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Very Low Carbohydrate Diet Enhances Human T Cell Immunity through Immunometabolic Reprogramming

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

30th Apr 2021

Dear Dr. Kreth,

Thank you for the submission of your manuscript to EMBO Molecular Medicine, and please accept my apologies for the delay in getting back to you. We have received feedback from two of the three reviewers who agreed to evaluate your manuscript. Should referee #1 provide a report, we will send it to you, with the understanding that we will not ask for an additional revision. As you will see from the reports below, the referees acknowledge the interest of the study but also raise important and partially overlapping concerns that should be addressed in a major revision.

Further consideration of a revision that addresses reviewers' concerns in full will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. However, we realize that the current situation is exceptional on the account of the COVID-19/SARS-CoV-2 pandemic. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

***** Reviewer's comments *****

Referee #2 (Comments on Novelty/Model System for Author):

The authors present interesting findings. However, their in vitro data showing that BHB-induces an increase in ROS is inconsistent with findings in vivo showing that BHB reduces ROS. The author's in vivo data support their main conclusions, but parts of their in vitro data do not. They will need to address the discrepancies in their revised manuscript. It is unclear if the immunometabolic reprogramming seen in activated T cells in vitro, which involves aerobic glycolysis, is similar to the metabolic reprogramming that occurs for these cells in vivo, which involves OxPhos. The authors will need to address these discrepancies.

Referee #2 (Remarks for Author):

Summary
The authors present data suggesting that very low carbohydrate diets can improve human T cell immunity. In vitro and in vivo data are presented showing that BHB enhances CD4+, CD8+ and regulatory T cell capacity and augmented T memory cell formation. Their RNAseq and functional metabolic analyses showed that BHB-induced immunometabolic reprogramming favoring mitochondrial oxidative metabolism. The BHB-enhanced increase in oxygen consumption did not occur at the expense of glycolytic capacity in either activated primary T cells or in activated T cells that were isolated from human subjects treated with the KD. The author's results emphasize the value of nutrition and dietary interventions in modern medicine. While the in vivo data support the authors conclusions, there are several anomalies associated with the in vitro data that will require reevaluation.

Major Comments
1. The authors present evidence showing that the BHB-enhanced increase in oxygen consumption did not occur at the expense of glycolytic capacity in either activated primary T cells, or in activated T cells isolated from human subjects treated with the KD. The authors state that "Upregulation of mitochondrial oxidative phosphorylation was not on the cost of glycolysis but on top, as we observed unchanged glycolytic capacity of human KD T cells". It is known that immune cells, activated in vitro, will generate energy through aerobic fermentation (glycolysis) with elevated lactate production (doi: 10.1074/jbc.M114.551051; doi:10.1126/sciimmunol.aas9822). This is a unique feature of the in vitro environment that does not occur in activated T cells in vivo (doi:10.1126/sciimmunol.aas9822). T cells can elicit an immune response independent of glycolysis or aerobic fermentation. The authors should also recognize that oxygen consumption is not always a marker for oxidative phosphorylation, especially when using the Seahorse instrument in cultured cells (https://doi.org/10.1016/j.isci.2020.101761). Aerobic fermentation together with oxygen consumption, is also seen in many proliferating cells that are grown in vitro, but is not seen in cells grown in vivo, as recently described (https://doi.org/10.1016/j.isci. 2020.101761). The authors will need to address these issues in citing references showing that OCR might not be indicative of oxidative phosphorylation when measured in cultured cells. They cannot therefore assume that OCR is synonyms with OxPhos. This should be mentioned.

2. The authors should consider the possibility that BHB might be increasing ATP synthesis through mitochondrial substrate level phosphorylation (mSLP) in the glutaminolysis pathway, as was recently presented (https://doi.org/10.1016/j.isci.2020.101761). The authors should also acknowledge this possibility.

3. It will be important for the authors to describe the BHB preparation they used in their experiments. Was this the D-BHB, the L-BHB, or a D/L racemic BHB? This is important, as only the D-BHB is produced naturally in vivo (doi:10.1016/j.plefa.2003.09.007).

4. The authors show that BHB increases ROS in the activated immune cells. This observation is not what is seen in intact tissue treated with D-BHB. The Veech group showed that D-BHB reduces ROS production by increasing the redox span of the CoQ couple (doi:10.1016/j.plefa.2003.09.007).

This increased redox span will reduce ROS production while increasing the efficiency of ATP hydrolysis (DOI 10.1002/iub.1627). The increased ROS seen in the in vitro-activated T cells is the result of the aerobic fermentation (Warburg effect) seen in these cells. Increased ROS is also seen in tumor cells, which use aerobic fermentation for ATP synthesis. The D'Agostino group showed that D-BHB will elevate ROS in tumor cells, but not in normal cells, which use OxPhos for ATP synthesis (DOI 10.1186/s12986-017-0178-2). The author's results in the activated T cells would be more in line with what is seen in tumor cells that also express aerobic fermentation than in normal cells that use OxPhos for ATP synthesis. The authors will need to address these issues, as the mechanisms by which BHB influences energy metabolism can be different between the in vitro and the in vivo environments.
5. The data in Table 1 show that BHB levels increased in blood during fasting. What was the effect of the fasting on blood glucose? The authors should discuss their findings in light of previous findings (PMCID: PMC2194504).

Referee #3 (Comments on Novelty/Model System for Author):

A dose response (in some of) the in vitro assays determine BHB effects on T cell cultures is recommended.

Referee #3 (Remarks for Author):

The paper by Hirschberger et al. deals with the effects of ketone bodies on human T lymphocytes, as well as with the effects of ketogenic diet in volunteers on T cell responses. Altogether, this is an interesting study, and the results are presented in a convincing and logical fashion. The authors might consider improving their paper in the following points:

Are the results obtained in Fig. 1 to 3 dose-dependent? The authors culture cells in the absence or presence (10 mM) of beta-hydroxybutyrate (BHB). What is the minimum concentration of BHB to obtain such effects? This appears important because ketogenic diet led to plasma BHB levels in the range of 1 to 2 mM, which is 5 to 10 times less than what the authors evaluated in vitro.

Would this perhaps explain why the in vitro results are somehow discrepant from the in vivo resuts. For example, in vitro TBET is downregulated by BHB while GATA3 is upregulated, while in vivo the ketogenic diet does not affet TBET but upregulates GATA3.

Fig.3A+B. Please indicate what was measured (CellROX dye and MitoSox) instead of FITC and PE. This remark applies to the rest of the figure where FITC measurements are indicated in an appropriate and inacurate fashion.

Would it make sense to calculate ratios such as Treg cells among total CD4 T cells to understand the net effects of BHB and the ketogenic diet?

In the Introduction or in the Discussion, the authors should cite a recent paper by Ferrere G et al.(JCI Insight. 2021 Jan 25;6(2):145207) showing that ketogenic diet and ketone bodies enhance anticancer immune responses in mice. Indeed, the statement " Adaptive immunity, however, has not been addressed so far..." in the Introduction is not correct.

RESPONSE TO REVIEWERS

Referee #2

Major Comments

1. The authors present evidence showing that the BHB-enhanced increase in oxygen consumption did not occur at the expense of glycolytic capacity in either activated primary T cells, or in activated T cells isolated from human subjects treated with the KD. The authors state that "Upregulation of mitochondrial oxidative phosphorylation was not on the cost of glycolysis but on top, as we observed unchanged glycolytic capacity of human KD T cells". It is known that immune cells, activated in vitro, will generate energy through aerobic fermentation (glycolysis) with elevated lactate production (doi: 10.1074/jbc.M114.551051; doi:10.1126/sciimmunol.aas9822). This is a unique feature of the in vitro environment that does not occur in activated T cells in vivo (doi:10.1126/sciimmunol.aas9822). T cells can elicit an immune response independent of glycolysis or aerobic fermentation.

Glucose is commonly known as essential substrate for T cell function (Chapman et al, 2020; Peng et al, 2016), and multiple studies have emphasized the importance of aerobic glycolysis for activated T cells both in vitro and in vivo (Michalek et al, 2011; Macintyre et al, 2014; Chang et al, 2013; Buck et al, 2016). Moreover, T cells also gain energy by other pathways such as OXPHOS, glutaminolysis and the pentose phosphate pathway (Wang & Green, 2012). Major metabolic differences, however, are not detected in in vitro cultivated versus in vivo primary T cells, but occur upon activation and differentiation both in vitro and in vivo (Ma et al, 2019; Klarquist et al, 2018; Tan et al, 2017; Tarasenko et al, 2017). ***We now have discussed this issue on page 14, last paragraph.***

The authors should also recognize that oxygen consumption is not always a marker for oxidative phosphorylation, especially when using the Seahorse instrument in cultured cells (https://doi.org/10.1016/j.isci.2020.101761). Aerobic fermentation together with oxygen consumption, is also seen in many proliferating cells that are grown in vitro, but is not seen in cells grown in vivo, as recently described (https://doi.org/10.1016/j.isci. 2020.101761). The authors will need to address these issues in citing references showing that OCR might not be indicative of oxidative phosphorylation when measured in cultured cells. They cannot therefore assume that OCR is synonyms with OxPhos. This should be mentioned.

We agree that OCR measured via Seahorse is not necessarily indicative of OXPHOS which particularly concerns cultured cancer cells, which can cover parts of their energy demands by mitochondrial substrate level phosphorylation (mSLP) linked ATP production through glutaminolysis (Ramanathan et al, 2005; Seyfried et al, 2017). The resulting succinate,

however, is not further processed in the TCA cycle but secreted due to a reduced Succinate Dehydrogenase (SDH) activity thus leading to ATP synthesis without increasing oxygen consumption (Seyfried et al, 2017, 2020). Conversely, T cells are capable of oxidatively metabolizing succinate thus producing further ATP by OXPHOS. Glutamine-derived ATP generated via oxidative phosphorylation (including mSLP) can be detected via OCR. Only mSLP-derived ATP generated during fermentation of glutamine is masked in Seahorse analysis (Seyfried, 2012). Thus, in the context of this study - using primary human T cells - the potential error in estimating ATP through Seahorse analysis can be considered far lower than compared to tumor cell culture. However, detailed analysis of ATP delivering pathways affected by KD requires further investigation. ***As suggested by the reviewer, we have added these important considerations to our manuscript on page 14, last paragraph and page 15, first paragraph.***

2. The authors should consider the possibility that BHB might be increasing ATP synthesis through mitochondrial substrate level phosphorylation (mSLP) in the glutaminolysis pathway, as was recently presented (https://doi.org/10.1016/j.isci.2020.101761). The authors should also acknowledge this possibility.

Thank you for this interesting comment. Indeed, T cells are known to perform glutaminolysis and to rely on glutamine for immune function (Wang et al, 2011; Wang & Green, 2012). Upon T cell activation, complex changes to substrate utilization occur (Ma et al, 2019). We agree that BHB might impact this complex process, due to altered substrate availability, negative feedback and/or allosteric regulation. For example, the activity of glutamate dehydrogenase is controlled through sirt4, which is inhibited by fasting and low blood glucose, thereby increasing glutaminolysis (Li et al, 2011; Min et al, 2018). The precise effects of KD on glutaminolysis and mSLP in human KD T-cells, however, require further investigation. ***As suggested, we now discuss this point in our manuscript on page 14, last paragraph and page 15, first paragraph.***

3. It will be important for the authors to describe the BHB preparation they used in their experiments. Was this the D-BHB, the L-BHB, or a D/L racemic BHB? This is important, as only the D-BHB is produced naturally in vivo (doi:10.1016/j.plefa.2003.09.007).

In our in vitro experiments, racemic D/L BHB has been used. As noted, only D-BHB is produced in vivo and is the only enantiomer that serves as a substrate for BHB dehydrogenase (Puchalska & Crawford, 2017). We performed in vitro dose finding experiments to determine the optimal BHB concentration for T cell culture (added to Appendix Figure S1). During a very short term BHB incubation of 48 hours, only 10mM (racemic) BHB displayed enhanced T cell immune capacity. In regard to these findings, we used 10mM D/L BHB in vitro, which is equivalent to approximately 5mM metabolically active D-BHB. This is in the range of endogenous levels of ketone bodies being maximally reached by a ketogenic diet, yet for only a very short incubation period without re-supplementation.

The use of racemic BHB and the dose finding experiments performed have been added to our manuscript (results, page 5, first paragraph | Appendix Figure S1).

4. The authors show that BHB increases ROS in the activated immune cells. This observation is not what is seen in intact tissue treated with D-BHB. The Veech group showed that D-BHB reduces ROS production by increasing the redox span of the CoQ couple (doi:10.1016/j.plefa.2003.09.007). This increased redox span will reduce ROS production while increasing the efficiency of ATP hydrolysis (DOI 10.1002/iub.1627). The increased ROS seen in the in vitro-activated T cells is the result of the aerobic fermentation (Warburg effect) seen in these cells. Increased ROS is also seen in tumor cells, which use aerobic fermentation for ATP synthesis. The D'Agostino group showed that D-BHB will elevate ROS in tumor cells, but not in normal cells, which use OxPhos for ATP synthesis (DOI 10.1186/s12986-017-0178-2). The author's results in the activated T cells would be more in line with what is seen in tumor cells that also express aerobic fermentation than in normal cells that use OxPhos for ATP synthesis. The authors will need to address these issues, as the mechanisms by which BHB influences energy metabolism can be different between the in vitro and the in vivo environments.

The role of ROS in T cell immunity is exceptional and markedly differs from tissues and particularly from cancer cells: ETC-derived ROS serve as a second messenger during T cell activation (Murphy & Siegel, 2013; Franchina et al, 2018), thus being considered pivotal for T cell immunity (Devadas et al, 2002; Jones et al, 2007; Sena et al, 2013). Of note, T cells diverge from NADPH-dependent GSH synthesis, insteading redirecting NADPH for ROS

production via NADPH oxidase to fulfill their demand on reactive oxygen species (Jackson et al, 2004; Kwon et al, 2010). These findings gave rise to the concept of mitohormesis, opposing the idea of ROS as solely detrimental byproducts of an imperfect oxidative system, but emphasizing the role of ROS as essential signaling molecules (Ristow, 2014). We found a balanced and mild increase of mitochondrial ROS in response to KD in activated human T cells, both in vitro and in vivo, which did not compromise cellular anti-oxidative systems. This augmentation of T cell second messengers may thus be contributing to enhanced T cell immune capacity on a KD.

In other words, in metabolic therapy of cancer (as described by Seyfried et al.), the different effects of BHB-induced elevated ROS on tumor cells and T cells might even work in the same direction: While oxidative stress compromises cancer cell viability, mild increase of ROS enhances T cell immune capacity which in turn further restrains tumor growth.

We have discussed these interesting points on page 15, second paragraph and page 16, last paragraph.

5. The data in Table 1 show that BHB levels increased in blood during fasting. What was the effect of the fasting on blood glucose? The authors should discuss their findings in light of previous findings (PMCID: PMC2194504).

Fasting blood glucose concentrations were also measured and are now depicted in Table 1 and Figure EV3b, as requested. No significant changes in blood glucose concentrations could be detected, which is in line with previous data and probably the result of constant hepatic gluconeogenesis (Cahill & Veech, 2003). However, significant effects of KD on blood glucose are likely to occur over longer KD periods.

We have added these considerations to our manuscript (Table 1, Figure EV3b | discussion, page 14, second paragraph).

Referee #3

1. Are the results obtained in Fig. 1 to 3 dose-dependent? The authors culture cells in the absence or presence (10 mM) of beta-hydroxybutyrate (BHB). What is the minimum concentration of BHB to obtain such effects? This appears important because ketogenic diet led to plasma BHB levels in the range of 1 to 2 mM, which is 5 to 10 times less than what the authors evaluated in vitro.

Thank you for this important remark. As we used racemic BHB, 10mM refers to only 5mM metabolically active D-BHB. This concentration was used due to dose finding experiments, showing superior results in contrast to lower BHB concentrations. 5mM D-BHB are considered a near-maximum blood ketone body concentration in humans on a KD. Since our in vitro results cover only a very short time frame of 48 hours, this high concentration was chosen. In vivo, far lower concentrations over a far longer time period have been analyzed. Whether low concentrations of D-BHB in vitro, cultured over a longer time period may result in similar immunometabolic alterations requires further investigation.

We have added the dose finding experiments (flow cytometry and qRT-PCR) to our manuscript (results page 5, first paragraph | Appendix Figure S1).

2. Would this perhaps explain why the in vitro results are somehow discrepant from the in vivo results. For example, in vitro TBET is downregulated by BHB while GATA3 is upregulated, while in vivo the ketogenic diet does not affect TBET but upregulates GATA3.

In our dose finding experiments, no significant alteration of Tbet/GATA3 was detected in response to varying concentrations of BHB. Of note, the ratio of Tbet/Gata3 mRNA expression displays a similar decline in BHB/NC in vitro and T1/T0 in vivo, resulting in an overweight of Th2 transcription factor expression under both conditions. The observed differences in isolated mRNA expression levels thus might rather be due to different time periods in vivo and in vitro.

We have added this finding to our manuscript (results page 5, first paragraph, and page 11, first paragraph | Appendix Figure S1 + Appendix Figure S2).

3. Fig.3A+B. Please indicate what was measured (CellROX dye and MitoSox) instead of FITC and PE. This remark applies to the rest of the figure where FITC measurements are indicated in an appropriate and inacurate fashion.

Thank you for this important remark.

As suggested, we have replaced FITC/ PE labeling with CellROX/ MitoSOX/ JC1/ ThioTracker throughout the manuscript to clearly indicate the measurement and to improve the clarity of the figures.

4. Would it make sense to calculate ratios such as Treg cells among total CD4 T cells to understand the net effects of BHB and the ketogenic diet?

We agree that the net effect of ketone bodies is best visible if regulatory T cells are calculated in regard to total CD4. Of note, in vivo Treg abundance is comparable to other studies (Churlaud et al, 2015; Rodríguez-Perea et al, 2015; Rueda et al, 2013).

Flow cytometry data of in vitro differentiated Treg (Figure 1) and in vivo Treg abundance (Figure 5) are depicted as % CD4+CD25+FoxP3+ of total CD4+ cells. Axis labeling and Figure captions have been updated accordingly.

5. In the Introduction or in the Discussion, the authors should cite a recent paper by Ferrere G et al.(JCI Insight. 2021 Jan 25;6(2):145207) showing that ketogenic diet and ketone bodies enhance anticancer immune responses in mice. Indeed, the statement " Adaptive immunity, however, has not been addressed so far..." in the Introduction is not correct.

We appreciate this important paper, as it consolidates the emerging evidence that KD impacts on T-cell immunity.

We have added the reference to our discussion (page 16, last paragraph) and we have updated the respective part of our introduction (page 3, second paragraph) as requested.

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1st Revision - Editorial Decision 27th May 2021

27th May 2021

Dear Prof. Kreth,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Figures: The number of datapoints plotted in the panels appears to differ from the 'n' in the legend in Figures 1A-G, 2B/C, 3D/F/J/K, 5A/C-F, 6C/D/G/H, EV1A, EV2A/B/G, EV4F/H-J/L/M. Please revise and correct.
2) In the main manuscript file, please do the following:
- Correct/answer the track changes suggested by our data editors by working from the attached/uploaded document.
- Remove font colour.
- Fig EV4I and J are called out as Fig EVI and J, please correct.
- In M&M, provide the antibody dilutions that were used for each antibody.
- In M&M, a statistical paragraph that should reflect all information that you have filled in the Authors Checklist, especially regarding randomization, blinding, replication.
- Please merge "Funding" section with "Acknowledgements".
- Add author contributions for Tingting Wu.
- Rename "Competing interest" to "Conflict of interest".
- Move "Author contributions", Acknowledgment", "Conflict of interest" and "Data availability" sections after Materials and Methods.
- Move Table 1, main Figure and EV Figure legends to the end of the manuscript. Remove Appendix table of content.
- Please use the following format to report the accession number of your data:

The datasets produced in this study are available in the following databases:
[data type]: [full name of the resource] [accession number/identifier] ([doi or URL or identifiers.org/DATABASE:ACCESSION])

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6) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts. This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication. Please note that the Authors checklist will be published at the end of the RPF.
7) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic

***** Reviewer's comments *****

Referee #2 (Remarks for Author):

The authors have done a good job in addressing previous concerns.

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 2nd Jun 2021

We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Simone Kreth, Simon Hirschberger
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2021-14323

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Power analysis was performed based on in vitro results. Interferon γ expression T1/T0 was designated as the primary study endpoint. A sample size of n=40 was calculated presuming an estimated effect size (Cohen's d) of 0.5, a level of significance α=0.05, a drop-out rate of 20% and a power level of 80%.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All healthy volunteers performed the diet, T cells were analyzed prior to start (T0) and at the end of the diet (T1). Thus, no randomization was necessary.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was performed since there have been control and intervention data from each study participant
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Statistical analysis was performed using GraphPad Prism 7.03 (GraphPad Software, Inc., USA). Paired t-test or Wilcoxon matched-pairs signed rank test, as appropriate, served for comparisons. Normal distribution was tested using D'Agostino & Pearson test and Shapiro Wilk test.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analysis was performed using GraphPad Prism 7.03 (GraphPad Software, Inc., USA). Paired t-test or Wilcoxon matched-pairs signed rank test, as appropriate, served for comparisons. Normal distribution was tested using D'Agostino & Pearson test and Shapiro Wilk test.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Estimate of variations has been performed using GraphPad Prism 7.03. Variation is represented as median, twenty-fifth and seventy-fifth percentiles and range, including dots indicating individual values.
Is the variance similar between the groups that are being statistically compared?	Yes. In case of non-similarity, Welch's correction has been applied.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies including catalogue number are depicted in the methods and materials section of the manuscript. Validation data and respective publications for each antibody are accessible through the manufacturer.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The study design and the study protocol were approved by the Institutional Ethics Committee of the Ludwig-Maximilian-University Munich, Germany (No. 19-523)
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Informed consent was obtained from all volunteers. Research was performed according to the Declaration of Helsinki (ethical principles for medical research involving human subjects) and the U.S. Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	The study was registered at the DKRS (German Clinical Trials Register; DRKS-ID: DRKS00023373).
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The accession number for the RNAseq analysis of CD4+/CD8+ T cells prior and post KD reported in this paper is GSE158407.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA