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## Supplemental information

# The immunotoxicity, but not anti-tumor efficacy, of anti-CD40 and anti-CD137 immunotherapies is dependent on the gut microbiota

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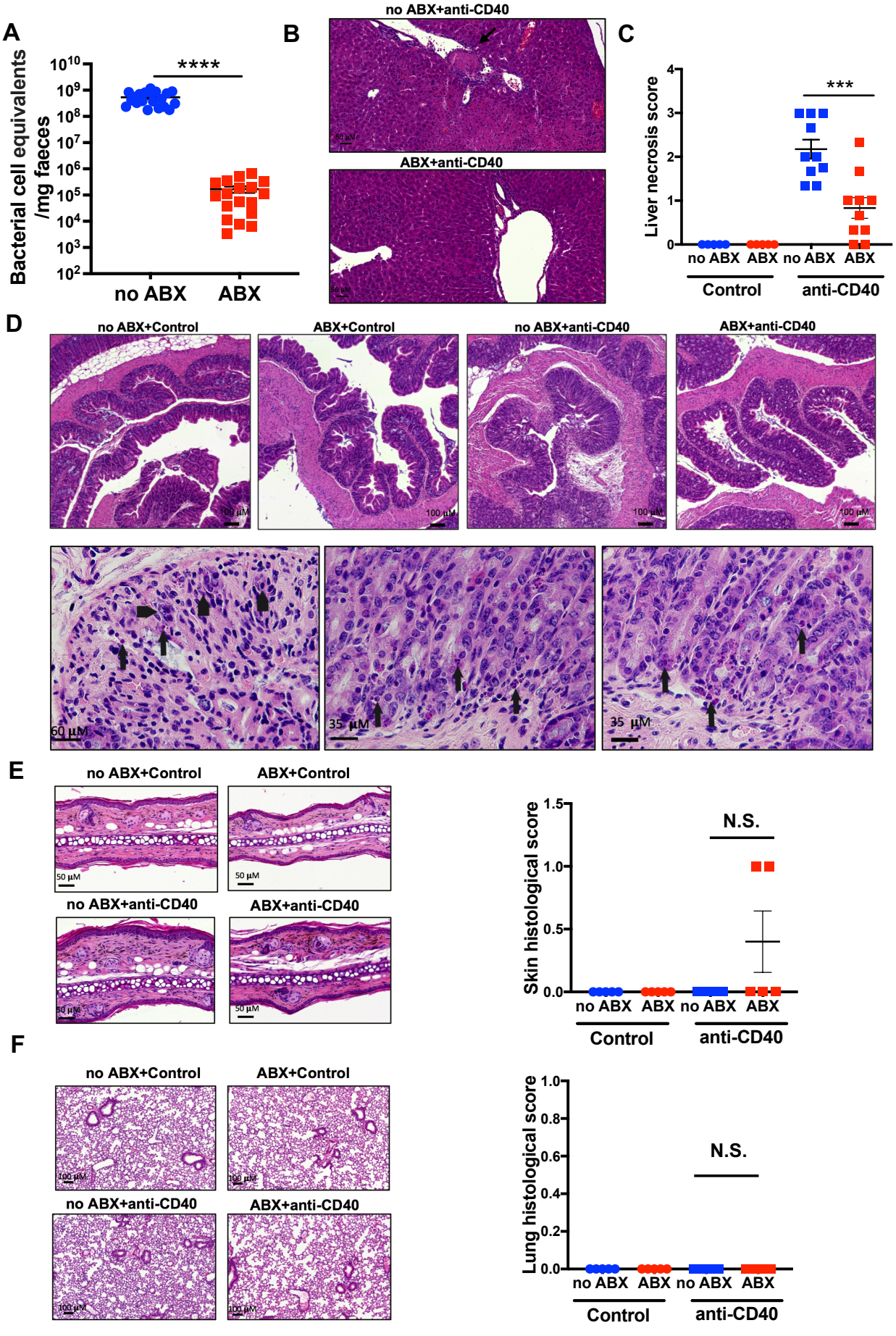
**SUPPLEMENTARY INFORMATION**

**SUPPLEMENTARY TABLES.**

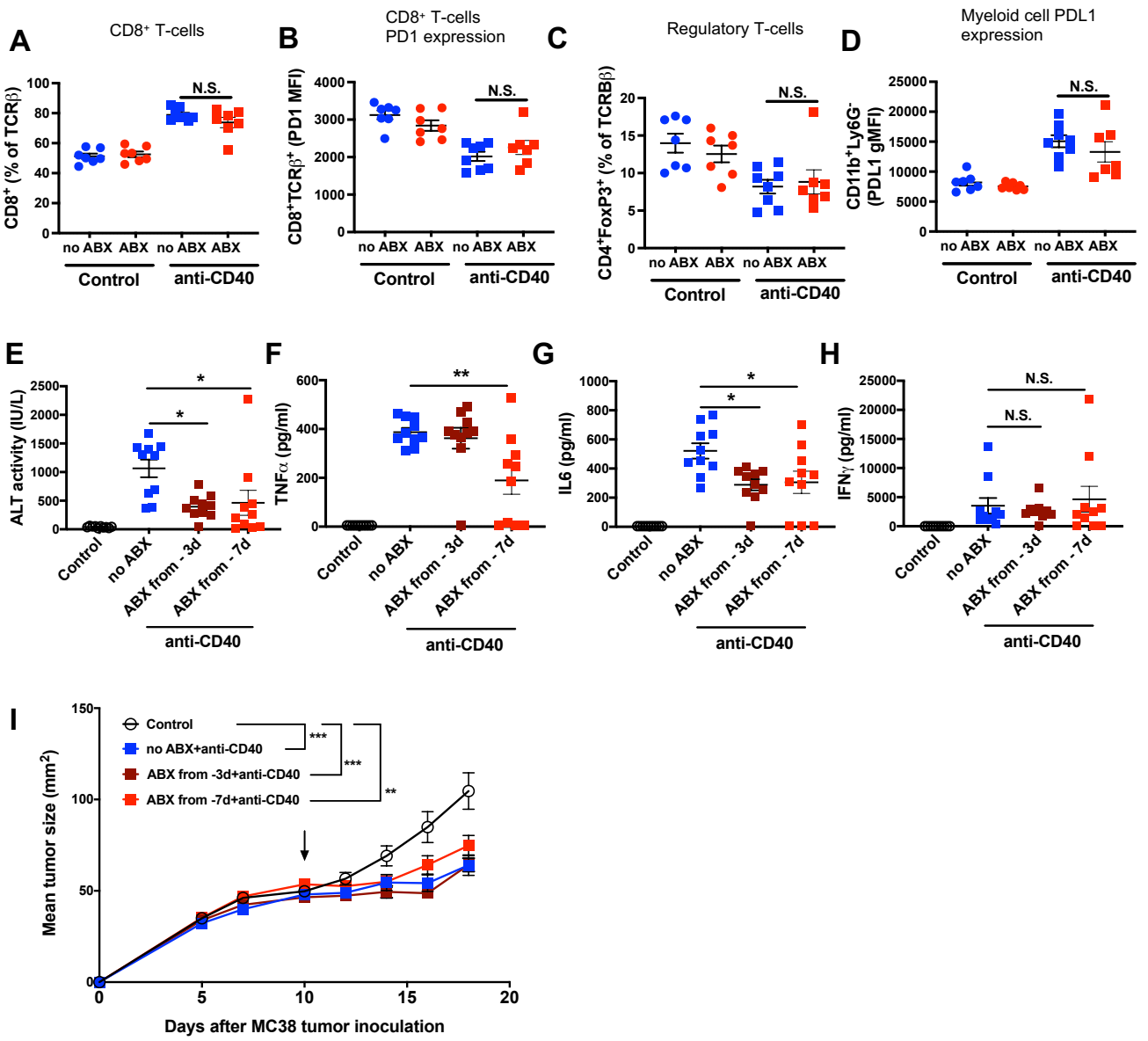
| Group | 16S rRNA copies/mg feces | Top hit in 16S rRNA database | Percent identity | Product length | Alignment length |
|---|---|---|---|---|---|
| GF+*Akkermanisa* cage #1 | 1.35E+07 | *Akkermansia muciniphilia* | 99.788 | 942 | 941 |
| GF+*Akkermansia* cage #2 | 1.51E+07 | *Akkermansia muciniphilia* | 99.046 | 943 | 940 |
| GF+*Enterobacter* cage#1 | 2.05E+08 | *Enterobacter cloace complex* | 99.787 | 941 | 940 |
| GF+*Enterobacter* cage#2 | 3.00E+08 | *Enterobacter cloace complex* | 99.787 | 940 | 939 |
| GF+*Clostridium* cage#1 | 3.29E+07 | *Clostridium scindens* | 99.6 | 973 | 932 |
| GF+*Clostridium* cage#2 | 2.69E+07 | *Clostridium scindens* | 99.6 | 972 | 932 |

**Supplementary Table 1. Confirmation of colonization of germ-free mice with bacterial monocultures presented in (Fig. 3K-M).** DNA was extracted from fecal samples pooled from co-housed mice. Colonisation with specific bacterial monocultures was confirmed by 16S rRNA gene qRT-PCR and SANGER sequencing of the 16S rRNA gene. Chromatograms were visually inspected to confirm that a single unique product was sequenced consistent with monocolonization. The species identity was assigned via a BLASTn search against the NCBI nr nucleotide database with top hit, percent alignment and product length shown.

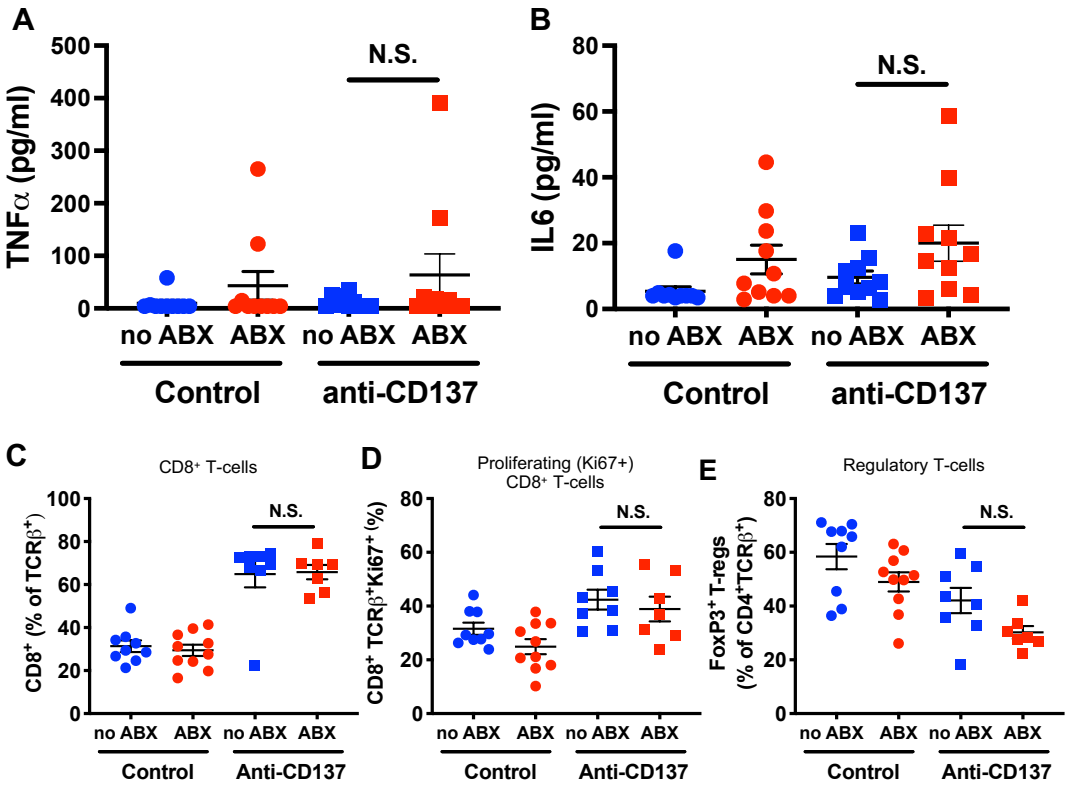
## SUPPLEMENTARY FIGURES



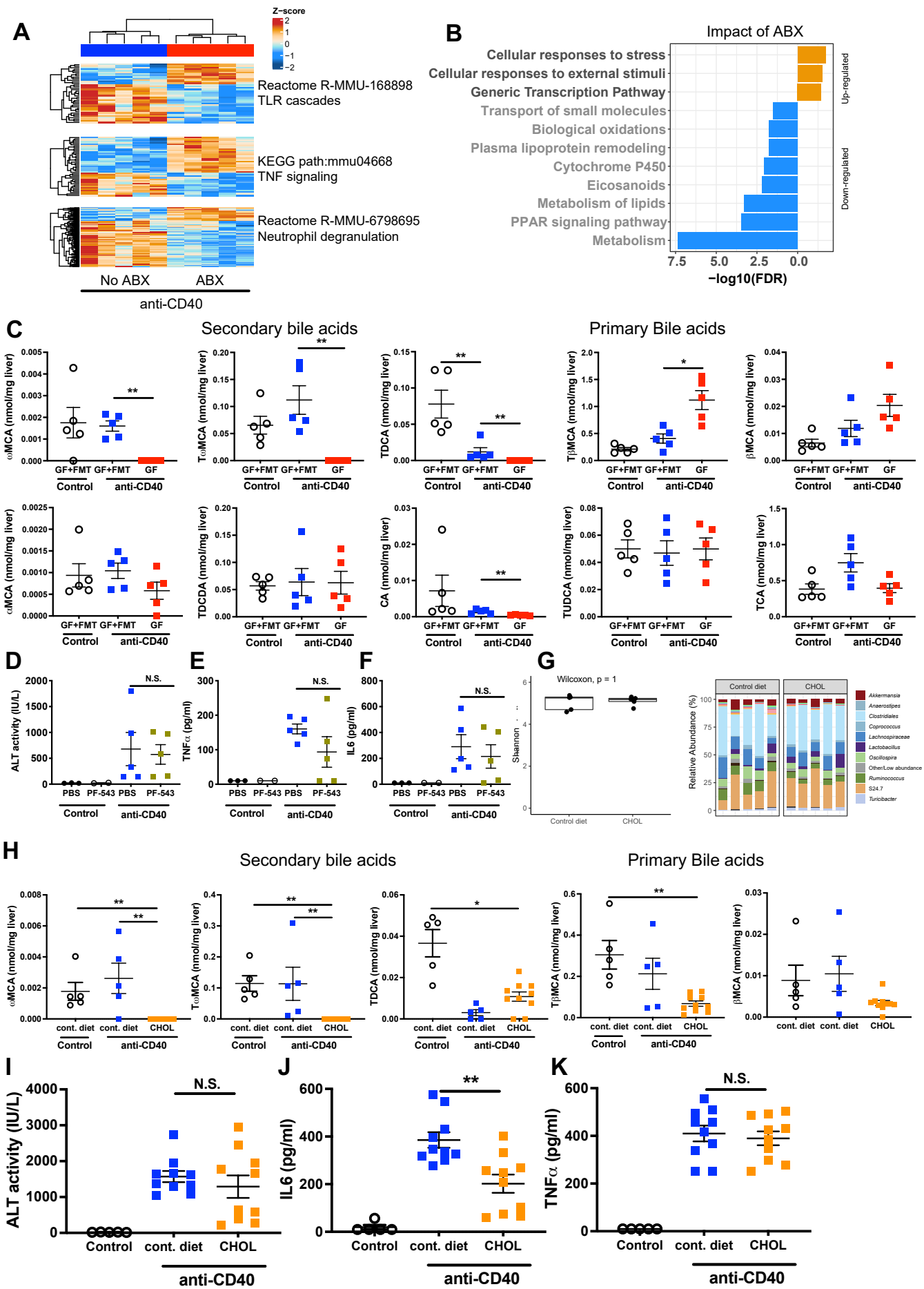
**Supplementary Figure 1. Impact of antibiotics on gut bacteria load and anti-CD40 induced damage to liver, colon, skin and lung. Related to Figure 1. (A)** Bacterial load in fecal samples collected from antibiotic treated (ABX) and untreated (no ABX) mice as determined by 16S rRNA gene qRT-PCR. **(B)** Representative H&E stained livers collected 24 hours after anti-CD40 (100μg i.p.) in no ABX and ABX mice demonstrating anti-CD40 induced liver necrosis and associated thrombi in liver portal vein (thrombi indicated by arrow) that were reduced in ABX mice. **(C)** Liver necrosis score in ABX and no ABX mice 24 hours after control (PBS) or anti-CD40 (100μg i.p.) treatment. **(D)** Representative H&E stained colon cross-sections from ABX and no ABX mice 24 hours after PBS or anti-CD40 treatment (top). (Bottom) Magnified areas of colon from no ABX mice at 24 hours after anti-CD40 treatment. Destruction and loss of colonic glands, with remnant damaged glands indicated by short thick arrows; apoptotic bodies are shown with long arrows. **(E)** Representative H&E stained skin cross-sections (left) and skin histological score (right) from ABX and no ABX mice 24 hours after PBS (control) or anti-CD40 treatment. **(F)** Representative H&E stained lung cross-sections (left) and lung histological score (right) from ABX and no ABX mice 24 hours after PBS (control) or anti-CD40 treatment. n=5-20 mice per group. Statistical significance was determined using a Mann-Whitney test. ***$P \leq 0.001$; ****$P \leq 0.0001$. Not significant (N.S.). Data are represented as mean ± SEM. Results shown are pooled from 3 independent experiments **(A)**, pooled from 2 independent experiment **(C)** or from single independent experiments **(B,D-F)**.



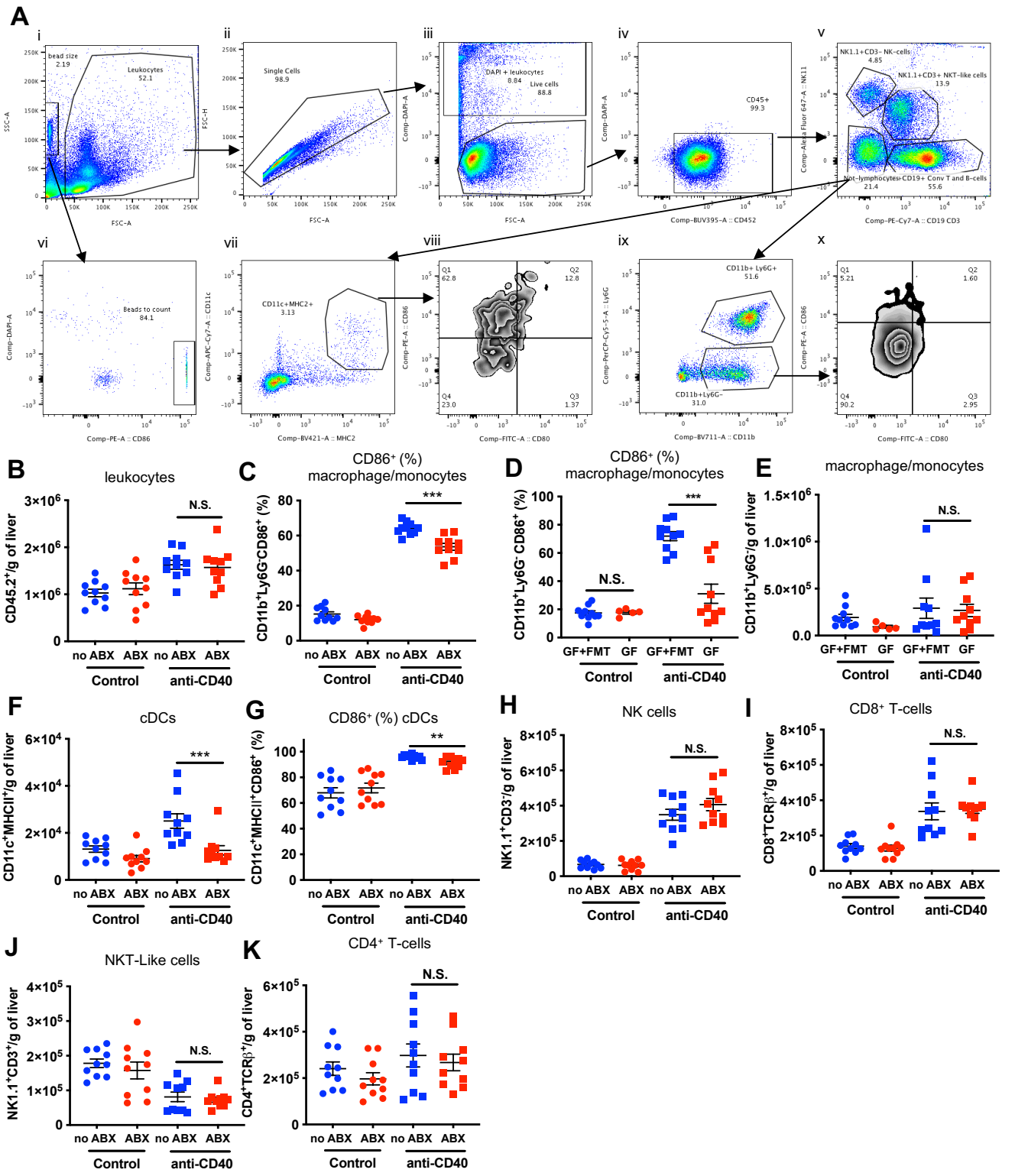

**Supplementary Figure 2. Impact of antibiotic treatment on anti-CD40 induced changes to tumor immune responses and immunotoxicity. Related to Figure 1. (A-D)** Antibiotic treated (ABX) or untreated (no ABX) mice were inoculated with MC38 tumors 2 weeks after the start of antibiotic/control treatment. Once tumors were established (~40-50mm$^2$) mice were treated with two doses of PBS (control) or anti-CD40 treatment (100µg i.p.) 4 days apart. 7 days after treatment initiation, tumors were removed and immune infiltration determined by flow cytometry. **(A)** Frequency of tumor infiltrating T-cells that were CD8$^+$, **(B)** expression of PD1 (gMFI) on CD8$^+$ T-cells, **(C)** expression of PDL1 (gMFI) on CD11b$^+$Ly6G$^-$ cells, **(D)** frequency of T-cells that were FoxP3$^+$ regulatory T-cells. Levels of **(E)** ALT, **(F)** TNFα, **(G)** IL6 and **(H)** IFNγ in serum collected 24 hours after control (PBS) or anti-CD40 treatment of control mice (no ABX) or in mice treated with antibiotics continuously via the drinking water, starting 3 or 7 days before administration of anti-CD40 or PBS. **(I)** MC38 tumor growth in ABX (starting day -3 or day -7) and no ABX mice injected i.p. every 4 days with 3 doses of PBS or anti-CD40 once tumors reached a size of ~40-50 mm$^2$. Treatment initiation indicated with an arrow. n=7-10 mice per group. Statistical significance was determined using a Mann-Whitney test **(A-D)** or one-way ANOVA with Dunnet's post-test analysis (final tumor sizes in each group analyzed) **(E-I)**. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Not significant (N.S.). Data are represented as mean ± SEM. Results shown are from independent single experiments.




**Supplementary Figure 3. Impact of antibiotic treatment on anti-CD137 induced changes to serum cytokines and tumor immune cells. Related to Figure 2.** Serum levels of **(A)** TNFα and **(B)** IL6 in serum assessed at 11 days after initiation of control (PBS) or anti-CD137 (100μg i.p., 3 doses 4 days apart) treatment of antibiotic treated (ABX) or untreated (no ABX) mice. **(C)** Percentages of tumor infiltrating T-cells that were $CD8^+$, **(D)** percentage of $CD8^+$ T-cells that were proliferating ($Ki67^+$) and **(E)** frequency of $CD4^+$ T-cells that were $FoxP3^+$ regulatory T-cells, 11 days after initiation of PBS or anti-CD137 treatment. n=7-10 mice per group. Statistical significance was determined by a Mann-Whitney test. Not significant (N.S.). Data are represented as mean ± SEM. Results shown are from a single experiment **(A-B)** or pooled from 2 independent experiments **(C-E)**.

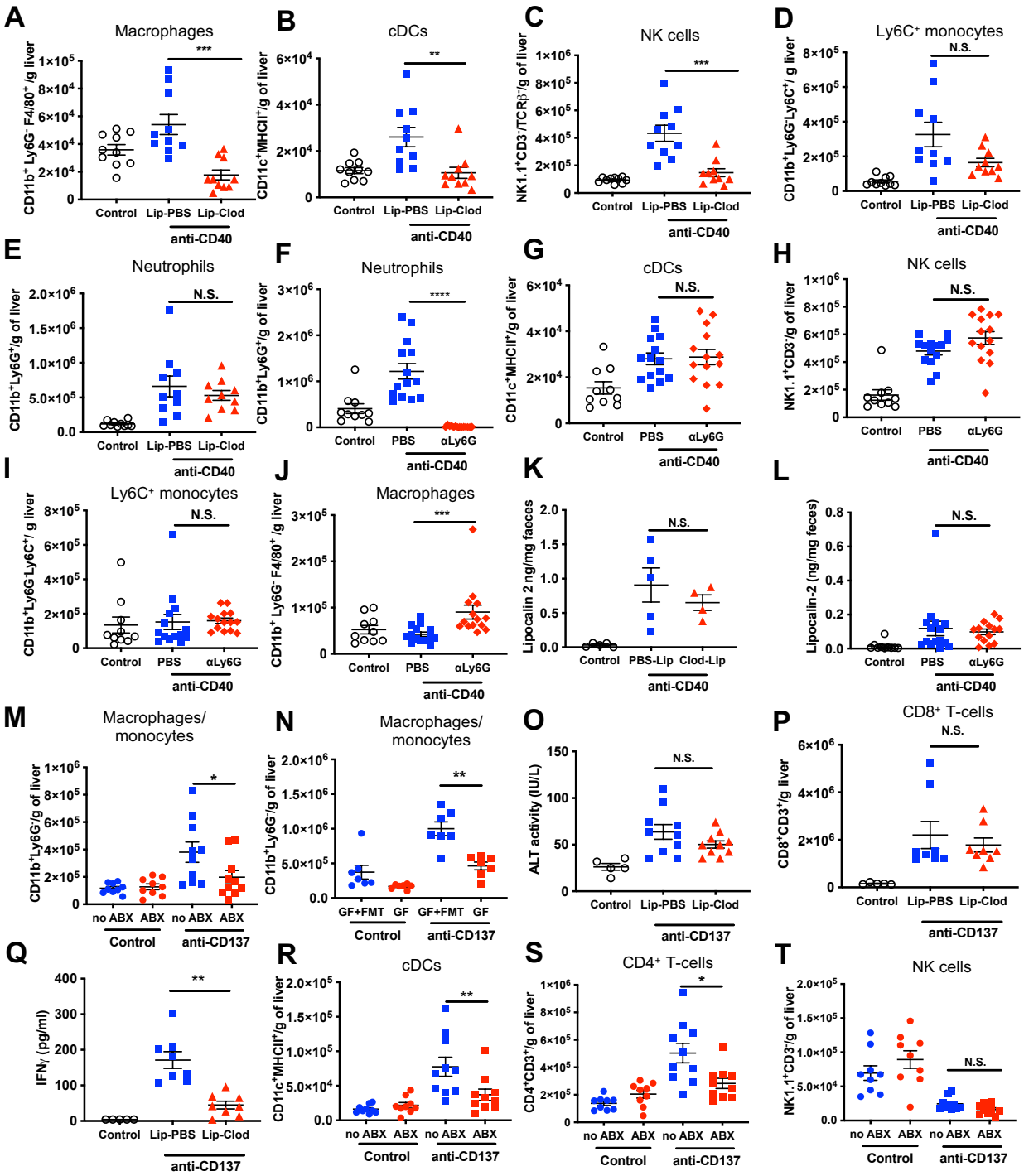

A
Z-score
Reactome R-MMU-168898
TLR cascades
KEGG path:mmu04668
TNF signaling
Reactome R-MMU-6798695
Neutrophil degranulation
No ABX
ABX
anti-CD40
B
Impact of ABX
Cellular responses to stress
Cellular responses to external stimuli
Generic Transcription Pathway
Transport of small molecules
Biological oxidations
Plasma lipoprotein remodeling
Cytochrome P450
Eicosanoids
Metabolism of lipids
PPAR signaling pathway
Metabolism
Up-regulated
Down-regulated
−log10(FDR)
C
Secondary bile acids
Primary Bile acids
ωMCA (nmol/mg liver)
TωMCA (nmol/mg liver)
TDCA (nmol/mg liver)
TβMCA (nmol/mg liver)
βMCA (nmol/mg liver)
αMCA (nmol/mg liver)
TDCDA (nmol/mg liver)
CA (nmol/mg liver)
TUDCA (nmol/mg liver)
TCA (nmol/mg liver)
GF+FMT GF+FMT GF
Control
anti-CD40
D
ALT activity (IU/L)
N.S.
PBS PF-543 PBS PF-543
E
TNFα (pg/ml)
F
IL6 (pg/ml)
G
Wilcoxon, p = 1
Shannon
Control diet
CHOL
Relative Abundance (%)
Akkermansia
Anaerostipes
Clostridiales
Coprococcus
Lachnospiraceae
Lactobacillus
Oscillospira
Other/Low abundance
Ruminococcus
S24.7
Turicibacter
H
Secondary bile acids
Primary Bile acids
cont. diet cont. diet CHOL
I
ALT activity (IU/L)
J
IL6 (pg/ml)
K
TNFα (pg/ml)
Control
cont. diet
CHOL
anti-CD40

**Supplementary Figure 4. The gut microbiota potently modulates liver lipid and bile acid pathways and bile acid sequestration modulates anti-CD40 induced CRS. Related to Figure 4.** RNA-Seq was used to profile gene expression in liver samples collected from untreated (no ABX) and antibiotic treated (ABX) mice, 24 hours after treatment with control (PBS) or anti-CD40 (100μg, i.p.). **(A)** Heatmap showing the expression of genes in 3 immune pathways of interest. Intensity represents the Z score of $\log_2$ library size normalized counts. The same heatmaps are also plotted individually in Fig. 5I, Fig. 6A and Fig. 7A. **(B)** Top pathways enriched among genes that were differentially expressed (FDR < 0.05) between no ABX and ABX mice (not anti-CD40 treated). Pathways which were increased in expression in ABX mice are indicated in orange, whereas those decreased in expression in ABX mice are shown in blue. **(C)** Levels of secondary bile acids; ωMCA (omega muricholic acid), TωMCA (tauro omega muricholic acid), TDCA (tauro deoxycholic acid), and primary bile acids; TβMCA (tauro beta muricholic acid), βMCA (beta muricholic acid), αMCA (alpha muricholic acid), TDCDA (tauro chenodeoxycholic acid), CA (cholic acid), TUDCA (tauro ursodeoxycholic acid), and TCA (tauro cholic acid) in the liver of germ-free (GF) and GF mice recolonized by a fecal microbiota transplant (GF+FMT) 24 hours after control (PBS) or anti-CD40 treatment (100μg i.p.). Levels of **(D)** ALT, **(F)** TNFα and **(G)** IL6 in serum 24 hours after treatment with control (PBS) or anti-CD40 (100μg i.p.) in mice injected i.p. daily, starting 2 days before anti-CD40/PBS treatment with control (PBS) or 100μg of sphingosine kinase 1 inhibitor (PF-543). (**G)** 16S rRNA gene sequencing was used to profile the composition of the fecal microbiota of SOPF mice treated for 7 days with a 2% cholestyramine diet (CHOL) or control. (**H)** Levels of secondary bile acids; ωMCA, TωMCA, TDCA or primary bile acids; TβMCA, βMCA in livers collected 24 hours after treatment with control or anti-CD40 in control diet (cont. diet) or CHOL treated mice. Levels of **(I)** ALT, **(J)** TNFα and **(K)** IL6 in serum collected 24 hours after treatment with control or anti-CD40 in cont. diet or CHOL treated mice. n=5-10 mice per group. Statistical significance was determined by Mann-Whitney test. *P ≤ 0.05; **P ≤ 0.01; Not significant (N.S.). Data are represented as mean ± SEM. Results shown are from single independent experiments.



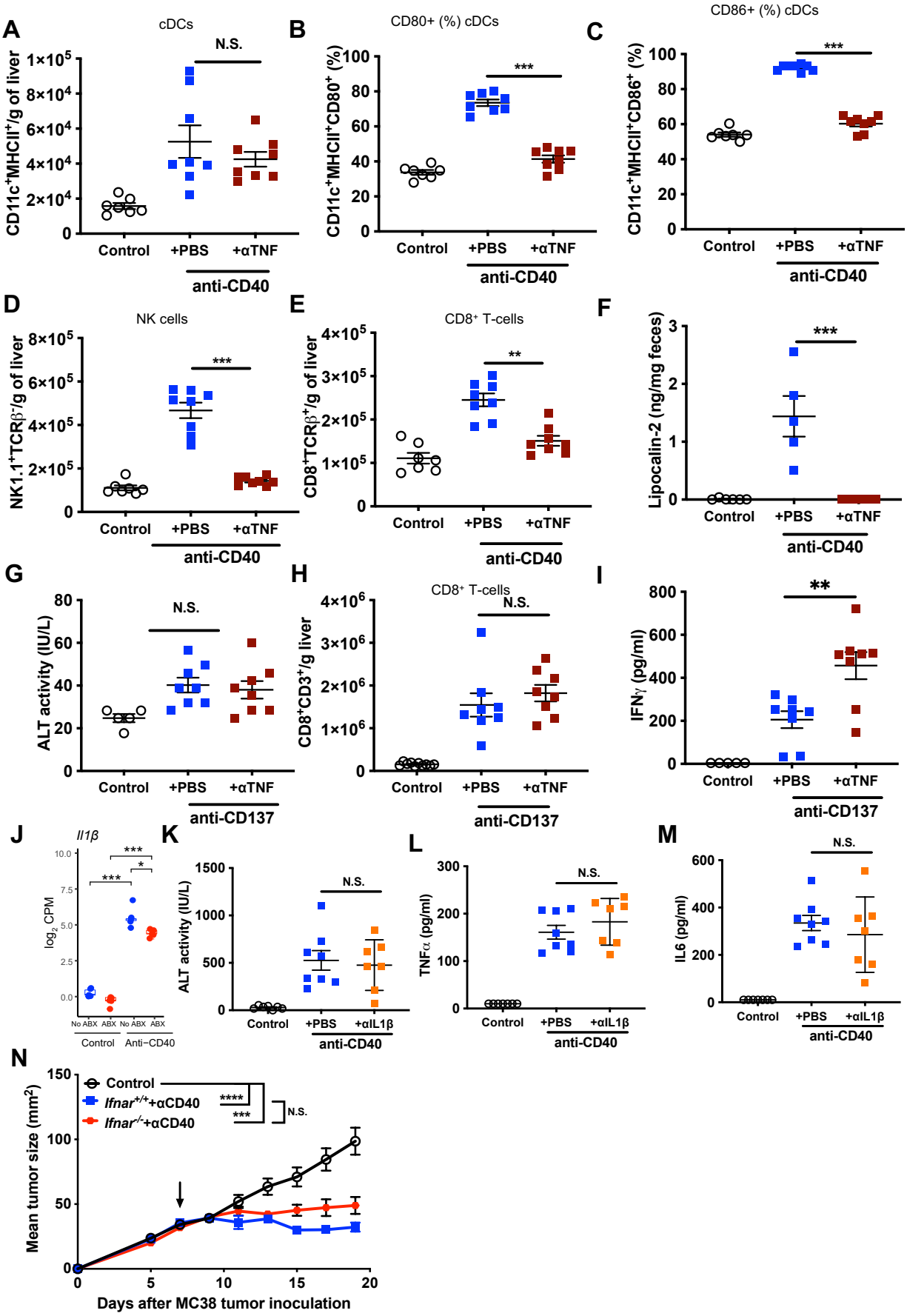

**Supplementary Figure 5. The gut microbiota modulate anti-CD40 induced immune infiltration and activation in the liver. Related to Figure 5.** **(A)** Flow cytometry gating strategy. Liver single cell suspensions (i) forward and side scatter, (ii) single cells, (iii) live cells gated as DAPI$^-$, (iv) leukocytes gated as CD45.2$^+$, (v) NK cells gated as NK1.1$^+$CD3$^-$, NKT-like cells gated as NK1.1$^+$CD3$^+$, non-lymphocyte cells gated as CD3$^-$CD19$^-$NK1.1$^-$, (vi) counting beads identified by tight fluorescence, (vii) cDCs gated as CD11c$^+$MHCII$^+$, (vii) cDC expression of activation markers CD80$^+$ and CD86$^+$, (ix) monocytes/macrophages gated as CD11b$^+$Ly6G$^-$ and neutrophils gated as CD11b$^+$Ly6G$^+$, (x) monocyte/macrophage expression of CD80$^+$ and CD86$^+$. **(B)** The number of leukocytes (CD45.2$^+$) per gram of liver 24 hours after control (PBS) or anti-CD40 treatment (100μg i.p.) in untreated (no ABX) or antibiotic treated (ABX) mice. Percentage of CD86$^+$ expressing

macrophages/monocytes ($CD11b^+Ly6G^-$) in **(C)** no ABX and ABX mice and **(D)** in germ-free (GF) and GF mice recolonized by a fecal microbiota transplant (GF+FMT) in the liver 24 hours after PBS or anti-CD40 treatment. **(E)** the number of macrophages/monocytes ($CD11b^+Ly6G^-$) per gram of liver 24 hours after control or anti-CD40 treatment in GF and GF+FMT mice. The number of **(F)** conventional DCs (cDCs; $CD11c^+MHCII^+$) and **(G)** frequency of cCDs expressing CD86 in the liver of no ABX and ABX mice 24 hours after control or anti-CD40 treatment. The number of **(H)** NK cells ($NK1.1^+CD3^-$), **(I)** $CD8^+$ T-cells ($CD8^+TCR\beta^+$), **(J)** NKT-like cells ($NK1.1^+CD3^+$) and **(K)** $CD4^+$ T cells ($CD4^+TCR\beta^+$) per gram of liver 24 hours after PBS or anti-CD40 treatment (100μg) in no ABX and ABX mice. n=5-10 mice per group. Statistical significance was determined by Mann-Whitney test. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. Not significant (N.S.). Data are represented as mean ± SEM. Results shown are pooled from 2 independent experiments.



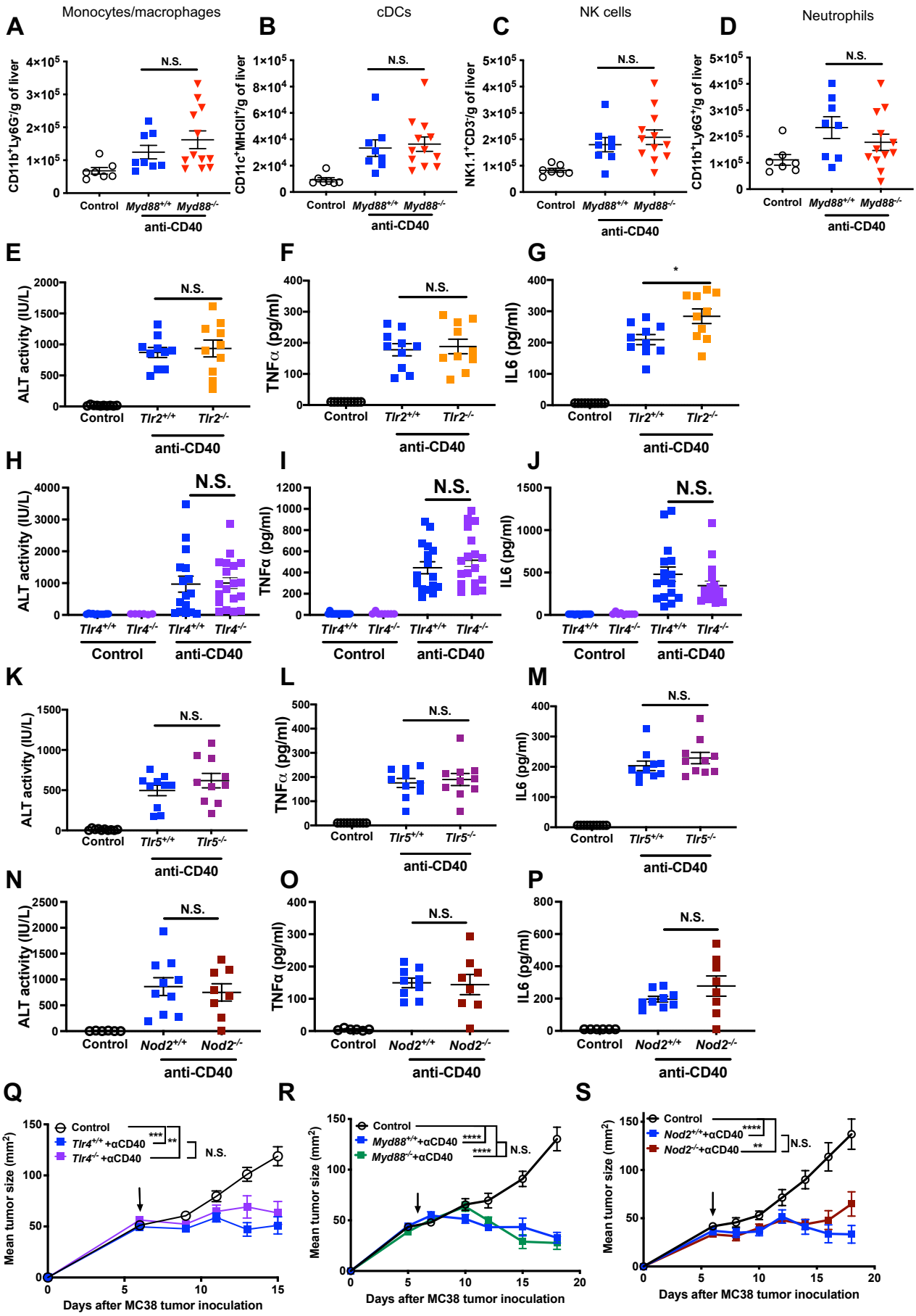

**Supplementary Figure 6. Impact of macrophage or neutrophil depletion on anti-CD40 and anti-CD137 induced toxicity and immune responses in liver. Related to Figure 5.** The number of **(A)** macrophages ($CD11b^+Ly6G^-F4/80^+$), **(B)** cDCs ($CD11c^+MHCII^+$), **(C)** NK cells ($NK1.1^+CD3^-/TCR\beta^-$), **(D)** inflammatory monocytes ($CD11b^+Ly6G^-Ly6C^+$) and **(E)** neutrophils ($CD11b^+Ly6G^+$) per gram of liver 24 hours after control (PBS) or anti-CD40 treatment (100µg i.p.) in control mice or mice treated with PBS loaded liposomes (Lip-PBS) or clodronate loaded liposomes (Lip-Clod) 24 hours prior to anti-CD40 treatment. The number of **(F)** neutrophils, **(G)** cDCs **(H)** NK cells ($NK1.1^+CD3^-$), **(I)** inflammatory monocytes and **(J)** macrophages per gram of liver 24 hours after control or anti-CD40 treatment of mice that were either treated with PBS or anti-Ly6G (500µg i.p.) 16 hours prior to anti-CD40 treatment. The number of macrophages/monocytes ($CD11b^+Ly6G^-$) per gram of liver from **(M)** untreated (no ABX) or antibiotic treated (ABX) mice and **(N)** germ-free (GF) and GF mice recolonized by a fecal microbiota transplant (GF+FMT) 11 days after treatment initiation with control or anti-CD137 (100µg i.p., 3 doses 4

days apart). (**O)** Serum ALT levels, **(P)** number of $CD8^+$ T-cells ($CD8^+CD3^+$) per gram of liver and **(Q)** serum IFNγ levels 11 days after treatment initiation with control or anti-CD137 (100μg i.p., 3 doses 4 days apart) in mice also treated with control (PBS), Lip-PBS or Lip-Clod 2 hours prior to anti-CD137 treatment initiation. Number of **(R)** cDCs, **(S)** $CD4^+$ T-cells ($CD4^+CD3^+$) and **(T)** NK cells per gram of liver 11 days after treatment initiation control or anti-CD137 (100μg i.p., 3 doses 4 days apart). n=5-14 mice per group. Statistical significance was determined using a Mann-Whitney. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Not significant (N.S.). Data are represented as mean ± SEM. Results shown are pooled from 2 independent experiments **(A-M, R-T)** or from a single experiment **(O-Q)**.




**Supplementary Figure 7. Impact of TNF, IL1β and IFNAR signalling blockade on anti-CD40 and anti-CD137 induced toxicity and immune responses in liver. Related to Figure 6. (A)** The number of cDCs ($CD11c^+MHCII^+$) per gram of liver and frequency of cDCs expressing **(B)** CD80 or **(C)** CD86, **(D)** number of NK cells ($NK1.1^+TCR\beta^-$), **(E)** number of $CD8^+$ T-

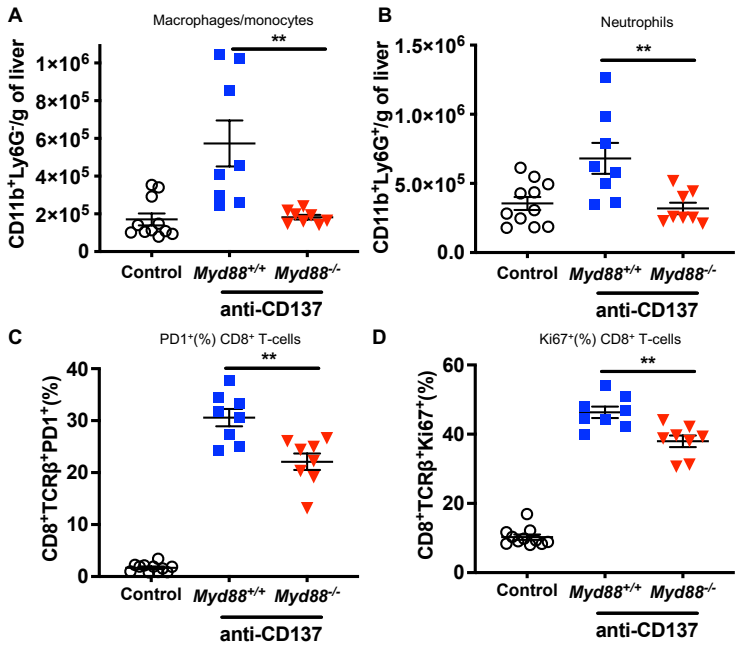
cells ($CD8^+TCR\beta^+$) per gram of liver and **(F)** fecal lipocalin-2 levels were determined 24 hours after treatment with control (PBS) or anti-CD40 (100μg i.p.). Indicated groups were also treated concurrently with PBS control or anti-TNF (200μg i.p.). **(G)** Serum ALT levels, **(H)** number of $CD8^+$ T-cells ($CD8^+CD3^+$) per gram of liver and **(I)** serum IFNγ 11 days after treatment initiation with control or anti-CD137 (100μg i.p., 3 doses 4 days apart). Indicated groups were also treated concurrently with PBS control or anti-TNF (200μg i.p., 3 doses 4 days apart). **(J)** *Il1β* normalized gene expression ($log_2$ count per million) in livers 24 hours after control or anti-CD40 treatment in untreated (no ABX) or antibiotic treated (ABX) mice. Levels of **(K)** ALT, **(L)** TNFα, and **(M)** IL6 in serum 24 hours after control or anti-CD40 treatment. Indicated groups were also treated concurrently with PBS control or anti-IL1β (200μg i.p.). Data from control and anti-CD40+PBS groups also plotted in **Fig. 6B-D**. **(N)** Growth of MC38 tumors in co-housed *Ifnar*$^{-/-}$ and wildtype C57BL/6 (*Ifnar*$^{+/+}$) mice that were injected i.p. every 4 days with 3 doses of PBS (control) or anti-CD40 (100μg i.p.). Treatment initiation indicated with an arrow. n=5-10 mice per group. Statistical significance was determined by Mann-Whitney test **(A-M)** or one-way ANOVA with Tukey's post-test analysis (final tumor sizes in each group analyzed) **(N)**. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Not significant (N.S). Data are represented as mean ± SEM. Results shown are from independent single experiments.




**Supplementary Figure 8. Responses to anti-CD40 in *Myd88*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr5*$^{-/-}$ and *Nod2*$^{-/-}$ mice. Related to Figure 7.**

The number of **(A)** monocytes/macrophages (CD11b$^{+}$Ly6G$^{-}$), **(B)** cDCs (CD11c$^{+}$MHCII$^{+}$), **(C)** NK cells (NK1.1$^{+}$CD3$^{-}$), and

**(D)** neutrophils **(**$CD11b^+Ly6G^+$) per gram of liver 24 hours after control (PBS) or anti-CD40 treatment of littermate *Myd88*$^{-/-}$ and wildtype (*Myd88*$^{+/+}$) mice. Levels of **(E)** ALT, **(F)** TNFα, and **(G)** IL6 in serum collected from co-housed *Tlr2*$^{-/-}$ and wildtype C57BL/6 (*Tlr2*$^{+/+}$) mice. Levels of **(H)** ALT, **(I)** TNFα, and **(J)** IL6 in serum collected from littermate *Tlr4*$^{-/-}$ and wildtype (*Tlr4*$^{+/+}$). Levels of **(K)** ALT, **(L)** TNFα, and **(M)** IL6 in serum collected from co-housed *Tlr5*$^{-/-}$ and wildtype C57BL/6 (*Tlr5*$^{+/+}$) mice. Levels of **(N)** ALT, **(O)** TNFα, and **(P)** IL6 in serum collected from co-housed *Nod2*$^{-/-}$ and wildtype C57BL/6 (*Nod2*$^{+/+}$) mice. Data in E-M 24 hours after control (PBS) or anti-CD40 treatment. Growth of MC38 tumors in **(Q)** littermate *Tlr4*$^{-/-}$ and wildtype (*Tlr4*$^{+/+}$) mice, **(R)** littermate *Myd88*$^{-/-}$ and wildtype (*Myd88*$^{-/-}$) mice or **(S)** co-housed *Nod2*$^{-/-}$ and wildtype C57BL/6 (*Nod2*$^{+/+}$) mice injected i.p. every 4 days with 3 doses of PBS (control) or anti-CD40 (100μg i.p.). Treatment initiation indicated with an arrow. n=5-19 mice per group. Statistical significance was determined by Mann-Whitney test **(A-P)** or one-way ANOVA with Tukey's post-test analysis (final tumor sizes in each group analyzed) **(Q-S)**. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Not significant (N.S). Data are represented as mean ± SEM. Results shown are from independent single experiments **(A-D, E-G, K-M, N-S)** or pooled from 2 independent experiments **(H-J)**.




**Supplementary Figure 9. Liver immune cell infiltration induced by anti-CD137 are significantly reduced in *Myd88*$^{-/-}$ mice. Related to Figure 7.**

Number of **(A)** macrophages and monocytes ($CD11b^+Ly6G^-$), **(B)** neutrophils ($CD11b^+Ly6G^+$) per gram of liver, **(C)** frequency of liver $CD8^+$ T-cells expressing PD1 and **(D)** frequency of liver $CD8^+$ T-cells expressing Ki67 determined in co-housed *Myd88*$^{-/-}$ and wildtype C57BL/6 (*Myd88*$^{+/+}$) mice, 11 days after initiation of control (PBS) or anti-CD137 treatment (100µg i.p. 3 doses 4 days apart). n=8-11 mice per group. Statistical significance was determined using a Mann-Whitney test. **P ≤ 0.01. Data are represented as mean ± SEM. Results shown are from a single experiment.