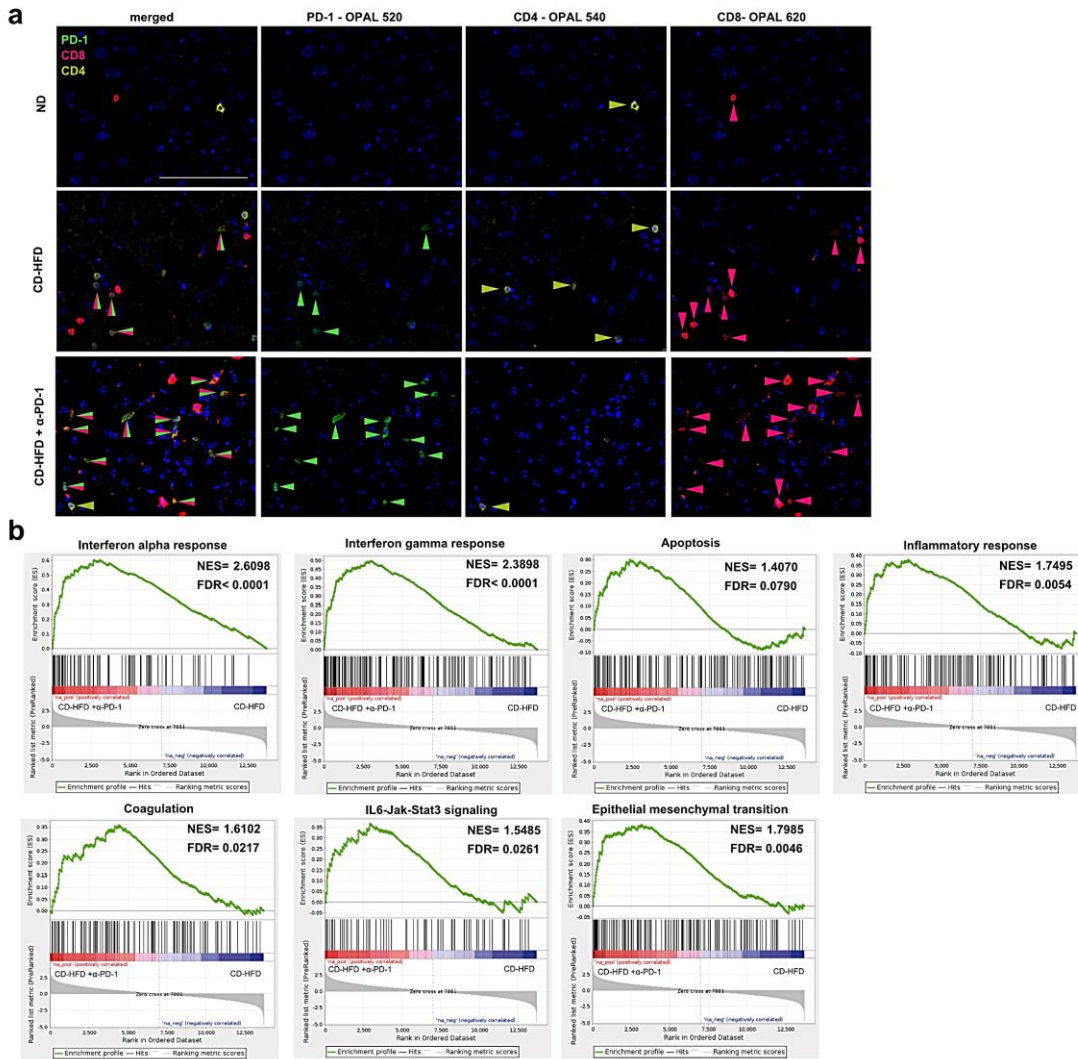
2317 CD-HFD +  $\alpha$ -PD-1 n= 7 mice). **(c)** Histological staining of hepatic tumor tissue by Collagen IV, cleaved Caspase 3,  
2318 CD8, Ki-67 of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (Collagen IV,  
2319 cleaved Caspase 3: CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice; CD8, Ki-67: CD-HFD n= 5 mice; CD-HFD  
2320 +  $\alpha$ -PD-1 n= 7 mice). Arrowheads indicate positive cells. Dotted line indicates tumor/lesion rim. Tumor area is  
2321 indicated by T. Scale bar: 100  $\mu$ m. **(d)** Quantification of Ki-67 staining of peri- and intra-tumoral hepatic tissue by  
2322 immunohistochemistry of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-  
2323 HFD n= 11 mice; CD-HFD +  $\alpha$ -PD-1 n= 10 mice). **(e)** Scoring of expression by immunohistochemistry staining of  
2324 intra- and **(d)** peri-tumoral hepatic tissue of 12 months CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-  
2325 1 antibodies (CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14 mice). Crossed out boxes indicate not sufficient tissue  
2326 for analysis. **(f)** Histological staining of intra-tumoral hepatic tissue by pSTAT1, or pSTAT3 of 12 months CD-HFD  
2327 or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (CD-HFD n= 13 mice; CD-HFD +  $\alpha$ -PD-1 n= 14  
2328 mice). Arrowheads indicate staining positive cells. Scale bar: 50  $\mu$ m **(g)** Quantification of **(h)** genomic aberrations  
2329 by array comparative genomic hybridization (aCGH) of tumor tissues of mice after 12 months on CD-HFD-fed mice  
2330 (n= 9) or 12 months on CD-HFD-fed mice + 8 weeks treatment with  $\alpha$ -PD-1 antibodies (n= 12).

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**Rebuttal Figure 34: Inflammation associated hepatic cytokine and chemokine environment in NASH**  
(a) and (b) multiplex ELISA of hepatic inflammation associated cytokines and (c) chemokines of 12 months ND or CD-HFD-fed mice (ND n= 10 mice; CD-HFD n= 14 mice).



**Rebuttal Figure 35:  $\alpha$ -PD-1 treatment causes enrichment of inflammation- and apoptosis-associated pathways in NASH**  
**(a)** Immunofluorescence microscopy of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n= 3 mice/group). Scale bar: 100  $\mu$ m. **(b)** Gene set enrichment analysis of RNA sequencing data of hepatic tissue comparing CD-HFD with CD-HFD-fed mice +  $\alpha$ -PD-1 of 12 months ND, CD-HFD or CD-HFD-fed mice + 8 weeks treatment by  $\alpha$ -PD-1 antibodies (n= 5 mice/group).

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**Reviewer Reports on the First Revision:**

Referee #1 (Remarks to the Author):

The authors have added an incredible amount of additional data from mouse models and clinical studies, and further strengthened their underlying hypotheses. The underlying message is important to the basic and clinical HCC communities. However, the concerns about manuscript with two underlying hypotheses, that are not sufficiently linked, remain.

I. The mouse studies in NASH-HCC models showing a tumor-promoting role of CD8+ PD-1+ T cells by increasing NASH, which is particularly strong when PD1-blockade is done early. This is corroborated by correlative human scRNA-seq, FACS and IHC studies showing immune alterations associated with NASH in patients confirming above data. These studies are well performed; however not strongly linked to the title of paper or the second part; as this is not relevant to immunotherapy which is given to patient with advanced unresectable HCC, and not to NASH patients or for HCC prevention. The concept of immunosurveillance would be an opportunity to link the two parts, but is not sufficiently investigated.

II. The mouse studies show a lack of response to checkpoint inhibition with a trend towards increased HCC as well as increased fibrosis when given as therapeutic treatment and increase HCC and NASH when given as prevention treatment. This is paralleled by human data showing lack of response to checkpoint inhibitors in patients with non-viral HCC. There are concerns about mouse studies as therapeutic checkpoint inhibitor approaches are performed when there is still underlying NASH activity; and there are limitations in the human section as all analyzed focus on non-viral HCC.

Main criticism:

1. The authors mingle above two concepts in the paper that are not necessarily linked and do not sufficiently separate these ideas: 1. The idea of immune-mediated NASH promotion. 2. The idea of failing restoration of anti-tumor immunity in NASH-HCC. The dual role of the immune systems in this long disease process is highlighted in the paper, but the fact that these are likely stage-specific functions, mentioned in the previous review, is not addressed. The authors failed to separate these in the mouse models, where they study the effect of checkpoint inhibitors in settings where NASH is still maintained via continued CD-HFD, both for prevention and therapeutic treatments. Not only does this not reflect the situation in most patients, who have advanced HCC and no to little ongoing NASH, but is also makes it difficult to interpret the data, i.e. is a potential beneficial of checkpoint inhibitors on tumors covered up by the increased NASH activity under checkpoint inhibition; or is it simply not present; or is there even the opposite?

2. The studies suggesting failure of checkpoint inhibition in NASH-HCC in mice and patients each have limitations. The provided information are incredibly important and potentially impactful but for this reason studies and data need to be very carefully performed, analyzed and interpreted to guide the field into the right direction.

2a. It is doubtful whether the employed mouse HCC studies are helpful to guide the field towards new concepts in NASH-HCC therapy - as the models first of all seem to rely on continued NASH with ongoing CD-HFD when therapies are given, making the factors driving HCC progression different from patients; it cannot be excluded that the HCC growth in these models still requires ongoing NASH; and second, these are not sufficiently established and characterized preclinical models, making it unclear whether the failure is due to model or species specifics, such as lacking similarities like sufficiently high mutational load and other characteristic that dictate response to immunotherapy in HCC patients. It also needs to be emphasized that all patients receiving checkpoint inhibitors have advanced HCC, usually outside of Milan criteria, which again may not be

achieved in mouse models. The authors provided additional data on their model, but mutational load is not specifically addressed. A key factor would be to have a model in which tumors continue to grow in the absence of NASH diet, within an environment that is similar to human HCC (cirrhotic). As this may be impossible and as mice could still respond differently, the strong focus on mouse models is not ideal to address the essence of this question, and more emphasis should have been on human trials. The main point of mouse models would be to address underlying mechanisms after having established key data in patients - the reverse approach is inherently less convincing and more difficult, requiring nearly perfect models.

2b. With the provided concept, suggesting that CD8+PD1+ cells promote NASH, and thereby driving HCC, but do not provide tumor-suppressing immune surveillance, the paper does not reflect that NASH carries a significantly lower risk for HCC development than other chronic liver diseases. The proposed concept of a failing immunosurveillance by CD8+PD1+ cells, based on mouse models, appears to not fully reflect clinical reality.

2b. The analysis of human checkpoint inhibitor trials is greatly expanded, but it is almost a paper within a paper that could be published on its own. Rather than trying to address some of the inherent human study weaknesses through analysis in mouse models, the authors should have attempted a deeper analysis of these data. One of the key points are whether the non-viral HCC is indeed mostly NASH-HCC; whether NASH-HCC has a different prognosis due to the underlying metabolic complications affecting other organ systems; moreover other differences such as age (a key factor in HCC outcomes) and gender are not consistently analyzed in Tables S9 and S10.

2c. Related to 2b, the authors did not ask and answer the question whether the divergent data on checkpoint inhibition in different patient groups are because of (A) presence of viral antigens that enhance anti-tumor immunity, especially in HBV-associated HCC; or whether (B) the lack of response in non-viral HCC is indeed related to specific NASH-HCC and its liver and immune environment as suggested by the entire paper.

2d. Subanalysis of cirrhotic NASH-HCC (lacking significant NASH activity) and non-cirrhotic NASH-HCC (where some patients may still have some NASH-activity) is not addressed, and admittedly extremely difficult (but the combination with a mouse model with NASH activity does trigger this question).

In summary, the data in the manuscript are relevant, highlighting the dual role of the immune system. However, mouse models are not sufficiently strong to answer questions on CD8+PD1+ cells and anti-tumor immunity independent of the associated NASH-driving functions; patient data lack in-depth analysis, lacking explanation of the data and addressing the question whether viral antigens may explain many of the observed differences rather than specifics of NASH-HCC and its environment; and the concepts of immune-mediated NASH promotion vs the failure of checkpoint inhibition in advanced NASH-HCC tumors lack a sufficient distinction due to the lacking analysis of stage-specific functions of these two underlying immune-mediated tumor-promoting and restricting mechanisms throughout the paper, due to lacking focus on this question as well as lacking mouse models that could satisfactorily answer this question. Hence, major overhaul and rewrite of the paper is needed, highlighting its strengths, and addressing above points so that the field benefits. One point that could link the two hypotheses would be failing immunosurveillance in the precancerous NASH liver. The paper will raise the important question whether NASH-HCC benefits from immunotherapy, but it will not be able to fully answer it (which is ok - it can be the basis for new prospective studies). However, the authors should make this message clearer to the audience by separating the two topics, i.e. (A) the role of the immune system in driving NASH and (B) the potential failure of checkpoint inhibitors in advanced NASH-HCC. Without this, the authors will not sufficiently reach and impact the basic or clinical research community.

Referee #3 (Remarks to the Author):

I do congratulate authors for an amazing effort to satisfy the many questions and concerns asked and posed by the reviewers. The revised version is much improved and in my view advances our knowledge on this matter a very considerable extent. The interconnection with the back-to-back paper is excellent. I am glad that my suggestion of TNF blockade leads to experimental postulation of a clinically actionable target. Moreover, in the companion manuscript this has been mechanistically explored as well.

Referee #4 (Remarks to the Author):

The rebuttal is very thorough. All of the technical points seem to have been addressed thoroughly. However, I'm left a bit concerned about the actual conclusions of the paper.

On the one hand there is a very thorough mechanistic study in mice which reveals a clear in vivo phenotype in vivo that CPI therapy promotes carcinogenesis. On the other there is clinical data - which is much improved - that different aetiologies of HCC respond differently to CPI. So it seems on the face of it that HCCs in NASH are harder to treat with the current monotherapy - that is a fine conclusion from the data and should certainly help design new stratified trials as suggested (and maybe would be the starting point for the paper). But almost all of the rest of the paper is about pathways to cancer development - there did not seem to be a strong signal from the human trial data or from the mouse in vivo experiment that CPI therapy of an established tumour made things worse.

It seemed to me currently the two bits are not that well connected - really addressing different issues - and the only experiment which clearly linked them is a negative result tucked away in extended figure 6/7 and is not really explored any further. For that it would be really helpful to show that this lack of response was linked to the CD8 phenotype seen. So a parallel experiment with a distinct non-NASH model of HCC which did show an impact of CPI would seem important. In other models published, even if responsive to dual checkpoint inhibition, there is limited response to PD1/PDL1 alone. So making the link between the underlying pathogenesis/CD8 phenotype and the responsiveness to CPI still needs to be clarified.



**Author Rebuttals to First Revision**

**(please note that the authors have quoted the reviewers in black and responded in blue)**

**Referee #1 (Remarks to the Author):**

The authors have added an incredible amount of additional data from mouse models and clinical studies, and further strengthened their underlying hypotheses. The underlying message is important to the basic and clinical HCC communities. However, the concerns about manuscript with two underlying hypotheses, that are not sufficiently linked, remain.

I. The mouse studies in NASH-HCC models showing a tumor-promoting role of CD8+ PD-1+ T cells by increasing NASH, which is particularly strong when PD1-blockade is done early. This is corroborated by correlative human scRNA-seq, FACS and IHC studies showing immune alterations associated with NASH in patients confirming above data. These studies are well performed; however not strongly linked to the title of paper or the second part; as this is not relevant to immunotherapy which is given to patient with advanced unresectable HCC, and not to NASH patients or for HCC prevention. The concept of immunosurveillance would be an opportunity to link the two parts, but is not sufficiently investigated.

We thank Referee #1 for appreciating our well performed studies and we agree with this Referee that the concept of immunosurveillance is appealing and could underline the connection between our mouse and the human data in a new light. To accommodate the message in this fresh light, we have changed the title of our manuscript into **"Immune-checkpoint blockade stalls immunosurveillance and anti-tumor effects in NASH-HCC"**.

We will further explain this concept in the text.

Of note, in the light of the IMbrave150 study PD-(L)1, targeted immunotherapy will most likely become the new standard of care first line therapy not only for advanced HCC, but also in a preventive fashion upon surgical intervention to avoid liver cancer recurrence – thus we believe that also the aspect of chemoprevention is an important issue.

II. The mouse studies show a lack of response to checkpoint inhibition with a trend towards increased HCC as well as increased fibrosis when given as therapeutic treatment and increase HCC and NASH when given as prevention treatment. This is paralleled by human data showing lack of response to checkpoint inhibitors in patients with non-viral HCC. There are concerns about mouse studies as therapeutic checkpoint inhibitor approaches are performed when there is still underlying NASH activity; and there are limitations in the human section as all analyzed focus on non-viral HCC.

We thank Referee #1 for highlighting ongoing feeding of NASH-inducing diet in our therapeutic PD-1-targeted immunotherapy scheme. One interesting concept for immunotherapy treatment for the future might be *"to first treat the metabolic disorder and normalize the hepatic and systemic dyslipidemia, then tackle HCC/tumor surveillance"*, which we will now address our revised manuscript.

We are in line with Referee#1, that the human non-viral HCC data leaves questions unanswered. Given that we did not have access to the raw data of the 3 phase III trials (led by

companies) included in the meta-analysis, we cannot provide information about the distribution of alcohol-related vs. NASH-related HCC within the non-viral HCC group.

Notably, we have added two 2 retrospective cohorts of HCC patients treated with immunotherapy, in which we addressed this issue by comparing NASH-related HCC vs. non-NASH etiologies. Here, we were able to demonstrate that NASH-related HCC was associated with a worse outcome in 2 independent cohorts. We agree that these retrospective cohorts still have their limitations, but will highlight that they could pave the way for prospective studies.

Main criticism:

1. The authors mingle above two concepts in the paper that are not necessarily linked and do not sufficiently separate these ideas:

1. The idea of immune-mediated NASH promotion. 2. The idea of failing restoration of anti-tumor immunity in NASH-HCC.

The dual role of the immune systems in this long disease process is highlighted in the paper, but the fact that these are likely stage-specific functions, mentioned in the previous review, is not addressed. The authors failed to separate these in the mouse models, where they study the effect of checkpoint inhibitors in settings where NASH is still maintained via continued CD-HFD, both for prevention and therapeutic treatments. Not only does this not reflect the situation in most patients, who have advanced HCC and no to little ongoing NASH, but is also makes it difficult to interpret the data, i.e. is a potential beneficial of checkpoint inhibitors on tumors covered up by the increased NASH activity under checkpoint inhibition; or is it simply not present; or is there even the opposite?

We thank Referee #1 for raising this point. The idea of immune-mediated NASH promotion can be indeed demonstrated by our data. To address the second point of failing restoration of anti-tumor immunity in NASH-HCC - we have analyzed liver tissue from established NASH-HCC that were treated with immune-check point blockade by flow cytometry markers of exhaustion, cytotoxicity, or re-activation. In none of the analyzed markers, we observed any differences between CD-HFD and CD-HFD + anti-PD-1 treatment, arguing against a tumor restricting role of CD8+ T-cells with ongoing CD-HFD feeding in the therapeutic setting. Like written in our previous response we saw a small but significant increase of CD44<sup>CD62L</sup>CD8 T cells (infiltrating memory cells), however we do not observe an anti-tumor effect.

This corroborates our immunohistochemistry data, in which we did not observe any difference of PD-1+ or CD8+ T-cells in peri- or intra-tumoral liver tissue. These data are now included in a revised manuscript (**Supplementary Information**).

**We have initially shown that over time CD8 T cells fuel steatosis and NASH development. Over time generation of hepatic, resident CXCR6+ PD1+CD8+ T cells that are dysfunctional for effective tumor-surveillance can be detected. However, these CD8 T**

cells are hyperactivated (as analyzed by single cell RNA Seq, flow cytometry) and auto-aggressive as shown by Dudek et al.. Thus, PD1CD8+ T cells are generated progressively filling up the pool of hepatic CD8+ T cells over time in the context of NASH. Experiments of CD8+ T cell depletion in established precancerous NASH show that the CD8+ T cell population contributes to liver cancer development – as CD8+ T cell depletion reduced HCC incidence.

Immune-checkpoint blockade either given at established NASH or at NASH with HCC exacerbates this phenotype by increasing the number of these hyper-activated PD1CD8+ T cells. At early stages of NASH/HCC immune check point blockade increases HCC incidence - at late stage HCC immune check point blockade cannot function anymore as the pre-existing T cells **cannot execute efficiently tumor surveillance**. Thus, a potential beneficial role of checkpoint inhibitors on tumors is overruled mainly by auto-aggressive T-cells. We cannot exclude on single cell level that there are single tumor specific cells present but if so they not only kill tumor cells and thus will induce also compensatory proliferation by randomly killing other cells (as shown by Dudek et al.).

Our single cell RNA seq. data as well as our flow cytometry analyses of PD1+ CD8+ T cells in the context of NASH as well as in the context of NASH/HCC clearly show that hepatic PD1CD8+ T cells do not change in quality but rather in number over time. Our velocity analysis indicates one final, endpoint CD8 +T cell population that increases over time progressively with NASH development which is a PD1+CD8+CXCR6+ T cell population - reminiscent of the auto-aggressive T cell population by Dudek et al. Moreover, in this co-submitted manuscript this stable character of auto-aggressive T cells over time is described, corroborating our findings. We will further discuss the concept in the revised manuscript that potential treatment of the underlying chronic inflammation (e.g. reverting NASH) may precede immunotherapy.

2. The studies suggesting failure of checkpoint inhibition in NASH-HCC in mice and patients each have limitations. The provided information are incredibly important and potentially impactful but for this reason studies and data need to be very carefully performed, analyzed and interpreted to guide the field into the right direction.

We thank Referee #1 for acknowledging the information of our data as “incredibly important and potentially impactful” and agree, that a careful handling needs to be done – this is what we have intended in the revised version of our manuscript.

2a. It is doubtful whether the employed mouse HCC studies are helpful to guide the field towards new concepts in NASH-HCC therapy - as the models first of all seem to rely on continued NASH with ongoing CD-HFD when therapies are given, making the factors driving HCC progression different from patients; it cannot be excluded that the HCC growth in these models still requires ongoing NASH; and second,

these are not sufficiently established and characterized preclinical models, making it unclear whether the failure is due to model or species specific, such as lacking similarities like sufficiently high mutational load and other characteristic that dictate response to immunotherapy in HCC patients. It also needs to be emphasized that all patients receiving checkpoint inhibitors have advanced HCC, usually outside of Milan criteria, which again may not be achieved in mouse models. The authors provided additional data on their model, but mutational load is not specifically addressed. A key factor would be to have a model in which tumors continue to grow in the absence of NASH diet, within an environment that is similar to human HCC (cirrhotic). As this may be impossible and as mice could still respond differently, the strong focus on mouse models is not ideal to address the essence of this question, and more emphasis should have been on human trials. The main point of mouse models would be to address underlying mechanisms after having established key data in patients - the reverse approach is inherently less convincing and more difficult, requiring nearly perfect models.

We thank Referee #1 for his/her comment and would like to state, that although CD8-depleted animals (former Figure 2, now Extended Data 7) have elevated NAS, and ALT and are metabolically impaired with all features of NAFLD/NAS, they lack liver cancer in the preventive treatment regimen. We now included distinct non-NASH liver cancer models into the manuscript to demonstrate that in the absence of NASH - immunotherapy does prolong animal survival and reduces liver cancer development.

Moreover, we cannot rule out entirely that species specific contribute to lack of response in the mouse models. However, our clinical data are in line with those obtained from our animal studies, as patients with NASH-related HCC treated with immunotherapy also had a poorer outcome.

Nevertheless, we acknowledge the limitations of such retrospective analyses and will clearly state in the discussion section that prospective validation of these findings is warranted. Our data could pave the way for the design of such prospective protocols. Additionally, we want to emphasize that currently there is no established and validated biomarker that predicts response to immunotherapy in HCC (as reviewed in Pinter M et al. JAMA Oncol 2020). Tumor mutational load is generally low in HCC and its use as a predictive biomarker in HCC is not supported by available clinical data (Ang C et al. Oncotarget 2019;10:4018. Harding JJ et al. Clin Cancer Res 2019; 25:2116. Wong CN et al. Liver Int 2020;Epub ahead of print). We agree with the reviewer that most patients with NASH-related HCC suffer from concomitant cirrhosis – but they also in general suffer from systemic dyslipidemia and obesity.

Thus, we do not believe that continuation of NASH-inducing diet in mice is in conflict with the clinical scenario in humans. On the contrary, patients with NASH usually remain exposed to metabolic risk factors (e.g., overweight, unhealthy diet, hypertension, hyperlipidemia, lack of exercise) even after they developed HCC. Only a minority of patients is able to dramatically

change their lifestyle. Thus, we agree that the mouse models are not perfect but they are still representative for our conducted clinical observation in large parts. We discuss this issue and potential complications in our discussion.

2b. With the provided concept, suggesting that CD8+PD1+ cells promote NASH, and thereby driving HCC, but do not provide tumor-suppressing immune surveillance, the paper does not reflect that NASH carries a significantly lower risk for HCC development than other chronic liver diseases. The proposed concept of a failing immunosurveillance by CD8+PD1+ cells, based on mouse models, appears to not fully reflect clinical reality.

We agree with Referee #1 that the used mouse model might only represent subsets of patients, as some metabolic-impaired patients with liver features reminiscent of NAFLD/NASH pathology react in a long-lasting manner to immunotherapy. **We rather even state that NASH does not allow CD8+ T cells to exert their function of immune-surveillance and this is exacerbated by immune check point blockade (see our point auto-aggression in human and mouse NASH).** So far, no clinical consensus could be achieved for biomarkers to discriminate those patients, who might benefit from immunotherapy. Moreover, we would like to draw attention to the suboptimal setup of the retrospective clinical design and the need for prospective validation of the proposed concept. We tone down our interpretation and discuss that the need for stratification of patients, who might benefit from immunotherapy, cannot only rely on the underlying inflammatory etiology.

2b. The analysis of human checkpoint inhibitor trials is greatly expanded, but it is almost a paper within a paper that could be published on its own. Rather than trying to address some of the inherent human study weaknesses through analysis in mouse models, the authors should have attempted a deeper analysis of these data. One of the key points are whether the non-viral HCC is indeed mostly NASH-HCC; whether NASH-HCC has a different prognosis due to the underlying metabolic complications affecting other organ systems; moreover other differences such as age (a key factor in HCC outcomes) and gender are not consistently analyzed in Tables S9 and S10.

We agree with the comment of Referee #1 and would like to raise awareness, that the clinical data were obtained retrospectively and outside of a prospective clinical trial protocol, biopsies before initiation of immunotherapy were not mandatory and thus are not available in the majority of patients. Therefore, we were not able to obtain data regarding the immune microenvironment with respect to different underlying etiologies. We now analyzed age and gender in our multivariate analysis (**Supplementary Table 9**) and these two factors were not a confounder of HCC treatment outcome.



2c. Related to 2b, the authors did not ask and answer the question whether the divergent data on checkpoint inhibition in different patients groups are because of (A) presence of viral antigens that enhance anti-tumor immunity, especially in HBV-associated HCC; or whether (B) the lack of response in non-viral HCC is indeed related to specific NASH-HCC and its liver and immune environment as suggested by the entire paper.

We thank Referee #1 for this important point and would like to refer to our answer previously given, that the human non-viral HCC data leaves unanswered questions behind. Given that we did not have access to the raw data of the 3 phase III trials included in the meta-analysis, we cannot provide information about the distribution of alcohol-related vs. NASH-related HCC within the non-viral HCC group, or level of viral antigens, that potentially enhance anti-tumor immunity. However, in our 2 retrospective cohorts of HCC patients treated with immunotherapy, we addressed this issue by comparing NASH-related HCC vs. non-NASH etiologies. We were able to demonstrate that NASH-related HCC was associated with a worse outcome in 2 independent cohorts. We agree that these retrospective cohorts still have their limitations, but they could pave the way for prospective studies.

2d. Subanalysis of cirrhotic NASH-HCC (lacking significant NASH activity) and non-cirrhotic NASH-HCC (where some patients may still have some NASH-activity) is not addressed, and admittedly extremely difficult (but the combination with a mouse model with NASH activity does trigger this question).

We agree with Referee #1, that analyses of non-cirrhotic NASH-HCCs might give valuable insights but is extremely difficult. Thus we were not able to perform further meaningful sub-analyses.

In summary, the data in the manuscript are relevant, highlighting the dual role of the immune system. However, mouse models are not sufficiently strong to answer questions on CD8+PD1+ cells and anti-tumor immunity independent of the associated NASH-driving functions; patient data lack in-depth analysis, lacking explanation of the data and addressing the question whether viral antigens may explain many of the observed differences rather than specifics of NASH-HCC and its environment; and the concepts of immune-mediated NASH promotion vs the failure of checkpoint inhibition in advanced NASH-HCC tumors lack a sufficient distinction due to the lacking analysis of stage-specific functions of these two underlying immune-mediated tumor-promoting and restricting mechanisms throughout the paper, due to lacking focus on this question as well as lacking mouse models that could satisfactorily answer this question. Hence, major overhaul and rewrite of the paper is needed, highlighting its strengths, and addressing above points so that the field benefits. One point that could link the two hypotheses would be failing immunosurveillance in the precancerous NASH liver. The paper will raise the important question whether NASH-HCC benefits from immunotherapy, but it will not be able to fully answer it (which is ok -

it can be the basis for new prospective studies). However, the authors should make this message clearer to the audience by separating the two topics, i.e. (A) the role of the immune system in driving NASH and (B) the potential failure of checkpoint inhibitors in advanced NASH-HCC. Without this, the authors will not sufficiently reach and impact the basic or clinical research community.

We thank Referee #1 for the constructive points and thus re-organized our manuscript to underline/highlighting its strengths while critically raising the point of prospective validation. **We will link the 2 hypotheses indicated above through failing immuosurveillance in the precancerous NASH liver in the revised version of our manuscript.**

***Referee #2 (Remarks to the Author):***

In their (too) lengthy rebuttal, Pfister et al., provide two key additions to their original manuscript. First, they increase the size of their original patient cohort and add a validation cohort and secondly, they perform in vivo depletion experiments to determine the involvement of several cell types and inflammatory mediators in establishing anti-PD-1-accelerated hepatocarcinogenesis. While the number of NAFLD patients included in this manuscript is still low (n=13 and n=11 respectively), it is encouraging to see that the data obtained was similar between the two datasets. I agree with the authors that this is an interesting observation.

We thank Referee #2 for acknowledging our work and for the notion that we have provided key additions to our original manuscript.

However, despite the overabundance of data, the mechanism by which NASH (and/or NAFLD) predisposes to anti-PD-1-accelerated hepatocarcinogenesis remains largely unclear. As I said in my original rebuttal, the data presented by the authors fail to demonstrate clear causal relationships. As an example, the authors present cytokine measurements after several antibody-based interventions in Extended Data 21, but fail to determine which of these are important. They state that liver inflammation is reduced upon CD8 depletion (which is a solid and interesting result), yet, for example, IFN $\gamma$ , IL-21 and IL-31 remain unchanged. What do the authors base their statements on?

We acknowledge the comment of Referee #2 but we would like to add that not all inflammatory mediators are reduced upon CD8 depletion - as indicted by our ICF signature analysis. We base our statement on immunohistochemistry describing reduced T-cell infiltration into the liver. We have now specified this further in the revised manuscript.

Are these mediators not inflammatory? Which mediators instead would indicate an inflammatory environment?

These are all inflammatory mediators, but many of them do not correlate with disease. We have now specified this further in our revised manuscript. IL31 for example does not correlate with NAS, ALT,



sirius red or tumor incidence. In contrast, our correlative analysis as well as our convolutional neural network analysis identified levels of TNF to correlate with liver cancer incidence.

Also, why can significant amounts of TNF still be found in conditions of TNF depletion? Similarly, why is TNF not significantly down in CD-HFD + anti-PD-1/anti-TNF when compared to CD-HFD + anti-PD-1? Does this not indicate that the authors' intervention did not work? And if that is the case, why is there a significant effect of anti-PD-1 + anti-TNF treatment on tumor lesions relative to anti-PD-1 only?

We politely disagree with Referee # 2. The TNF inhibition has worked as we have observed significant decrease of CD8+ T cells, PD1+ T cells, MHCII+ and F480+cells.

Along the same lines, I had asked in my original review about the involvement of CD4 T cells. The authors have now performed CD4 depletion experiments, and they state that this 'did not decrease liver pathology or liver inflammation (lines 471-472)', yet they show that TNF, the molecule they say is responsible for causing liver inflammation, is in fact significantly less abundant (Extended Data 23c, CD-HFD + anti-PD-1 vs CD-HFD + anti-PD-1/anti-CD4), as is IL-21, IL-33, IL-1B and IL-13 (amongst others). Are these mediators not inflammatory? Which mediators then indicate an inflammatory environment?

We thank Referee #2 for this notion and we are happy that this Referee has acknowledged our data on the role of CD4 T cells. Again as stated above we will be more precise when stating the term inflammatory environment and will write this more specifically in the main text and discussion.

To compound these issues, I believe the authors have actually stumbled upon an interesting finding regarding these CD4 cells, which they seem to have overlooked. While tumor incidence is similar upon depletion of CD4 cells in the context of CD-HFD + anti-PD-1 (Fig. 4n), the number of tumors per liver and the individual lesion size is actually reduced (Extended Data 24a, b). This would imply that the CD4 cells actually do play a role in the authors' proposed mechanism. It is unclear to me why the authors would not follow up on this important aspect of their mechanism, especially since they put a lot of emphasis on the CD4 cells when discussing their patient data.

We thank Referee# 2 for this statement and agree that this is an interesting finding and might be in line with CTLA4 mediated T-reg depletion (a particular subset of T cells) - and indeed we had already noted and discussed this in the revised paper that CD4 T cells might contribute to tumor progression – but not initiation – as tumor incidence was unchanged in a CD4 T cell depletion setting. Thus, we believe that in the mouse and the human setting CD4 T cells might play a role in tumor progression and we have now re-enforced this statement in the discussion section.

Lastly, the authors remain highly selective in the data they choose to discuss and how they interpret

it (also see my points before). To illustrate more examples:  
line 247 and Figure 1e, more populations are affected than just the CD8 compartment;  
line 250 Extended Data 3g, CD4 cells actually increase significantly;  
line 264-266 and Figures 1i-j and Extended Data 5a-d, CD8 cells both lose and gain cytotoxic function by RNA seq (see *Gzma/Gzmb* for example) and the effect size of CD4 cells seems the same if not larger than the CD8 T cells;  
line 273-276 and Extended Data 5e, not everything was validated by mass spec including importantly the finding of enhanced *Tox* expression;  
line 342-343 and Extended Data 11f, CD4 cells do also change significantly,  
line 343-344 and Extended Data 11k-l, While NKT and NK cells do not increase effector cytokine production neither do CD8 T cells;  
line 431-433 and Extended Data 18e, the authors actually present significant data;  
line 434-436 and Extended Data 18f, the authors actually present significant data;  
line 456-457 and Extended Data 19f-h, the authors present significant data for CD4 cells and largely in the same order of magnitude as their findings for CD8 cells

We thank Referee# 2 for the statements. As the functional data show a very strong effect with CD8 T cells we focus on this cell population in this manuscript – still we do cite and discuss the other populations as well. We thank Referee# 2 for these points and will adjust those accordingly.

**Referee #3 (Remarks to the Author):**

I do congratulate authors for an amazing effort to satisfy the many questions and concerns asked and posed by the reviewers. The revised version is much improved and in my view advances our knowledge on this matter a very considerable extent. The interconnection with the back-to-back paper is excellent. I am glad that my suggestion of TNF blockade leads to experimental postulation of a clinically actionable target. Moreover, in the companion manuscript this has been mechanistically explored as well.

We thank Referee #3 for his insights and constructive comments throughout the review process.

**Referee #4 (Remarks to the Author):**

The rebuttal is very thorough. All of the technical points seem to have been addressed thoroughly. However, I'm left a bit concerned about the actual conclusions of the paper. On the one hand there is a very thorough mechanistic study in mice which reveals a clear in vivo phenotype in vivo that CPI therapy promotes carcinogenesis. On the other there is clinical data - which is much improved - that different aetiologies of HCC respond differently to CPI. So it seems on the face of it that HCCs in NASH are harder to treat with the current monotherapy - that is a fine conclusion from the data and should certainly help design new stratified trials as suggested (and maybe would be the starting point for the paper). But almost

all of the rest of the paper is about pathways to cancer development - there did not seem to be a strong signal from the human trial data or from the mouse in vivo experiment that CPI therapy of an established tumour made things worse.

We agree that our clinical data clearly indicate that immunotherapy seems to be less effective in patients with NASH-related HCC. We acknowledge that these retrospective analyses still have their limitations and need prospective validation. Our data could pave the way for the design of such prospective protocols.

It seemed to me currently the two bits are not that well connected - really addressing different issues - and the only experiment which clearly linked them is a negative result tucked away in extended figure 6/7 and is not really explored any further. For that it would be really helpful to show that this lack of response was linked to the CD8 phenotype seen. So a parallel experiment with a distinct non-NASH model of HCC which did show an impact of CPI would seem important. In other models published, even if responsive to dual checkpoint inhibition, there is limited response to PD1/PDL1 alone. So making the link between the underlying pathogenesis/CD8 phenotype and the responsiveness to CPI still needs to be clarified.

We thank Referee 4 for this important comment. We have now added distinct non-NASH liver cancer models that do respond to CPI – as a positive control and have included this into the Supplemental material.

**Reviewer Reports on the Second Revision:**

Referee #1 (Remarks to the Author):

The authors have addressed my concerns through rewriting of the manuscript, which has become more coherent, with mouse and human parts now fitting much better together. I only have two remaining comments/suggestions, which can be mostly addressed through editorial changes, but which are important for the overall interpretation of the manuscript:

1. The paper is not sufficiently clear on whether anti-PD1 affects injury (which promotes HCC) and thereby might overshadow its anti-tumor effects when given as “therapeutic” approach in NASH-HCC mice at later stages (13 months). The authors state author state that there is more pronounced liver damage - but show unaltered NAS score and decreased serum ALT in the anti-PD1-treated group in Extended Data 2F, i.e. no increase in damage. This data is in fact supporting a failure of checkpoint inhibitors in a therapeutic setting, and not just an increased injury (which is likely not occurring in most patients receiving checkpoint inhibitor therapy) overshadowing anti-tumor immunity in this mouse model. This would in the end be more similar to what is seen in patients and should be clearly pointed out in the results and discussion (and the statement on damage should be corrected). As there is more fibrosis, which is often the result of more injury but could also be the result of more inflammation in response to checkpoint inhibitors, the authors might want to additional check injury by TUNEL staining to be 100% sure.

1b. The authors have shown several additional mouse models in Extended Data 3, in which which anti-PD1 was effective, i.e. different from the NASH model - but the comparison is not fair as none of these mouse models had a chronic injury component. Fortunately, a recent paper addressed this point (Chung et al, PMID: 32839204), showing decreased HCC in a DEN+CCl4 HCC model when anti-PD1 was given in a preventative manner from week 10-20. This paper should be cited and discussed. The discussion of the Chung et al paper in the context of PD1 inhibitor-mediated NASH injury promotion and lack of therapeutic effects is important and will further improve the paper, providing more evidence that these are NASH-specific phenomena.

Referee #2 (Remarks to the Author):

The authors have improved the paper significantly, with better explanations, fewer overstatements and more focus.

A few final remarks:

-as far as I could see, the %age of TNF producing cells was not quantified, only images were shown (Figure 1n,o; Extended Data 2i-n).

-same for TNF expression (Extended Data 5a-g).

Referee #4 (Remarks to the Author):

The comments have all been addressed. In the title could the authors maybe use impacts on or something similar rather than precludes as that sounds a bit absolute.

**Author Rebuttals to Second Revision**

**(please note that the authors have quoted the reviewers in black and responded in blue)**



Referees' comments:

**Referee #1** (Remarks to the Author):

The authors have addressed my concerns through rewriting of the manuscript, which has become more coherent, with mouse and human parts now fitting much better together. I only have two remaining comments/suggestions, which can be mostly addressed through editorial changes, but which are important for the overall interpretation of the manuscript:

1. The paper is not sufficiently clear on whether anti-PD1 affects injury (which promotes HCC) and thereby might overshadow its anti-tumor effects when given as “therapeutic” approach in NASH-HCC mice at later stages (13 months). The authors state author state that there is more pronounced liver damage - but show unaltered NAS score and decreased serum ALT in the anti-PD1-treated group in Extended Data 2F, i.e. no increase in damage. This data is in fact supporting a failure of checkpoint inhibitors in a therapeutic setting, and not just an increased injury (which is likely not occurring in most patients receiving checkpoint inhibitor therapy) overshadowing anti-tumor immunity in this mouse model. This would in the end be more similar to what is seen in patients and should be clearly pointed out in the results and discussion (and the statement on damage should be corrected). As there is more fibrosis, which is often the result of more injury but could also be the result of more inflammation in response to checkpoint inhibitors, the authors might want to additional check injury by TUNEL staining to be 100% sure.

We thank Reviewer #1 for his positive and constructive input and his help to make this paper more conclusive and focused. We agree with the point of Referee#1 that a failure of checkpoint inhibitors is strengthened by the reduced liver damage. Indeed, we did not observe an increase in liver damage (by Cl. Casp 3 staining) upon anti-PD1 treatment - corroborating the argument of Referee#1. We have now underlined this in the text.

1b. The authors have shown several additional mouse models in Extended Data 3, in which which anti-PD1 was effective, i.e. different from the NASH model - but the comparison is not fair as none of these mouse models had a chronic injury component. Fortunately, a recent paper addressed this point (Chung et al, PMID: 32839204), showing decreased HCC in a DEN+CCI4 HCC model when anti-PD1 was given in a preventative manner from week 10-20. This paper should be cited and discussed. The discussion of the Chung et al paper in the context of PD1 inhibitor-mediated NASH injury promotion and lack of therapeutic effects is important and will further improve the paper, providing more evidence that these are NASH-specific phenomena.

We thank Reviewer #1 for this comment. We have now cited the manuscript by Chung et al., and have discussed it in the main text.

**Referee #2**

The authors have improved the paper significantly, with better explanations, fewer overstatements, and more focus.

We thank Reviewer #2 for commenting that our manuscript has improved significantly.



A few final remarks:

-as far as I could see, the %age of TNF producing cells was not quantified, only images were shown (Figure 1n,o; Extended Data 2i-n).

We thank Referee #2 for the comment. We have highlighted the quantification in Figure 1n and added a quantification for former Figure 1o (which was now moved into the Extended data 2) - which is now also included in Extended data 2.

-same for TNF expression (Extended Data 5a-g).

TNF expression was quantified and is indicated in Extended data 5.