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Triggering MSR1 promotes JNK-mediated inflammation in IL-4 activated macrophages

Manman Guo, Anetta Härtlova, Marek Gierliński, Alan Prescott, Josep Castellvi, Javier Hernandez Losa, Sine K. Petersen, Ulf A. Wenzel, Brian D. Dill, Christoph H. Emmerich, Santiago Ramon Y Cajal, David G. Russell and Matthias Trost

Review timeline:

Submission date:	1st Aug 2018
Editorial Decision:	24th Sep 2018
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Editorial Decision:	22nd Mar 2019
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Editor: Karin Dumstrei

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 24th Sep 2018

Thank you for submitting your manuscript to The EMBO Journal. Sorry for the delay in getting back to you with a decision, but I have now received the input from two referees and their comments are provided below.

As you can see the referees appreciate that the analysis, but also find that some further experiments are needed to fully support the key conclusions. Should you be able to address the raised concerns in full then I would like to invite you to submit a revised version. Let me know if we need to discuss any specific experiments further.

REFEREE REPORTS:

Referee #1:

This study appears technically well done. Conceptually, it may provide a new insight into a particular pathway potentially capable of or contributing to switching M2s to M1 macrophages. Since this pathway was tapped through unbiased MS data, it appears that it may be important. This is further supported by some data from ovarian cancer tissues.

I nevertheless have mixed impressions of this work:

A) I find this work technically solid (with some exceptions re missing techniques) and replete with informative sets of data, albeit not necessarily coming fully together in a comprehensive story as one would hope. Regarding how these data are combined and presented it seems that there may be a

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need or rather an opportunity for better organization of "why" certain experiments were done and "what" the conclusions were.

B) This reviewer has very little issues with technical aspects of this study but is frankly overwhelmed by the flow or rather lack of it and - with all due respect and apologies - non sequiturs in interpretations, which appear too many to list them all, with just a subset highlighted below. It is incumbent upon the authors to either take this into account and adjust, and then apply similar measures throughout, or to ignore altogether. Only after such a comprehensive overhaul can this be re-reviewed properly. Below is a sampling of issues (note: not a comprehensive and full coverage).

1. The abstract is OK but it also can be improved in terms of conceptual line of thought. It is a bit confusing to start with phagosomal maturation and then switch to M2/M1 reprogramming (which also seems not to be clearly stated). Please modify.
2. Fig. 2B. TAK1 was on the volcano plot in a very "gray" area of lesser standouts (by fold change and p values it is at the intersection of the cutoffs). Why focus on it then?
3. Fig. 1 A-B. The uptake of apoptotic vs necrotic cells. How does that fit with the main focus of the study? Why is in this study the charge analyzed? Will this contribute to the overall main storyline of this work? Are the differences in uptake (albeit labeled statistically significant) really that different? They are presented as different, but than the figure title says that phagocytosis is not affected.
4. Real time acidification, proteolysis, lipolysis.... This needs to be better explained in the legend so that the reader does not need to wade through methods. Needs to be clear what method was used and how were these measurements done (Fig. 1C-E), rather then "real time measurements". Also appropriate repeats and stats need to be shown along with the tracings.
5. The polyubiquitination of MSR1: Is this the only receptor whose polyubiquitination affects the outcome? The authors need to use mutants in a more coherent way to test whether this is a correlate or a driver of processes.
6. The authors state in the discussion that MSR1 is not important for inflammatory switching, as TLR and IL1R is more important. So what is this important for? This is definitely not clear.
7. Related to the above point (6): There may be something else that the authors missed to think of that's downstream of TAK1.

Etc., etc....

Referee #2:

The manuscript by Guo et al., entitled "Triggering MSR1 promotes JNK-mediated inflammation in IL-4 activated macrophages" demonstrates a phagosomal signaling pathway in M2 macrophages. The authors found that activated MSR1 caused ubiquitin induced TAK1/MKK7/JNK signaling in IL-4 activated M2 macrophages, thereby allowing macrophage phenotypic switching from the M2 (anti-inflammatory) toward the M1 (inflammatory) state.

The studies were conducted using multiple different biological approaches and provided novel scientific evidence on a molecular level, establishing the phagosomal MSR1-JNK signaling pathway in macrophages. However, I have some general concerns that should be addressed. Moreover, some additional studies are required to more fully document the presented conclusions.

Detailed comments are as follows:

1. The authors used an IL4-induced M2 polarization model in this study, but did not examine other M2 polarization conditions, for example IL13-induced M2 polarization or other types of polarization, including M2b and M2c. The authors need to conduct phagocytosis assays in these different M2 polarization conditions. These experiments will clarify whether the authors' conclusions are restricted to only IL4-mediated M2 polarization or whether the authors' conclusions are relevant to other types of M2 polarization.

2. In Figure 3A, JNK protein expression in the IL4-treated phagosomal fraction is unclear. The authors should provide more convincing data. In Figure 3B, the representative quantification data do not seem to correlate with the data presented in Figure 3A. The authors should provide the

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quantification method. Why does the TCL fraction not contain MKK7 protein?

3. The authors showed that ubiquitylated MSR1 is recruited to the Tab1/Tab2/Tak1/MKK7 complex using the UBC13 inhibitor in Figure 3D. The authors should also provide immunocytochemistry data for theTab1, Tab2, and MKK7 proteins using the UBC13 inhibitor in Figure S3.

4. In Figure S4, the authors showed MSR1 protein expression in M0 and M2 macrophages by western blot. The author should provide western blot data for MSR1 protein expression in IFNg-treated M1 macrophages in Figure S4.

5. The authors state " MSR1 showed diminished MSR1 K63-polyubiquitylation" according to Figure 5B. However, the MSR1 ubiquitylation data are unclear. The authors should provide better data to support this conclusion. In addition, the authors should provide p-MKK7 western blot data. Regarding Figure 5B, do Fucoidan or OxLDL alone activate the JNK signaling pathway? The authors should include data for Fucoidan and OxLDL alone treatments in Figure 5A, 5B, 5C, and 5D.

6. In Figure 5C & 5D, the authors need to provide data for additional M1 marker expression. This manuscript would be much more convincing for the conclusion that MSR-1 promotes pro-inflammatory M1 markers expression.

7. The authors do not show CD86 data in Figure 5E. The authors should provide these data.

8. In Figure S5, the authors should provide quantification graphs.

9. The authors should co-stain for a macrophage marker (e.g. F4/80) and MSR1 in Figure 6A.

10. The authors' conclusion that the JNK pathway promotes M1 polarization has been reported previously and should be cited (PMID:23223452).

There are also several minor comments:
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2. In line 4, page 7, "Figure 1A" should be "Figure 2A".
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1st Revision - authors' response 6th Feb 2019

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We thank both referees for their insightful comments.

Referee #1:

This study appears technically well done. Conceptually, it may provide a new insight into a particular pathway potentially capable of or contributing to switching M2s to M1 macrophages. Since this pathway was tapped through unbiased MS data, it appears that it may be important. This is further supported by some data from ovarian cancer tissues.

I nevertheless have mixed impressions of this work:

A) I find this work technically solid (with some exceptions re missing techniques) and replete with informative sets of data, albeit not necessarily coming fully together in a comprehensive story as one would hope. Regarding how these data are combined and presented it seems that there may be a need or rather an opportunity for better organization of "why" certain experiments were done and "what" the conclusions were.

We thank the reviewer for their encouraging comments. We decided to present the data in the same order as we identified them. This makes it more like a "detective story". We decided against making up a hypothesis and telling the story the other way around, as we believe it is important for readers to see that unbiased proteomics data can result in surprising and exciting new findings.
*We took the reviewer's concerns on board and described better **why** we did certain experiments.*

B) This reviewer has very little issues with technical aspects of this study but is frankly overwhelmed by the flow or rather lack of it and - with all due respect and apologies - non sequiturs in interpretations, which appear too many to list them all, with just a subset highlighted below. It is incumbent upon the authors to either take this into account and adjust, and then apply similar measures throughout, or to ignore altogether. Only after such a comprehensive overhaul can this be re-reviewed properly. Below is a sampling of issues (note: not a comprehensive and full coverage).

1. The abstract is OK but it also can be improved in terms of conceptual line of thought. It is a bit confusing to start with phagosomal maturation and then switch to M2/M1 reprogramming (which also seems not to be clearly stated). Please modify.

We re-wrote the abstract intensively. We hope it is now satisfactory for the reviewer.

2. Fig. 2B. TAK1 was on the volcano plot in a very "gray" area of lesser standouts (by fold change and p values it is at the intersection of the cutoffs). Why focus on it then?

We focused on TAK1 and MKK7 as they were significantly changing, known to be forming a complex and as it was rather surprising to find a pro-inflammatory signalling complex increased on the phagosome of anti-inflammatory macrophages.

3. Fig. 1 A-B. The uptake of apoptotic vs necrotic cells. How does that fit with the main focus of the study? Why is in this study the charge analyzed? Will this contribute to the overall main storyline of this work? Are the differences in uptake (albeit labeled statistically significant) really that different? They are presented as different, but than the figure title says that phagocytosis is not affected.

This has been changed. We added a sentence that negatively charged particles are taken up through scavenger receptors, thereby linking the data better to later results.

4. Real time acidification, proteolysis, lipolysis.... This needs to be better explained in the legend so that the reader does not need to wade through methods. Needs to be clear what method was used and how were these measurements done (Fig. 1C-E), rather then "real time measurements". Also appropriate repeats and stats need to be shown along with the tracings.

We added more detail to the figure legend as requested. SEM traces are shown for figures 1C &D (very small as did many, many replicates). Figure 1E is a representative of 3 independent replicates.

5. The polyubiquitination of MSR1: Is this the only receptor whose polyubiquitination affects the outcome? The authors need to use mutants in a more coherent way to test whether this is a correlate or a driver of processes.

There are a few other receptors in the list of ubiquitylated proteins (Table EV2/EV3). However, MSR1 was the most enriched receptor with 129-fold increase between TAB2-TUBE and the control-mutant Tab2-TUBE. The peptide with the ubiquitylation site on K27 of MSR1 was only identified in the TUBE pull-down and not in control. Finally, it was the 3rd most intense Gly-Gly peptide after ubiquitin itself and Fc-receptor gamma, which was only 6-fold enriched and thus not further followed up.
We have now done additional experiments to address the role of MSR1 ubiquitylation. The data shows that point mutation of MSR1 at K27R abrogated the activation of JNK in response to IL-4+Fucoidan indicating that MSR1 polyubiquitylation is required for JNK signalling activation (Figure 5C).

6. The authors state in the discussion that MSR1 is not important for inflammatory switching, as TLR and IL1R is more important. So what is this important for? This is definitely not clear.

We have changed this part to make it clearer. The signalling is not as strong as acute LPS/TLR signalling, which is clearly a very strong inflammatory signal. However, the signal is strong enough to switch macrophages to an inflammatory phenotype and it will play an important role in chronic conditions. We believe that this will play an important role in conditions where MSR1 will be continuously triggered by lipids. We have started a project in which we could confirm that chronic inflammation triggered by obesity is dependent on this MSR1 signalling. Including this early data would be out of scope for this manuscript, though. We would like to invite the reviewer to watch out for another paper from our group in the future.

7. Related to the above point (6): There may be something else that the authors missed to think of that's downstream of TAK1.

We have tested the activation of NF-kB as suggested by testing for IkBa degradation (Figure EV8). Under our experimental settings, WT and MSR1 KO BMDMs stimulated either with Fucoidan alone or with IL-4 followed by Fucoidan stimulation do not exhibit the activation of NF-kb signalling pathway. It appears likely that the proinflammatory switch is only mediated through the JNK pathway.

Etc., etc....

Referee #2:

The manuscript by Guo et al., entitled "Triggering MSR1 promotes JNK-mediated inflammation in IL-4 activated macrophages" demonstrates a phagosomal signaling pathway in M2 macrophages. The authors found that activated MSR1 caused ubiquitin induced TAK1/MKK7/JNK signaling in IL-4 activated M2 macrophages, thereby allowing macrophage phenotypic switching from the M2 (anti-inflammatory) toward the M1 (inflammatory) state.

The studies were conducted using multiple different biological approaches and provided novel scientific evidence on a molecular level, establishing the phagosomal MSR1-JNK signaling pathway in macrophages.

However, I have some general concerns that should be addressed. Moreover, some additional studies are required to more fully document the presented conclusions.

Detailed comments are as follows:

1. The authors used an IL4-induced M2 polarization model in this study, but did not examine other M2 polarization conditions, for example IL13-induced M2 polarization or other types of polarization, including M2b and M2c. The authors need to conduct phagocytosis assays in these different M2 polarization conditions. These experiments will clarify whether the authors' conclusions are restricted to only IL4-mediated M2 polarization or whether the authors' conclusions are relevant to other types of M2 polarization.

We have now included additional data showing that IL13-activate macrophage behave like IL-4 activated macrophages. Both M2 type cytokines, IL-4 and IL-13, increased the uptake of apoptotic cells and negatively charged carboxylated beads, while the uptake of necrotic cells and positively charged amino beads was comparable to untreated control.

2. In Figure 3A, JNK protein expression in the IL4-treated phagosomal fraction is unclear. The authors should provide more convincing data. In Figure 3B, the representative quantification data do not seem to correlate with the data presented in Figure 3A. The authors should provide the quantification method. Why does the TCL fraction not contain MKK7 protein?

We provided a more representative Western blot from three biological replicates. This is cut out of a blot with other conditions and we have added the full blot in Figure EV4C. Please note that for one sample for a western blot of latex bead phagosomes ~21 10 cm dishes of BMDMs are required.
The quantification method (imageJ) was added to the figure legend of Figure 3.

3. The authors showed that ubiquitylated MSR1 is recruited to the Tab1/Tab2/Tak1/MKK7 complex using the UBC13 inhibitor in Figure 3D. The authors should also provide immunocytochemistry data for theTab1, Tab2, and MKK7 proteins using the UBC13 inhibitor in Figure S3.

We have now included an additional experiment. The Ubc13 inhibitor treatment, as expected, significantly decreased the enrichment of MKK7 on the phagosome of IL-4 treated macrophages.

4. In Figure S4, the authors showed MSR1 protein expression in M0 and M2 macrophages by western blot. The author should provide western blot data for MSR1 protein expression in IFNg-treated M1 macrophages in Figure S4.

We have added mass spectrometry data from phagosome proteomics experiments of IFN-g, IL-4, IL-13 and IL-10 activated macrophages. These data show that all these activation states increase the amount of MSR1 on phagosomes to about the same levels (~1.7 to 2.4-fold).

5. The authors state " MSR1 showed diminished MSR1 K63-polyubiquitylation" according to Figure 5B. However, the MSR1 ubiquitylation data are unclear. The authors should provide better data to support this conclusion. In addition, the authors should provide p-MKK7 western blot data. Regarding Figure 5B, do Fucoidan or OxLDL alone activate the JNK signaling pathway? The authors should include data for Fucoidan and OxLDL alone treatments in Figure 5A, 5B, 5C, and 5D.

We think we did not make it clear enough that the blot in 5A shows a TUBE pull-down specific for K63 ubiquitylated proteins. Thus, the pulled-down MSR1 for which we blot is polyubiquitylated. We have changed the figure accordingly.

We have now included new data. Under our experimental settings, WT BMDMs stimulated with Fucoidan exhibit lower activation of Mkk7 and JNK compared to WT BMDMs co-stimulated with IL-4 followed by Fucoidan stimulation. As briefly noted in the discussion, we hypothesized that IL-4 induces specific E3 ligase activation that is involved in the regulation of signalling protein complex formation and promotes MKK7-JNK activation. (Figure EV8).

6. In Figure 5C & 5D, the authors need to provide data for additional M1 marker expression. This manuscript would be much more convincing for the conclusion that MSR-1 promotes pro-inflammatory M1 markers expression.

We performed additional experiments and included qPCR data for Ccl2 in Figure 5D &E and flow data for CD86 in Figure 5F.

7. The authors do not show CD86 data in Figure 5E. The authors should provide these data.

We performed additional experiments and included flow data for CD86 in Figure 5F.

8. In Figure S5, the authors should provide quantification graphs.

Done. We provided the additional quantification of the Facs data in Figure EV7.

9. The authors should co-stain for a macrophage marker (e.g. F4/80) and MSR1 in Figure 6A.

Unfortunately, these cancer slides are not available for us anymore and thus, we cannot do as requested. But considering that MSR1 (CD204) is considered a solid marker for tumour associated macrophages (Li et al, Lung Cancer. 2018; Sun et al, Biomed Res Int, 2018, Kawachi et al, Cancer Sci, 2018; Miyasato et al, Cancer Sci, 2017 and many more), we are convinced that the stained cells are indeed macrophages.

10. The authors' conclusion that the JNK pathway promotes M1 polarization has been reported previously and should be cited (PMID:23223452).

We apologise for not including this reference, which slipped out from a previous version. We included it.

There are also several minor comments:
1. In line 5 of page 4, "Keizer, S.J." should be removed.
Done.
2. In line 4, page 7, "Figure 1A" should be "Figure 2A".
Done.
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10. In Figure 4 legend, "(F)" should be "(E)".
Done.

2nd Editorial Decision 13th Mar 2019

Thank you for submitting your revised manuscript to The EMBO Journal. Your revised manuscript has seen by referee #2 and the comments are provided below. As you can see, the referee appreciates the introduced changes and support publication here. There are a few issues that need to be sorted out please also make sure to discuss the Han et al citation in a better manner.

REFEREE REPORTS:

Referee #2:

The revised paper has been improved. The conclusion that MSR1 triggers JNK-mediated inflammation in IL4-treated macrophages is supported by the data shown. The paper provides an advance that deserves to be published. However, two remaining issues need to be addressed:

a) I saw no Figure legends in the version of the manuscript that I reviewed. This omission made it difficult to evaluate the paper. This must be corrected.

b) The authors corrected an omission in the previous version of the manuscript by including a citation to Han et al. (lines 393-395). This is an improvement. However, my impression is that this could have been done in a more forthright manner that more accurately reflects this literature. While Han et al. did study obesity-associated inflammation (noted by the current authors in their citation), Han et al. also reported the effects of compound JNK-deficiency in BMDMs under M1 and M2 polarizing conditions in vitro, which appears to be directly relevant to the current study and is surprisingly unstated.

2nd Revision - authors' response 22nd Mar 2019

The authors performed all requested editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Matthias Trost
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2018-100299R

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 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 $\chi 2$ 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 repeated 3-6 times according to experiences with effect sizes.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	no animal studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	no animal studies.
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.	no animal studies.
For animal studies, include a statement about randomization even if no randomization was used.	no animal studies.
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.	immunohistochemistry was performed in a blinded fashion
4.b. For animal studies, include a statement about blinding even if no blinding was done	no animal studies.
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.	for proteomics experiments, normal distibution of data was assessed by histograms.
Is there an estimate of variation within each group of data?	no.
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
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
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-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

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).	catalog numbers have been provided.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were from ATCC. They are authenitcated by proteomics and phagocytic activity. We test every 4-6 weeks for mycoplasma and the cells were negative.

* 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.	no animals. BMDMs from were made from femurs of both male and female mice.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	no animals.
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.	no animals.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	the immunohistochemistry of human cancers was approved by the local committee of Hospital Universitario Vall d´Hebron, Barcelona
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.	Patients gave informed consent and the experiments conformed to the principles set out in the WMA declaration.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	no patient photos
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	no restrictions.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	none
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.	none
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.	none

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	We have uploaded our mass spectrometric raw data to the PRIDE repository. Reviewers can access the raw data using the following identifiers PXD010478 (TUBE data; Username: reviewer61442@ebi.ac.uk Password: GDRWhOkY) and PXD004520 (phagosome proteomics data; Username: reviewer26301@ebi.ac.uk Password: PSZ2j2Kz).
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).	done.
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).	n/a
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.	n/a

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.	no.