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Branched-chain ketoacids secreted by glioblastoma cells via MCT1 modulate macrophage phenotype

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Transaction Report:

(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 March 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, both referees indicate that the data on BCKA transport by MCT proteins represents only a minor advance, since it has been reported before. On the other hand, the experimental part showing an effect on phagocytosis is currently very preliminary. Referee 1 suggests several experiments how this part could be strengthened and referee 2 indicated upon further discussion that s/he would be supportive of publication in EMBO reports if this part was strengthened.

From the referee comments it is clear that, as it stands, the study does not represent a sufficiently striking conceptual advance to consider its publication in our journal. However,
given the potential interest of the findings on phagocytosis, I would like to give you the opportunity to revise your study and to strengthen the part on how BCKA modulates phagocytosis by tumor-associated macrophages. Please address this point and also the other referee concerns in your revision and supply a complete point-by-point response.

I would of course understand if you prefer to seek rapid publication elsewhere, but should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to

allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section can stay as it is now. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

REFEREE REPORTS

Referee #1:

This manuscript, "MCT1-mediated excretion of glioblastoma cell branched-chain ketoacids modulates macrophage phagocytosis" by Santos Silva et al. demonstrates that the branched-chain ketoacid (BCKA) derivatives of branched-chain amino acids (BCAA) are being secreted from glioblastoma cell lines. They further demonstrate that MCT1 and MCT4 enhance BCKA secretion from Xenopus oocyte and that inhibition of MCT1 but not MCT4 decreases BCKA secretion from various glioblastoma cell lines. MCT1 and MCT4 localize subcellularly in proximity to BCAT1, suggesting potential substrate channeling between enzyme and transporter. Lastly, the authors show that exogenously added BCKAs suppress phagocytic activity of macrophages.

Metabolic features of cancer cells and in particular metabolic crosstalk between tumor cells and non-transformed cells in the tumor microenvironment are an area of active research. BCKA generation by various cancer types and transport of some BCKA species by MCT proteins have been demonstrated previously. The results of this study consolidate previous findings, but are a rather modest advance over the current knowledge. The data are of overall good quality and the combination of Xenopus oocytes and cultured mammalian cells as experimental systems is appreciated. Modulation of macrophage phagocytosis by tumor cell-secreted BCKA is an interesting concept that could prove important for the understanding of the glioblastoma microenvironment. However, the part on macrophage modulation is underdeveloped and further experiments are required to justify its emphasis in both title and abstract.

Major points:

1. Figure 7D, E: Do BCKAs at 300 µM specifically suppress phagocytosis or influence macrophage function more broadly? This issue is of particular relevance because BCKAs have been shown to exert various cytotoxic effects. How does exogenous addition of BCKAs at such levels affect proliferation and viability of glioblastoma cells?

2. Figure 7D, E: The authors could greatly strengthen their conclusion about the immunosuppressive effects of BCKAs by demonstrating that BCKAs are secreted by glioblastoma cells at sufficient levels to suppress phagocytosis. This can easily be tested e.g. by co-culture of glioblastoma cells with macrophages or treatment of macrophages with glioblastoma cell-conditioned media.

Minor points:

1. For clarity of language, increased dependence of cancer cells on amino acid metabolism is not part of the Warburg effect, but another metabolic trait observed in many cancer types (introduction, first paragraph).

2. Along the same lines, pyruvate is contrasted with alpha-ketoacids. However, pyruvate is an alpha-ketoacid (introduction, second page, first paragraph).

3. Figure 4: It would be good to confirm the phenotypes of pharmacological MCT1 inhibition with genetic loss of function experiments.

3. Figures 4, 5: Does pharmacological/genetic perturbation of MCT1 or MCT4 affect BCAT1 expression levels?

4. Figure 6: To put the finding that BCAT1 colocalizes with MCT proteins into context, it would be interesting to know where LDH localizes. Can the preference of MCT1 and MCT4 for BCKAs and lactate, respectively, be explained by preferential co-localization with BCAT1 and LDH?

5. Figure 7A-C: The authors report that macrophages do not take up BCKAs. Do macrophages under identical conditions cause a measurable depletion of BCAAs (or other nutrients) in the medium? How do MCT1 and MCT4 levels compare between macrophages and glioblastoma cell lines?

Referee #2:

This study demonstrates the release of BCKA from glioma cells, which may affect phagocytosis of stroma macrophages. The title is misleading as the effect on phagocytosis is hardly investigated at all. The study appears incomplete at this stage, while much space is given to already established data.
Credit should be given where due. BCKA transport via MCT1 and MCT4 has been reported by Broer et al. 1998 (Biochemical Journal) and Dimmer et al., Dimmer et al., 2000; Tr

1st Revision - authors' response 20 June 2017

To both reviewers

We would like to thank the reviewers for their insightful comments which we have tried to address to the fullest.

To strengthen the part of the manuscript dealing with the effects of (tumor-derived) BCKAs on macrophages as requested by both reviewers and the editor, we repeated the ^{13}C tracing experiments and extended the analyses of both data sets. In this new analysis we detected labeling of the BCAAs (which we had not analyzed before) in macrophages in both experiments. The data show that, in contrast to our original conclusions, macrophages actually do take up BCKAs and reaminate them to BCAAs. Consistent with this finding BCKA-treated macrophages reduced their consumption of

BCAAs from the media. In addition, the macrophages also reduced their uptake of pyruvate and hexoses, but did not show any signs of reduced viability. Altogether these new data show that uptake of BCKAs is associated with changes in macrophage metabolism which might be affect their phagocytic capacity. To our knowledge, uptake of BCKAs by macrophages is described here for the first time.
We hypothesize that this BCKA uptake had not been detected in our UPLC analysis of macrophage culture media (Figures 7A-C in the manuscript originally submitted) due to concurrent excretion of unlabeled, BCAA-derived BCKAs by the macrophages that compensated for the uptake of labeled BCKAs. Because of this complication, we decided to no longer include the data on determination of BCKAs in supernatants from BCKA-treated macrophages in the new version of the manuscript. Instead, we can now unequivocally demonstrating BCKA uptake based on our ^{13}C tracing data.

The reviewers of course are correct that it already is known that KIC and KIV can be transported by MCT1 and MCT4. Following the suggestion of Reviewer 2, we now are citing the original research articles instead of review articles referring to them. Furthermore, we have moved the *Xenopus* data to the supplementary material and made clear how our analysis approach and method differs from the one originally published. First, we're using direct determination of the concentrations of all three BCKAs, rather than indirectly inferring BCKA transport from changes in intracellular pH caused by influx of protons co-transported with BCKAs. Second, in the heterologous expression in *Xenopus* oocytes, we have modeled the situation in tumor cells, by measuring the concentrations of BCKAs that were generated by BCAT1 inside the oocytes and excreted to the media by either MCT1 or MCT4. The respective revisions in the new version of the manuscript are detailed in the point by point response to the reviewers. Further, we would like to stress that our data on glioblastoma cells show that MCT1 mainly is responsible for BCKA excretion. This conclusion could not have been derived from the *Xenopus* data and demonstrates that it is important to study the function of the MCTs in glioblastoma cells.

By measuring BCKA excretion from mammary carcinoma cells, we have strengthened our hypothesis that the phenotype of MCT1 knockdown reported by others might be due not only to the reduction of the excretion of pyruvate but also of BCKA excretion. This is the first time that excretion of BCKAs from glioblastoma and mammary carcinoma cells was demonstrated.

In the text of the revised manuscript we have colored in green the changes regarding the major points pointed out by Reviewer 1 as well as the comments of Reviewer 2 and in blue the changes regarding minor points pointed out by Reviewer 1.

Reviewer 1

Major points

1. Figure 7D, E: Do BCKAs at 300 µM specifically suppress phagocytosis or influence macrophage function more broadly? This issue is of particular relevance because BCKAs have been shown to exert various cytotoxic effects. How does exogenous addition of BCKAs at such levels affect proliferation and viability of glioblastoma cells?

The exogenous addition of 100 µM or 300 µM BCKAs to glioblastoma cells do not affect cell proliferation assessed by Click-iT proliferation assay or cell viability assessed by propidium iodide staining followed by flow cytometric analysis. These new data are now presented in **Figure EV2** and the first paragraph of the Results section.
To analyze if BCKAs exert cytotoxic effects on macrophages we performed a viability assay using propidium iodide staining and analysis of positive (non-viable) and negative (viable) cells using flow cytometry. We observed that exogenous addition of 300 µM BCKAs to macrophages did not affect the percentage of viable cells. These novel data are included in **Figure EV5A** and on page 18 of the main text. Proliferation was not tested since differentiated macrophages do not proliferate *in vitro*.

2. Figure 7D, E: The authors could greatly strengthen their conclusion about the immunosuppressive effects of BCKAs by demonstrating that BCKAs are secreted by glioblastoma cells at sufficient levels to suppress phagocytosis. This can easily be tested e.g. by co-culture of

glioblastoma cells with macrophages or treatment of macrophages with glioblastoma cell-conditioned media.

The levels of BCKAs that were used in the uptake and phagocytosis assays were based on our initial observation that cultured glioblastoma cells can excrete large amounts of BCKAs (accumulating to close to 100 µM in the cell culture media) in a short time (24 h) (see **Figure 1**). Considering that (1) per cell the volume of media in cell culture is considerably larger than the volume of the extracellular space in the tissue, and (2) BCKA concentrations of up to 4.6 mM (more than 10-fold higher than what we used) have been observed in the serum of MSUD patients, the BCKA concentrations we used probably are well within the physiologic range for glioblastoma. Furthermore, we now show that the BCKA concentrations we used do not negatively impact survival or proliferation of macrophages or tumor cells **(Figures EV2 and EV5A)** and page 18. We intentionally did not use a co-culture system since glioblastoma and other tumor cells are excreting many cytokines and other soluble factors that can affect the phenotype of neighboring cells. It was our intention to focus our study on the effect of BCKAs. Co-culture therefore was not a suitable experimental approach.

Minor points:

1. For clarity of language, increased dependence of cancer cells on amino acid metabolism is not part of the Warburg effect, but another metabolic trait observed in many cancer types (introduction, first paragraph).
We rephrased the first paragraph of the introduction. It now reads: "Rapidly proliferating types of cancers have been shown to exhibit characteristic alterations of metabolism including a shift away from oxidative phosphorylation and towards aerobic glycolysis, which is known as the "Warburg effect", and an increased dependence on amino acid metabolism [1, 2]."

2. Along the same lines, pyruvate is contrasted with alpha-ketoacids. However, pyruvate is an alpha-ketoacid (introduction, second page, first paragraph).

Thank you for pointing out this mistake. We changed the sentence to: "In the cancer field, studies so far have mostly focused on the role of MCTs in the transport of lactate and pyruvate, but others have reported that MCTs also can mediate the influx of other hydroxy and ketoacids into Xenopus oocytes [20-22] and that an MCT protein is required for the transport of α ketoisocaproate (KIC) in neurons [23]." (Introduction, top of page 4).

3. Figure 4: It would be good to confirm the phenotypes of pharmacological MCT1 inhibition with genetic loss of function experiments.
The siRNA-mediated knockdown of MCT1 confirmed the decrease in BCKAs excretion in glioblastoma cells observed using MCT1 inhibitor AR-C155858. These new data are shown in **Figure EV3** and referred to in the main text at the top of page 17.

3. Figures 4, 5: Does pharmacological/genetic perturbation of MCT1 or MCT4 affect BCAT1 expression levels?
The pharmacological as well as genetic perturbation of MCT1 and MCT4 did not lead to consistent downregulation of *BCAT1* expression suggesting that the observed reductions in BCKA excretions are not due to reduced BCKA production but indeed are reflecting reduced transmembrane transport. These data are shown in **Appendix Figure S3**, and referred to on page 17.

4. Figure 6: To put the finding that BCAT1 co-localizes with MCT proteins into context, it would be interesting to know where LDH localizes. Can the preference of MCT1 and MCT4 for BCKAs and lactate, respectively, be explained by preferential co-localization with BCAT1 and LDH?
Thank you for pointing out this additional mechanism of regulating transport specificity. We re-examined our data and indeed found that BCAT1 was significantly more often associated with MCT1 than with MCT4 in the U87-MG and U251-MG cell lines. This analysis is now reported in the context of **Figure 5** (page 18, first paragraph). In addition, we now also performed PLA of MCT1 or MCT4 and LDHA, the enzyme that generates lactate **(Appendix Figure S7)**. These new data (described in the Discussion section near the bottom of page 21) showed that LDHA was significantly more often associated with MCT1 than with MCT4 in U87-MG cells. Suggesting that there is no clear preferential co-localization of LDHA with MCT4 in U87-MG cells. In U251-MG

cells, which do not express LDHA, we tested for, but could not detect any preferential association of LDHB with either of the MCTs. We conclude that the selectivity of MCT1 for BCKAs might partly be explained by the preferential association of BCAT1 and MCT1 as detected by PLA. We do not have any evidence for a similar mechanism regulating lactate excretion.

5. Figure 7A-C: The authors report that macrophages do not take up BCKAs. Do macrophages under identical conditions cause a measurable depletion of BCAAs (or other nutrients) in the medium? How do MCT1 and MCT4 levels compare between macrophages and glioblastoma cell lines?

We did additional analyses to address the reviewer's comment. Macrophages cultured without BCKAs indeed are depleting BCAAs, indicating that they are metabolically active. Interestingly, addition of BCKAs to the media leads to uptake and amination of BCKAs (as described in the response to the reviewers) and decreased consumption of BCAAs by the macrophages without affecting their viability. Consumptions of pyruvate and hexoses were reduced, as well. These data suggest that exposures of macrophages to BCKAs initiate alterations of their metabolism. The novel data are provided in **Figure EV5B and EV5C** and discussed on pages 18 and 19.
Macrophages either differentiated with M-CSF or glioblastoma-conditioned medium express similar or higher levels of MCT1 and MCT4 compared to U87 cells. These data indicate that MCT expression is not limiting transport. The data are now shown in **Appendix Figure S5** (top of page 19).

Reviewer 2

The title is misleading as the effect on phagocytosis is hardly investigated at all.
We rephrased the title and it now reads: "MCT1-mediated excretion of glioblastoma cell branched-chain ketoacids modulates macrophage phenotype".
The study appears incomplete at this stage, while much space is given to already established data.
In the revised version of the manuscript we have tried to better explain the added value of our data on BCKA transport in *Xenopus* oocytes and glioblastoma cells. Corresponding text can be found in the Results section near the bottom of page 15 and on page 16, and in the Discussion section on page 20. For additional details please see section addressed to both reviewers at the beginning of this letter.

Credit should be given where due. BCKA transport via MCT1 and MCT4 has been reported by Broer et al. 1998 (Biochemical Journal) and Dimmer et al., Dimmer et al., 2000; Transfer of nitrogen between glutamate/glutamine and BCKA/BCAA was proposed by Yudkoff et al., 1994 J. Neurochem.
Thank you for pointing out our mistake. Instead of citing more recent review articles we are now citing the original research as you suggested. These citations can be found in the following places: introduction near the bottom of page 3, and on page 4; results, page 15, last paragraph; discussion, first paragraph; discussion, pages 20 and 22.

2nd Editorial Decision 24 July 2017

Thank you for the submission of your revised manuscript to our editorial offices. Martina is currently on vacation, thus I handle the manuscript for the time being. We have now received the report from the referee that was asked to re-evaluate your study (you will find enclosed below). As you will see, the referee supports the publication of your manuscript in EMBO reports. However, s/he has still a major and a minor concern we ask you to address in a final revised version.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORT

Referee #1:

The authors have submitted a revised manuscript that has addressed the majority of the reviewers' suggestions. However, there remains a concern about the modulation of macrophage phagocytosis by tumor cell-secreted BCKAs. The authors argue that the media volume in cell culture is substantially larger than the volume of pericellular fluids in tissue. While this is correct, BCAA concentrations are vastly higher in cell culture than in vivo, which provides BCAT1 with supraphysiological substrate levels. BCKA could thus actually accumulate to higher concentrations in culture media. The reference to MSUD is misleading in this context. This disease is caused by mutations in the BCKA dehydrogenase complex, which catalyzes an irreversible step downstream of BCAT1, which differs from the situation in glioblastoma overexpressing BCAT1. Extracellular BCKA levels in tumors are thus unknown and it is unclear how they relate to those in tissue culture models. It would strengthen the manuscript if a demonstration that a biological source can produce sufficient BCKAs levels to inhibit macrophage phagocytosis was documented. The author's point that co-culture experiments can be difficult to interpret is taken. However, the confounding issue of cytokine production can be circumvented by using heat-inactivated conditioned media. At the very least, the authors should demonstrate that BCKAs over a range of concentrations inhibits phagocytosis, including 100 µM, which is the concentration measured in glioblastoma culture supernatants.

Minor Point: The measurements of nutrient consumption by macrophages treated with BCKAs in the revised manuscript are seemingly unrelated to the phagocytosis inhibition but interesting. Unfortunately, glutamine is missing from this analysis. This nutrient would be particularly instructive, because BCKA uptake might concomitantly increase glutamine consumption as a nitrogen source for the transamination reaction to BCAAs. This might also shed light on the decreased consumption of pyruvate and hexoses. I am surprised that the authors detect succinate uptake - DMEM normally does not contain this metabolite.

2nd Revision - authors' response 21 August 2017

EMBOR-2017-44154V3

Point-by-point responses to reviewers

Reviewer 1
(emphasis added by corresponding author B.R.)

The authors have submitted a revised manuscript that has addressed the majority of the reviewers' suggestions. However, there remains a concern about the modulation of macrophage phagocytosis by tumor cell-secreted BCKAs. The authors argue that the media volume in cell culture is substantially larger than the volume of pericellular fluids in tissue. While this is correct, BCAA concentrations are vastly higher in cell culture than in vivo, which provides BCAT1 with supraphysiological substrate levels. BCKA could thus actually accumulate to higher concentrations in culture media. ***The reference to MSUD is misleading in this context.*** *This disease is caused by mutations in the BCKA dehydrogenase complex, which catalyzes an irreversible step downstream of BCAT1, which differs from the situation in glioblastoma overexpressing BCAT1.* ***Extracellular BCKA levels in tumors are thus unknown and it is unclear how they relate to those in tissue culture models****.*

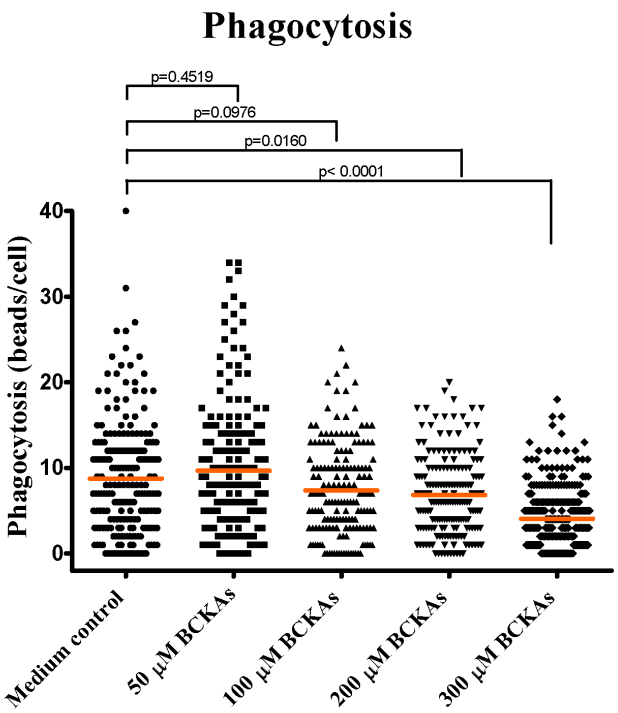
Glioblastoma and MSUD share the characteristic feature of increased cellular BCKA excretion due to aberrant BCKA metabolism. We do not find this comparison misleading. However, we do agree with the reviewer that it is difficult to compare extracellular volumes and BCAA concentration *in vitro* and *in vivo*. We therefore have modified the corresponding text in the discussion section (p10). It now reads:
"Our analysis showed that glioblastoma cells are excreting BCKAs, resulting in their accumulation to concentrations of up to 85 µM in the culture media within 24 hours. Extracellular BCKA levels in tumors are unknown but BCKA concentrations of 0.4-4.6 mM have been observed in patients with maple syrup urine disease (MSUD), a heritable defect of BCAA catabolism which is associated with increased cellular BCKA excretion [34]."

It would strengthen the manuscript if a demonstration that a biological source can produce sufficient BCKAs levels to inhibit macrophage phagocytosis was documented. The author's point that co-culture experiments can be difficult to interpret is taken. However, ***the confounding issue of cytokine production can be circumvented by using heat-inactivated conditioned media.***

We agree with the reviewer that heat treatment could inactivate cytokines; however, it would not alter concentrations of some tumor-excreted metabolites that could influence macrophage phenotype. Since we specifically wanted to study the effects of BCKAs on macrophage phenotype, we intentionally treated the macrophages by controlled supplementation of the media with BCKAs. Treatment with tumor-conditioned media (heat inactivated, or not), or co-culture with tumor cells would have exposed the macrophages to unwanted, undefined additional factors.

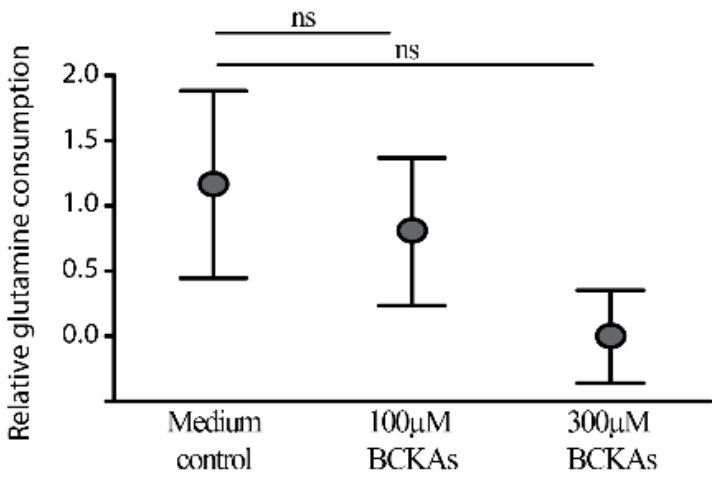
At the very least, the authors should demonstrate that BCKAs over a range of concentrations inhibits phagocytosis*, including 100 µM, which is the concentration measured in glioblastoma culture supernatants.*

As suggested by the reviewer, we now demonstrate that BCKAs inhibit phagocytosis in a concentration-dependent manner. **These new data were added in (Fig. 6D).** While there seems to be no effect at a concentration of 50 µM BCKAs, inhibition increases when further increasing BCKA concentration:
100µM: $p<0.1$; 200µM: $p<0.05$; 300µM; $p<0.0001$.



Minor Points:
The measurements of nutrient consumption by macrophages treated with BCKAs in the revised manuscript are seemingly unrelated to the phagocytosis inhibition but interesting. ***Unfortunately, glutamine is missing from this analysis.*** *This nutrient would be particularly instructive, because BCKA uptake might concomitantly increase glutamine consumption as a nitrogen source for the transamination reaction to BCAAs. This might also shed light on the decreased consumption of pyruvate and hexoses.*

We now also analyzed glutamine consumption. There was no significant change in glutamine consumption between control and BCKA-treated macrophages.



I am surprised that the authors detect succinate uptake - DMEM normally does not contain this metabolite.

We are sorry for the misunderstanding. The detected succinate likely is contained in the FCS supplement, not the DMEM. To make things more clear, we replaced the labels “DMEM” with “medium control” in Figure 6 and Figure EV5 and the corresponding figure legends.

3rd Editorial Decision 14 September 2017

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, also former referee 1 is now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- Please review the statistical analysis in your manuscript. The number of independent biological replicates has to be listed in all figure legends. Please note that it is not possible to calculate significance and mean values from technical replicates as in this case only the technical variability is measured but not the reproducibility of the observed effect in independent experiments. Please show the individual data points as scatter blots in case the number of independent experiments is smaller than 3 and remove the p-values.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORT

Referee #1:

The revised manuscript now satisfactorily addresses all of the concerns raised in the original review.

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: Bernhard Radlwimmer
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44154V2

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 justified
- 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?	experiments were always performed in technical triplicates and independently repeated 2 to 3 times
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.	NA
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.	in situ Proximity ligation assay and phagocytosis assay results were assessed in a blinded manner.
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?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes. Tested using graphpad prism software
Is there an estimate of variation within each group of data?	F test to compare variances
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum

http://datadryad.org

http://figshare.com

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	α-tubulin (clone DM1A, #T9026, Sigma-Aldrich), anti-MCT4 (sc50329, Santa Cruz), anti-MCT1 (AB3538P, Millipore), anti-BCAT1 (rabbit polyclonal, Insight Biotechnology limited (Wembley, UK)),anti-PGK1 (GTX107614, GeneTex), anti-LDHA (SAB1100050, Sigma), anti-LDHB (PA527505, Invitrogen), anti-MCT1 (ab90582, Abcam), anti-MCT4 (376140, Santa Cruz),HRP-conjugated to mouse IgG (#7076, Cell Signaling Technology), HRP-conjugated to rabbit IgG (#7074, Cell Signaling Technology)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Human cell lines used were recently authenticated using The Multiplex human Cell line Authentication Test (MCA).

* 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.	Xenopus laevis females were purchased from Xenopus Express. Segments of ovarian lobules were removed surgically under sterile conditions from frogs anesthetized with 1 g/L of 3-amino-benzoic acid ethyl ester and rendered hypothermic. Stage V Xenopus oocytes were used as described in methods section - Page 8
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The procedure was approved by the Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany (23 177–07/A07-2–003 §6) - Page 8
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.	NA
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.	NA
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.	NA
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	NA
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