EMBO reports

TLR4-dependent shaping of the wound site by MSCs accelerates wound healing

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

1st Editorial Decision 14 August 2019

Thank you for the submission of your research manuscript to EMBO reports. I am sorry for the delayed decision, but I had contacted a third referee that had agreed to assess the manuscript, who did not return his/her report, despite several reminders. However, we have received reports from two referees, which can be found at the end of this email.

As you will see, both referees think that the findings are of high interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore 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 me if a 3-months time frame is not sufficient so that we can discuss the revisions further.

When submitting your revised manuscript, please also carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision. When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that the changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript

text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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 should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. the RNA-seq. data) are deposited in an appropriate public database. See: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We 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. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please format the references according to our journal style. See: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Please move the acknowledgements, the COI statement and the author contributions after the discussion, before the M&M part.

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

REFEREE REPORTS

Referee #1:

In this study, authors have investigated the exposure to LPS in MSCs and the related consequences in the modulation of wound inflammation. The team has extensive experience in this area and on the importance of neutrophil infiltration and activity in cutaneous wound healing and on the isolation and application of MSC to skin wounds.

Here they explore the hypothesis that MSCs are able to sense their microbial environment and adapt their anti-inflammatory response to environmental and especially microbial cues. The experimental design explores MSCs influence on neutrophil Netosis in vivo and in vitro. MSCs are primed or not with LPS to model the bacterial environment. Although the general conclusion is supported by the data there remains important details to clarify.

1- MSCs are primed with LPS and then washed before being tested with neutrophils. Although neutrophils are activated with PMA, I wonder whether they would behave differently in the presence of LPS. Indeed Neutrophils will be exposed to the same environment as MSCs. Are the differences in Netosis still relevant in an LPS rich culture medium?

2- Gene expression changes suggest a lot of cytokine related genes are being expressed by MSCs upon LPS exposure. Could experiments be conducted with MSC conditioned medium?

3- The classical mixed lymphocytic assay is often used to evaluate the degree of immunosuppressive activity by MSCs. Could LPS primed MSCs be used in this assay to more formally and quantitatively demonstrate their pro-inflammatory activity.

4- Figure 3a, I suppose the immunostains for IL1, CXCL6 are human specific? If so, it looks like there is more MSC engraftment in primed MSCs versus non-primed. Can you please report on the count of beta2m+ MSCs in addition to reporting on percentages.

5- The siRNA experiment clearly shows good targeting of TLR4. Authors should also demonstrate that LPS stimulation does not lead to CXCL6/IL1/IL8 expression upon TLR4 silencing.

6- siRNA and scrambled siRNA can activate TLR3 in MSCs. It si therefore essential to use a group treated with scrambled MSCs rather than control MSCs for the wound closure experiment. Authors should explore gene expression after siRNA treatment to ensure absence of significant TLR3 activation.

7- There doesn't seem to be a link between netosis and wound healing speed according to the data. Indeed, LPS primed MSCs restore netosis in neutrophils to the degree observed in the absence of MSCs, yet they accelerate healing as do non-primed MSCs that reduce netosis. Clearly netosis is not the most important element. Although I accept that higher levels of TGFbeta-expressing macrophages are present in LPS-primed MSC treated wounds, it is difficult to accept the link to neutrophils as both numbers and activation do not differ in PBS control versus LPS-primed MSCs treated wounds.

Referee #2:

In the present manuscript Munir and colleagues present data on the role of MSC sensitization by bacteria components on the immune function and the wound healing process. The authors show that LPS can activate MSCs which further activate neutrophils thus enhancing the healing process in vivo. This process is controlled by the activation of the TLR4 pathway. Furthermore, the activated neutrophils are engulfed by macrophages, thus releasing TGF-β1 which most probably plays a role in myofibroblast formation. This is a very interesting, well-performed and well-written work, which supports a new role of the MSCs, i.e. under specific conditions to provoke neutrophil activation towards pathogen elimination and tissue repair.

Comments:

- The activation of neutrophils by MSCs (shown in Fig. 1) can be achieved in the absence of a continuous PKC activation?

- Are there any data on the levels of ROS in the described system?

- Which are the kinetics of TLR4 levels and activation after LPS treatment?

1st Revision - authors' response 7 January 2020

Response to Comments of Referee #1

In this study, authors have investigated the exposure to LPS in MSCs and the related consequences in the modulation of wound inflammation. The team has extensive experience in this area and on the importance of neutrophil infiltration and activity in cutaneous wound healing and on the isolation and application of MSC to skin wounds.

Here they explore the hypothesis that MSCs are able to sense their microbial environment and adapt their anti-inflammatory response to environmental and especially microbial cues. The experimental design explores MSCs influence on neutrophil Netosis in vivo and in vitro. MSCs are primed or not with LPS to model the bacterial environment. Although the general conclusion is supported by the data there remains important details to clarify.
We are grateful to this Reviewer for her/his encouraging comments and we wish to clarify her/his constructive critical comments below.

1.1- MSCs are primed with LPS and then washed before being tested with neutrophils. Although neutrophils are activated with PMA, I wonder whether they would behave differently in the presence of LPS. Indeed Neutrophils will be exposed to the same environment as MSCs. Are the differences in Netosis still relevant in an LPS rich culture medium?

We followed this Reviewer´s suggestion and experimentally addressed her/his question whether PMA activated neutrophils would behave differently in co-cultures with MSCs in the presence of LPS. No difference in the attenuated suppression of NET formation was observed in co-cultures of PMA activated neutrophils with MSCs in the presence of LPS in the supernatant (Figure 1., lower row, right panel.) when compared to PMA activated neutrophils co-cultured with MSCs which had been primed with LPS which was washed away prior to co-culture (lower row, left panel). Non-primed MSCs, by contrast, led to a significantly stronger suppression of Net formation (upper row, outer right panel). Incubation of neutrophils with PMA alone served as positive control and DMSO treated neutrophils as negative control. The fact that in a LPS rich medium no suppression of Netosis of LPS exposed neutrophils occurred ensures that a potential infection is most efficiently counteracted by Netosis. The injection of LPS primed MSCs into wounds is correlated with a transcriptomic shift in MSCs, significantly enhanced Net formation and profoundly accelerated wound closure. Therefore, independent of combating infection, injection of primed MSCs with LPS apparently will be highly valuable for a refined MSC-based treatment strategy for wounds to accelerate wound repair

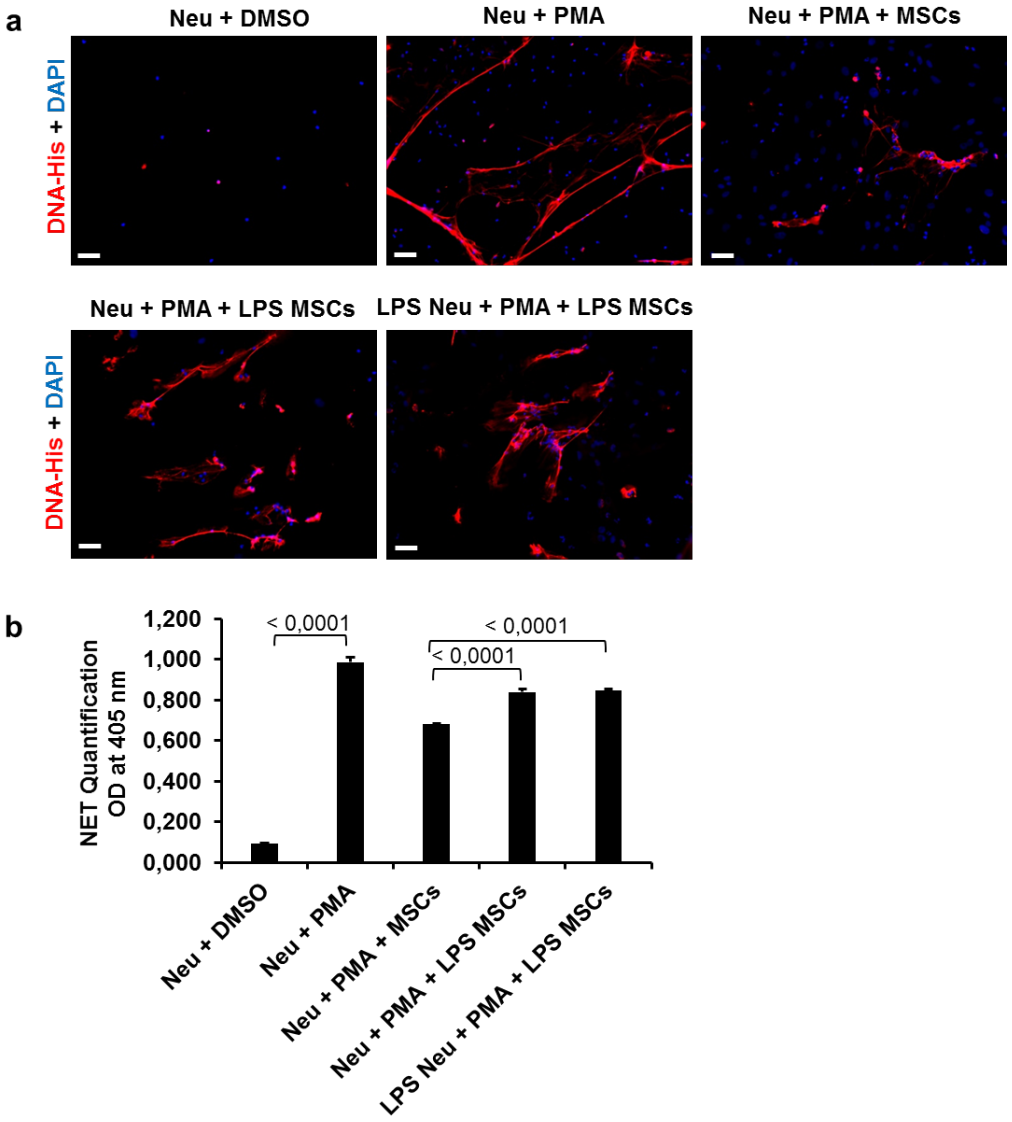


Figure 1. MSCs depict reduced suppression of Net formation in the presence of LPS in co-cultures with neutrophils. (a) PMA activated neutrophils were co-cultured with MSCs in the presence of LPS at a concentration of 100 ng (lower row, right panel). No difference in the attenuated suppression of NET formation was observed compared to PMA activated neutrophils co-cultured with LPS primed MSCs (lower row, left panel). Non-primed MSCs, by contrast, led to a significantly stronger suppression of Net formation (upper row, outer right panel). Incubation of neutrophils with PMA alone served as positive control and DMSO treated neutrophils as negative control. Scale bars: 50µm. A representative experiment out of 3 independent experiments is depicted. **(b)** Quantification of NET bound elastase indicative of NET formation. Statistical analysis was performed using one way ANOVA, values are represented as mean ± SEM, n=3.
We have included data into the result section **Supplementary figure 2** as highlighted in red.

1.2- Gene expression changes suggest a lot of cytokine related genes are being expressed by MSCs upon LPS exposure. Could experiments be conducted with MSC conditioned medium?
We thank this reviewer for this considerate suggestion which is related to her/his previous point. We have addressed this question (Figure. 2 below). First, we have incubated neutrophils directly with 100 ng LPS with a clear activation and Net formation (Fig.2a, upper row, right panel and figure 2b). Also transferring the supernatants from MSCs primed with LPS for 24 h onto non-primed neutrophils induced a significant Netosis (Figure 2a, lower row, outer panel and Fig.2b). This implies that supernatants of LPS primed MSCs can activate neutrophil Net formation.

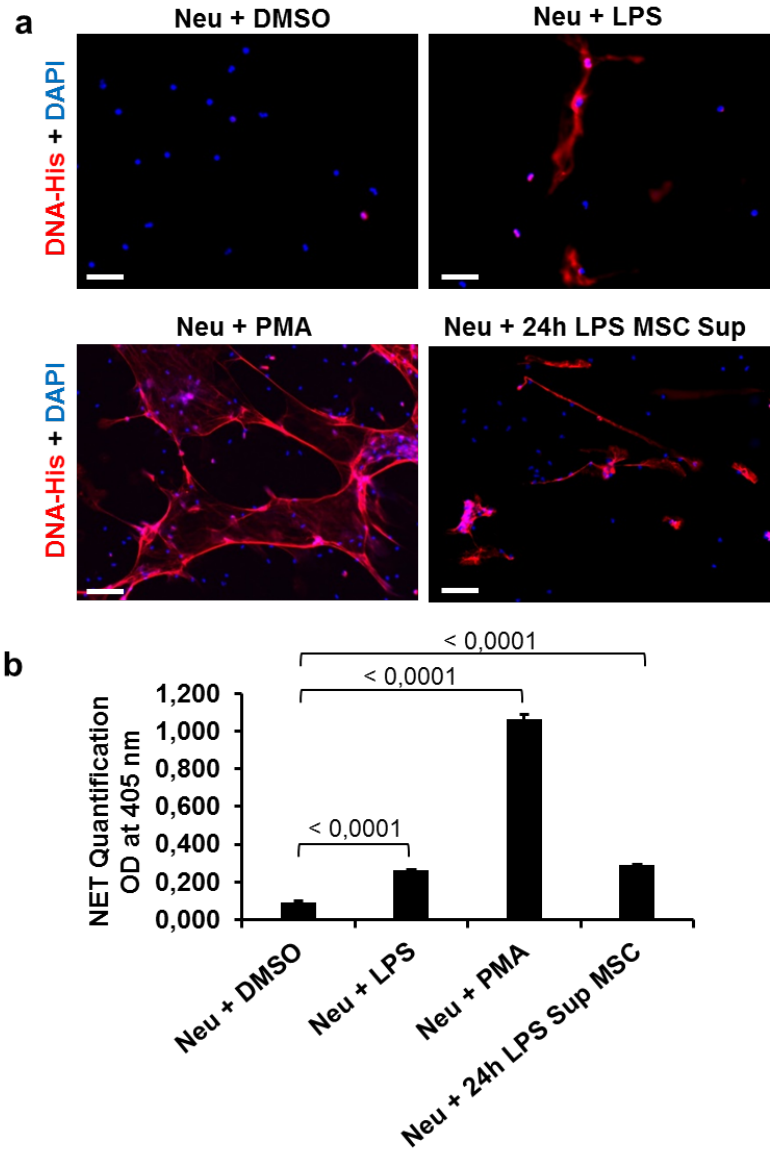


Figure 2. Supernatants collected from LPS primed MSCs stimulate netosis. (a) Non-activated neutrophils were exposed to the supernatants collected from MSCs treated with 100ng/ml LPS for 24h. A significant increase in Net formation was observed. Incubation of neutrophils with PMA treated neutrophils served as positive control and DMSO treated neutrophils as negative control. Scale bars: 50µm. **(b)** Quantification of NET bound elastase indicative of NET formation. Statistical analysis was performed using one way ANOVA, values are represented as mean ± SEM, n=3.

1.3- The classical mixed lymphocytic assay is often used to evaluate the degree of immunosuppressive activity by MSCs. Could LPS primed MSCs be used in this assay to more formally and quantitatively demonstrate their pro-inflammatory activity.
This reviewer made an interesting suggestion that the degree of immunosuppressive MSC activity may be also assessed by a mixed lymphocyte reaction. We initially felt that the response of the MSC following LPS priming may be rather specific for the innate immune system. However, since it is a very interesting request and we do not know the outcome, we followed this reviewer´s suggestion and performed a classical mixed lymphocyte reaction in the presence and absence of LPS primed and non-primed MSCs. We found that MSCs - independent of being primed with LPS or left non-primed - depict a suppressive effect on effector T-cells (Figure 3). We included these data in the result section of the revised manuscript and into the ***Supplementary figure 4****, the procedure of MLR into the Material & Method section (as shown below) and discussed the specificity of the LPS response of MSCs on neutrophils.*

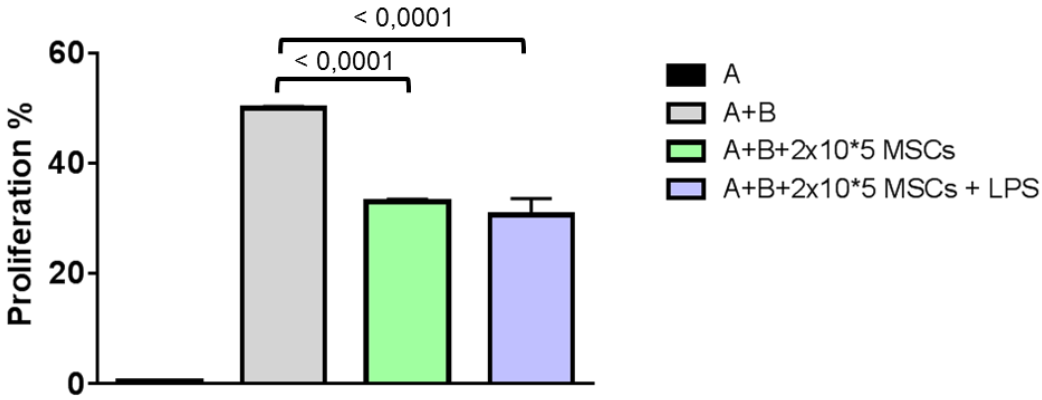


Figure 3. Both non-primed MSCs and LPS primed MSCs suppress T cell proliferation in a mixed lymphocyte reaction. *CFSE labeled PBMCs from buffy coat of healthy, unrelated, non-HLA-matched donor (effector cells A) was stimulated with irradiated PBMCs (stimulator cells B) in the absence or presence of untreated or LPS-treated MSCs. After 7 days, cells were stained for CD2 and 7-AAD. Live cells were identified by 7-AAD negativity and proliferation of $CD2^+$ T cells was defined by CFSE dilution. Statistical analysis was performed using one way ANOVA, values are represented as mean ± SEM, n=6.*

Lymphocyte isolation and mixed lymphocyte reaction (MLR)
PBMCs were obtained from peripheral blood of healthy donors. Isolation was performed by density centrifugation on Biocoll Separating Solution (1.077g/ml, Biochrom). MLRs were carried out in RPMI 1640, 5% fetal calf serum (Sigma Aldrich), 2 mM L-glutamin, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin and 0.05 mM 2-mercaptoethanol at 37°C and 7.5% CO_2. $2x10^6$ PBMCs/ml were labeled with 5µM CFSE (ThermoFisher Scientific) for 10 min at 37°C followed by several washing steps with ice-cold PBS-5%FCS. $2.5x10^5$ CFSE-labeled PBMCs were stimulated with $2.5x10^5$ irradiated allogeneic PBMCs (30Gy) in the presence or absence of $2.5x10^5$ MSCs. After 7 days cells were stained for CD2-APC (cl. RPA-2.10, eBiosciences) and 7-AAD (Sigma) und proliferation was determined on $CD2^+$ $7\text{-}AAD^-$ cells.

1.4- Figure 3a, I suppose the immunostainings for IL1, CXCL6 are human specific? If so, it looks like there is more MSC engraftment in primed MSCs versus non-primed. Can you please report on the count of $\beta 2M^+$ MSCs in addition to reporting on percentages?

As to the reviewer´s question whether immunostaining has been performed with human specific antibodies against IL1C and CXCL6, we have used antibodies which recognize human CXCL6 and human IL1β. To specifically detect human MSCs which release either CXCL6 or IL1β, we performed double staining with an antibody which specifically detects human β2 microglobulin (β2m+). The overlay of CXCL6 (red) and human β2m+ (green) is yellow and the overlay of CXCL6 (red) and human β2m+ (green) is yellow. We only counted yellow cells. Thus, we are confident to have only selected for human MSCs which release either CXCL6 or IL1β. In addition, the reviewer raised the question whether there is more engraftment in primed MSCs versus non-primed MSCs. In addition, the reviewer raised the question whether there is more engraftment in primed MSCs versus non-primed MSCs. As we study a xenograft model, there is no long term engraftment of the MSCs. We previously have shown by quantification of either human specific Alu sequences (Jiang et al., 2013) or by human-specific beta actin sequence PCR (Vander Beken et al., 2019) that under different experimental conditions after injection of MSCs into wounds was comparable with a gradual decline of MSCs after 2 to 5 days after injection. Nevertheless, we followed the reviewer´s request and counted β2m+ human MSCs in non-primed versus LPS primed MSCs. There is a non-significant difference in the counts of β2m+ human MSCs in non-primed versus LPS primed MSCs (Figure 4). We attached the results below (Figure 4) in ***Supplementary figure 6*** *in the revised version of the manuscript.*

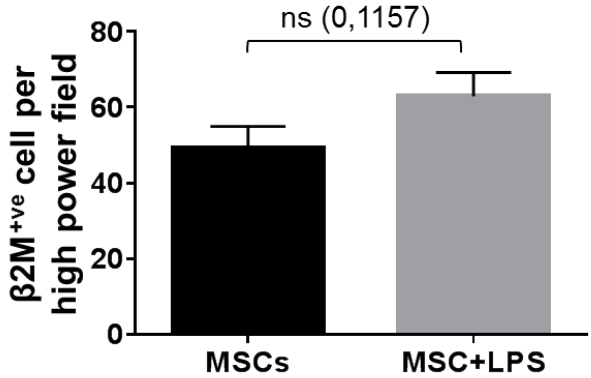


Figure 4. No significant difference in β2M$^+$ MSC numbers in wounds injected with non-primed versus LPS-primed MSCs.
Quantification of human β2M$^+$ MSCs in the skin wounds that were treated either with PBS or LPS primed MSCs for 24h. Counting of β2M$^+$ MSCs in wound sections was performed 24h after the administration of MSCs. Statistical analysis was performed using unpaired t-test, values are represented as mean ± SEM, n=3. ns, non-significant.

1.5- The siRNA experiment clearly shows good targeting of TLR4. Authors should also demonstrate that LPS stimulation does not lead to CXCL6/IL1/IL8 expression upon TLR4 silencing.
We thank the reviewer for her/his constructive comment. We have assessed CXCL6, IL-1β and IL-8 on mRNA level upon silencing LPS-primed TLR4 silenced MSCs. We found that in TLR4 silenced MSCs the mRNA expression of CXCL6, IL-1β and IL-8 was markedly reduced as compared to LPS-primed scrambled MSCs. These data confirm that TLR4 is mainly responsible for the release of CXCL6 and other cytokines.

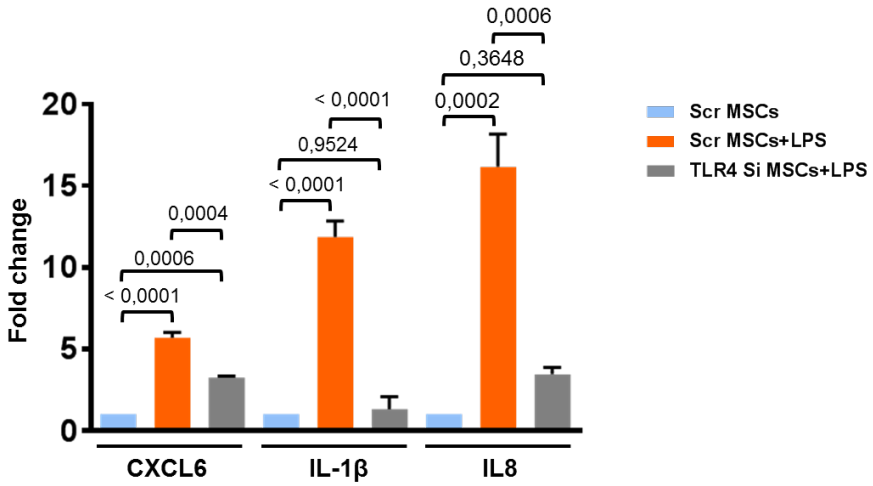


Figure 5. Supplementary figure S8. LPS stimulation reveal repressed stimulation of CXCL6/IL-1β/IL-8 expression in TLR4 silenced MSCs. qPCR results demonstrating mRNA expression of CXCL6, IL-1β and IL-8 upon silencing of TLR4 in LPS-primed silenced MSCs. Comparisons were made between 'Scr MSCs vs Scr MSCs + LPS', 'Scr MSCs vs TLR4 Si MSCs + LPS', and 'Scr MSCs + LPS vs TLR4 Si MSCs + LPS'. Statistical analysis was performed using one way ANOVA, values are represented as mean ± SEM, n=3.

We have included data into the result section **Supplementary figure 8** as highlighted in red.

1.6- siRNA and scrambled siRNA can activate TLR3 in MSCs. It is therefore essential to use a group treated with scrambled MSCs rather than control MSCs for the wound closure experiment. Authors should explore gene expression after siRNA treatment to ensure absence of significant TLR3 activation.

We found this reviewer´s suggestion very stimulating, though not easy to address, in particular as there is no a clear separation between TLR3 and TLR4 target genes. This is due to the fact that both TLR4 and TLR3 share at least at the level of the Toll-IL-1 receptor domain-containing adaptor the same pathway with the induction of IFN-β (TRIF)-dependent signaling leading to IFN regulatory factor 3 (IRF3) activation (Hu et al, 2015). In addition, similar to TLR4 activation TLR3 can lead to expression of NFκB-dependent genes (Jamamoto et al., 2003; Jeyaseelanet al., 2007). To circumvent this difficulty, we followed two independent strategies:

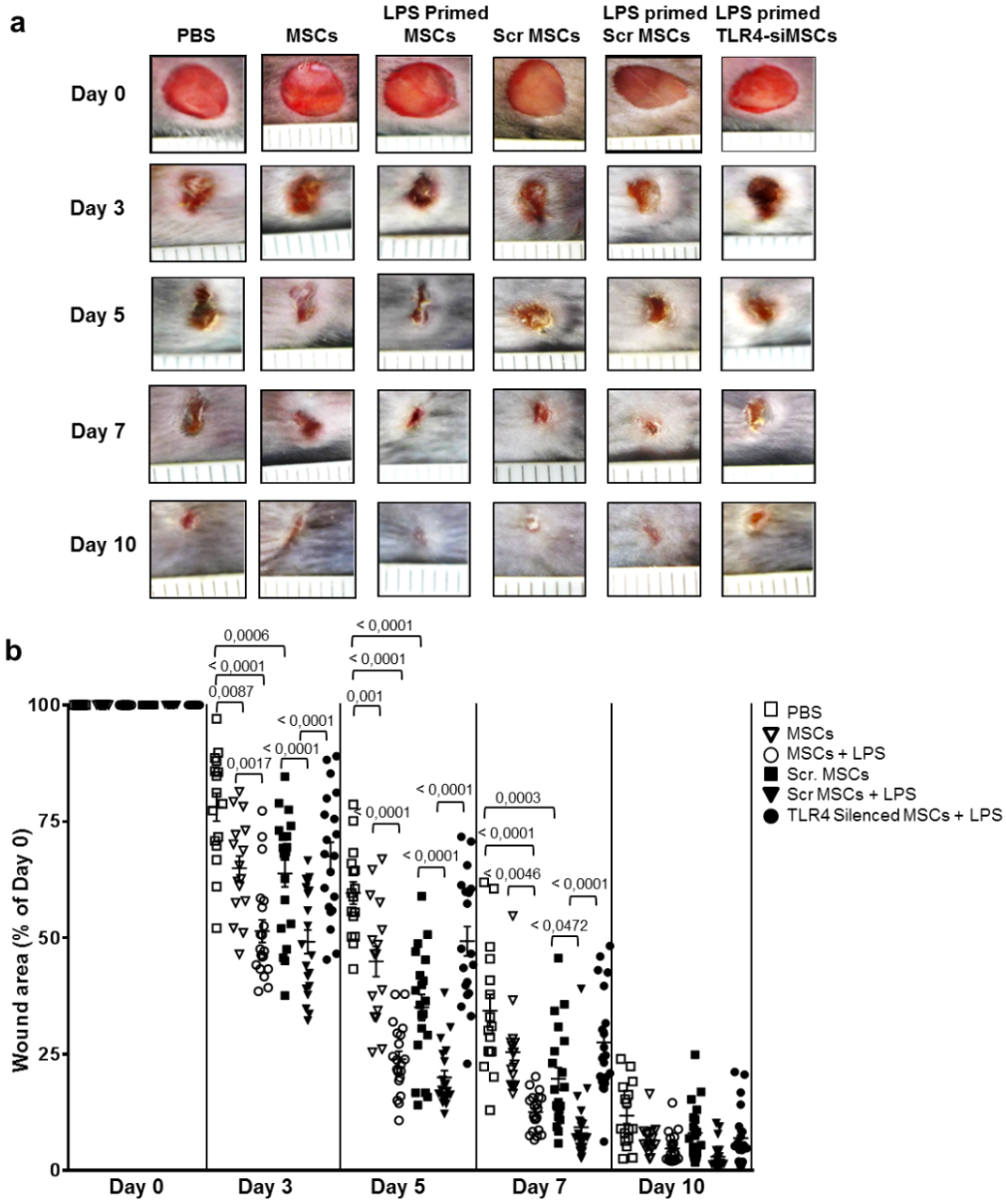
First, we performed the in vivo experiment and introduced - apart from all other experimental groups - the scrambled siRNA MSC groups as suggested by this reviewer. This experimental design allowed to approach the question whether injection of MSCs treated with scrambled siRNA (scr) may - via TLR3 activation - accelerate wound healing similar to that observed in wounds injected with LPS treated MSCs. As shown in Figure 6a and b (below), the acceleration of wound healing injected with LPS-primed MSCs is significantly better (Fig.6a, fifth row from left, and 6b open circles) when compared to non-primed scr siRNA treated MSCs (Fig. 6a , fourth row from left, and Fig. 6b black squares). In addition, LPS primed TLR4 silenced MSCs when injected into wounds cannot accelerate wound healing (Fig.6a, 6th row from left and Figure 6b, closed black circles) in comparison to injection of src treated MSCs (Fig. 6a, fourth row from left, and Fig. 6b black squares) or LPS and scr treated MSCs (Fig.6a, fifth row from left, and 6b black triangles). These data clearly show that TLR4 activation in MSCs plays a major though possibly not an exclusive role in enhancing wound healing.

Secondly, as already mentioned, the upregulation of IRF3 with a strong interferon response and up-regulation of NF-κB dependent genes downstream of TRIM do not reliably help to discriminate between TLR4 and TLR3 activation because both can activate these pathways (Hu et al, 2015; Jamamoto et al., 2003; Jeyaseelanet al., 2007). To explore TLR3 activation, we therefore employed in TLR4 siRNA treated MSCs to study phosphorylation of the TLR3 receptor at TYR 759 which is an essential upstream step in TLR3 receptor activation (Yamashita et al., 2012). These experiments may contribute to clarify this reviewer´s question whether siRNA or scrambled siRNA activate the TLR3 pathway in MSCs. We found that neither TLR4 silenced MSCs nor scramble siRNA treated MSCs reveal phosphorylation of the TRLR3 receptor at the TYR 759 site.

In addition, we found that silencing of TLR4 in LPS primed MSCs fully abrogated the beneficial effect of LPS treated MSCs on accelerating wound healing in many aspects (wound closure not any longer accelerated, NET formation not any longer enhanced, α-SMA^{+} myofibroblasts responsible for

wound contraction and closure not any longer up-regulated, CD31 positive cells indicative of vessel formation not any longer increased in number to name only a few downstream effector mechanisms enforced by TLR4). The impact of TLR4 silencing on so many aspects of wound healing is a very interesting observation. Even if TLR4 specific siRNA by itself non-specifically up-regulates TLR3, we still find a complete attenuation of the beneficial up-speeding of wound closure when injecting LPS primed TLR4 silenced MSCs into wounds. In the case siRNA upregulates TLR3 – which may be the case – this up-regulation, however, cannot overrun the effect of TLR4 silenced LPS primed MSCs. These data imply that even if siRNA or scrambled siRNA induce TLR3, it does not significantly contribute to the acceleration of wound healing in the case TLR4 is silenced in LPS primed MSCs. Also our data, indirectly suggest that even if LPS by itself can activate TLR3, it does not override the effect of LPS primed TLR4 silenced MSCs and does not contribute to accelerate healing.

We include our data for this reviewer and will shortly discuss the potential activation of TLR3 which –does not predominantly contribute to wound closure. However, in our results, though TLR4 is the major driver for accelerated wound healing in the described experimental setting, we do not claim that LPS activated TLR4 is exclusively responsible for accelerated wound healing.



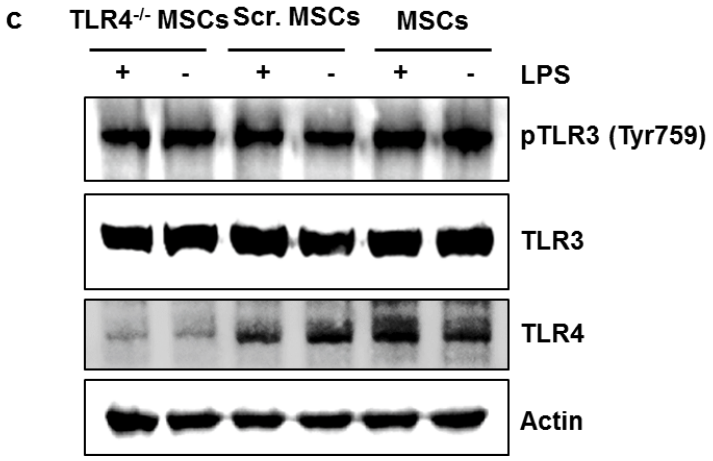


Figure 6. LPS primed MSCs accelerate wound healing in a TLR4 dependent manner.
(**a**) Representative clinical pictures of murine wounds at 0, 3, 5, 7 and 10 days after wounding. Enhanced wound healing in LPS primed MSCs group as opposed to all other groups. (**b**) Statistical analysis of 20 wound areas per group at the indicated time points, expressed as percentage of the initial wound size (day 0), for PBS control, non-primed MSCs, LPS primed MSCs, non-primed ScrMSCs, LPS primed ScrMSCs and LPS primed TLR-4 silenced MSCs. Results are mean ± SD representing 1 of 3 independent experiments. Statistical analysis was performed using one way ANOVA. (**c**) The transfection with TLR4-specific siRNA or scramble siRNA does not induce TLR3 receptor activation in MSCs. The representative Western blot analysis depicting expression of indicated proteins in different experimental groups (n=3).
We have included data into the result section **Figure 5** as marked in red.

1.7- There doesn't seem to be a link between netosis and wound healing speed according to the data. Indeed, LPS primed MSCs restore netosis in neutrophils to the degree observed in the absence of MSCs, yet they accelerate healing as do non-primed MSCs that reduce netosis. Clearly netosis is not the most important element. Although I accept that higher levels of TGFβ -expressing macrophages are present in LPS-primed MSC treated wounds, it is difficult to accept the link to neutrophils as both numbers and activation do not differ in PBS control versus LPS-primed MSCs treated wounds.
This reviewer is right that there may not be a direct link between netosis and enhanced wound contraction as we initially claimed. As to bacteria infested wounds both a microbicidal gene program enhancing netosis as a major strategy counteracting bacteria, and a gene program accelerating wound closure which serves to rapidly seal the wound site and, in consequence, prevents further invasion of pathogens into the wound. Both programs represent two aspects of the adaptive MSC response to LPS exposure which enforce complementary protection of the organisms from infection. Apparently, this is an evolutionary very refined program which contributes to enhance the survival of organisms.

We have toned down our claim that netosis is causally linked to enhanced wound closure and introduced parts of the above arguments into the discussion.

We agree with this reviewer that the data depicting enhanced numbers of macrophages which express TGF-β1 are strong and enhanced TGF-β1 promotes angiogenesis (Roberts et al., 1986) and accelerates wound contraction via induced myofibroblast differentiation (Desmouliere et al., 1993). Though our evidence arguably is correlative, we still are confident that neutrophils - at least in part - contribute to enhance TGF-β1 release from macrophages and, in consequence, to enhance wound contraction. We now more distinctly embrace other possibilities for enhanced TGF-β1 release from macrophages in our discussion. Let me first forward some arguments for the likely possibility that, indeed, neutrophils but not only neutrophils do play a role in enhanced wound closure following injection of LPS primed MSCs.

First, in wounds injected with LPS primed MSCs neutrophils are significantly higher in number when compared to wounds injected with PBS and MSCs (Figure 6c). Second, double immunostaining for active caspase indicative for apoptosis is higher in neutrophils in wounds injected with LPS primed MSCs when compared to PBS injected or wounds injected with non-

primed MSC ***(Supplemental figure 3)***. *We now included the significance calculation between the group of LPS primed MSCs vs non-primed MSCs. These data clearly show that there is more neutrophil apoptosis in LPS primed MSCs. Third, confocal microscopy of wound sections double stained for neutrophils and macrophages depict more double positive cells indicative of close attachment – a step prior to the formation of the phagocytotic cup – or already phagocytosed apoptotic neutrophils in wounds injected with LPS primed MSCs (Figure 6 a, b). Engulfment of apoptotic neutrophils is a strong signal for macrophages to release TGF-β1 (Fadok et al., 1998; Peters et al., 2006), and this in consequence, leads to differentiation into contractile α-SMA positive myofibroblasts which are responsible for enhanced wound contraction (Desmouliere et al., 1993). This set of correlative data would favor a contribution of neutrophils in acceleration of wound healing. However, alternative scenarios are possible or the combination of the above mentioned with alternative scenarios. Our data, would also fit to a scenario, where LPS primed MScs release enhanced IL-1β which may recruit macrophages to the wound site (Rider et al., 2017), where they - upon exposure of other paracrine factors or exosomes released from LPS primed MSCs - undergo a switch from pro-inflammatory M1 macrophages to anti-inflammatory and characteristically TGF-β1 releasing M2 macrophage. In fact, earlier let-7, a micro-RNA, released from LPS primed MSCs contributed to the switch of M1 to M2 macrophages in diabetic mice (Ti et al., 2015).*
In the revised manuscript, we toned down the claim for the initially envisioned pathogenic sequence and introduced an alternative - which may explain enhanced TGF-β1 release from macrophages after enhanced engulfment of neutrophils which, in consequence, contributes to the enhanced wound closure in wounds injected with LPS primed MSCs. Accordingly, we modified our graphical summary and the legend (marked in red) in the revised version of our manuscript.

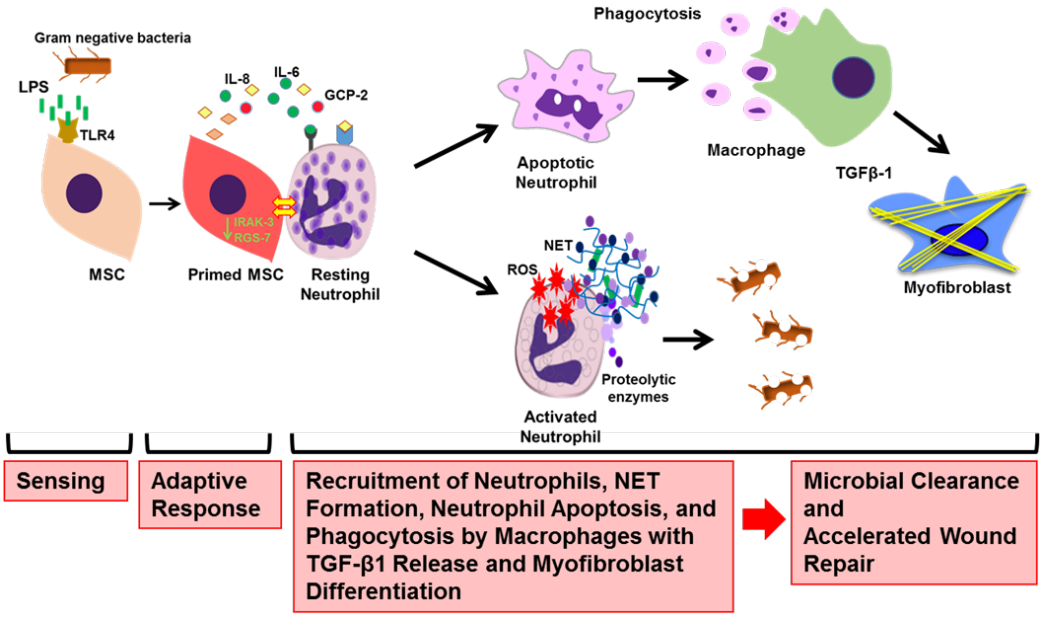


Figure 7. LPS sensing of MSCs shapes the function of neutrophils at the wound site.
Graphical summary, depicting the molecules involved in sensing, signaling and raising an adaptive response in MSCs. MSCs sense bacterial intruders as modelled by the key molecule LPS, a widely distributed PAMP and wall component of Gram negative bacteria. LPS is sensed via the TLR4 receptor and the signal is relayed via MyD88 to downstream effectors which, in consequence, shape the adaptive MSCs response. This response is enforced by fundamental transcriptomic reprogramming which is responsible for the release of factors critical for neutrophil and macrophage recruitment to the wound site. The adaptive response of LPS primed MSCs depicts a bifurcation with neutrophil activation leading to enhanced microbicidal NET formation and ROS release to directly counteract invading bacteria. In addition, in a second line of complementary tissue protection and repair, apoptotic neutrophils, are phagocytosed by macrophages. Neutrophil engulfment constitutes a strong signal for macrophages to release TGFβ1 which subsequently enhances differentiation of myofibroblasts and accelerates wound contraction and thus wound closure. Acceleration of wound closure together with enhanced NET formation and ROS release

effectively counteract microbial invasion. These data may stimulate new avenues to refine MSC-based therapies for difficult-to-heal wounds and/or infected wounds.

Response to Comments of Referee #2

In the present manuscript Munir and colleagues present data on the role of MSC sensitization by bacteria components on the immune function and the wound healing process. The authors show that LPS can activate MSCs which further activate neutrophils thus enhancing the healing process in vivo. This process is controlled by the activation of the TLR4 pathway. Furthermore, the activated neutrophils are engulfed by macrophages, thus releasing TGF-β1 which most probably plays a role in myofibroblast formation. This is a very interesting, well-performed and well-written work, which supports a new role of the MSCs, i.e. under specific conditions to provoke neutrophil activation towards pathogen elimination and tissue repair.
We are grateful to this reviewer´s favorable comments on our manuscript. We do appreciate it, and we are very willing to follow his advice.

Comments:

2.1- The activation of neutrophils by MSCs (shown in Fig. 1) can be achieved in the absence of a continuous PKC activation?
PKC is the strongest inducer of neutrophil activation. LPS, fMLP and IL-8 did not work as strong (Figure. 8). As we wish to show strong suppression of neutrophil activation by non-primed MSCs as opposed to LPS primed MSCs, we do need a strong inducing agent. This allowed us to dissect the role of LPS primed MSCs.

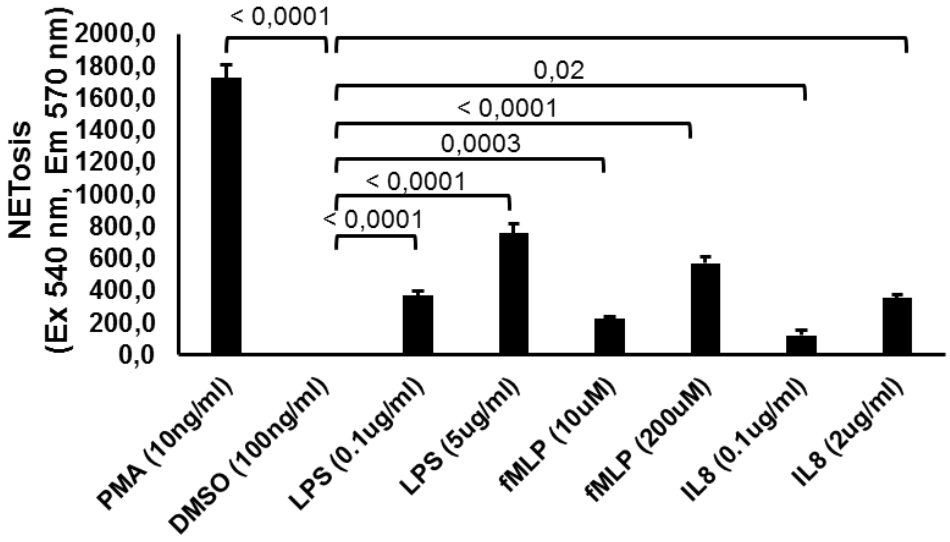


Figure 8. LPS, fMLP and IL-8 stimulates NETosis even in a PKC-independent manner. The freshly isolated human neutrophils were incubated with indicated compounds and then the NETosis was quantified by measuring absorbance at 405nm. Statistical analysis was performed using one way ANOVA, values are represented as mean ± SEM, n=3

2.2- Are there any data on the levels of ROS in the described system?
Yes, we select ROS concentrations as a read out for microbicidal neutrophil activation (see below, Figure 9).

We included this data in the revised manuscript in ***Supplementary figure 1****.*

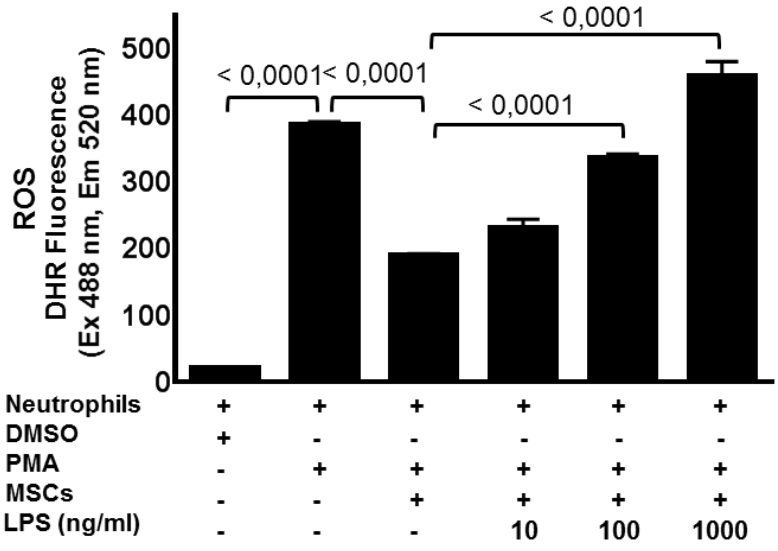


Figure 9. LPS-treated MSCs augment neutrophil activation and enhanced ROS production. Graph shows quantification of ROS by measuring fluorescence intensity of DHR123 dye. The MSCs were primed with increasing concentrations of 10ng/ml, 100ng/ml and 1000ng/ml LPS followed by co-culture with neutrophils. The MSCs were then incubated with ROS-specific dye DHR123 and the fluorescence was read after 60 minutes of incubation at 488/520 nm with spectrophotometer. Incubation of neutrophils with PMA alone served as positive control and DMSO treated neutrophils as negative control. Statistical analysis was performed using one way ANOVA, values are represented as mean ± SEM, n=3.

2.3- Which are the kinetics of TLR4 levels and activation after LPS treatment?
According to this reviewer, we performed a full time kinetic both in terms of TLR4 concentrations, and in terms of TLR4 activation. For TLR4 activation we employed IL-6 and IL-8 levels as well as the suppression of IRAK, we need to find something independent of TLR3. We have included this kinetic in ***Supplementary figure 5****.*

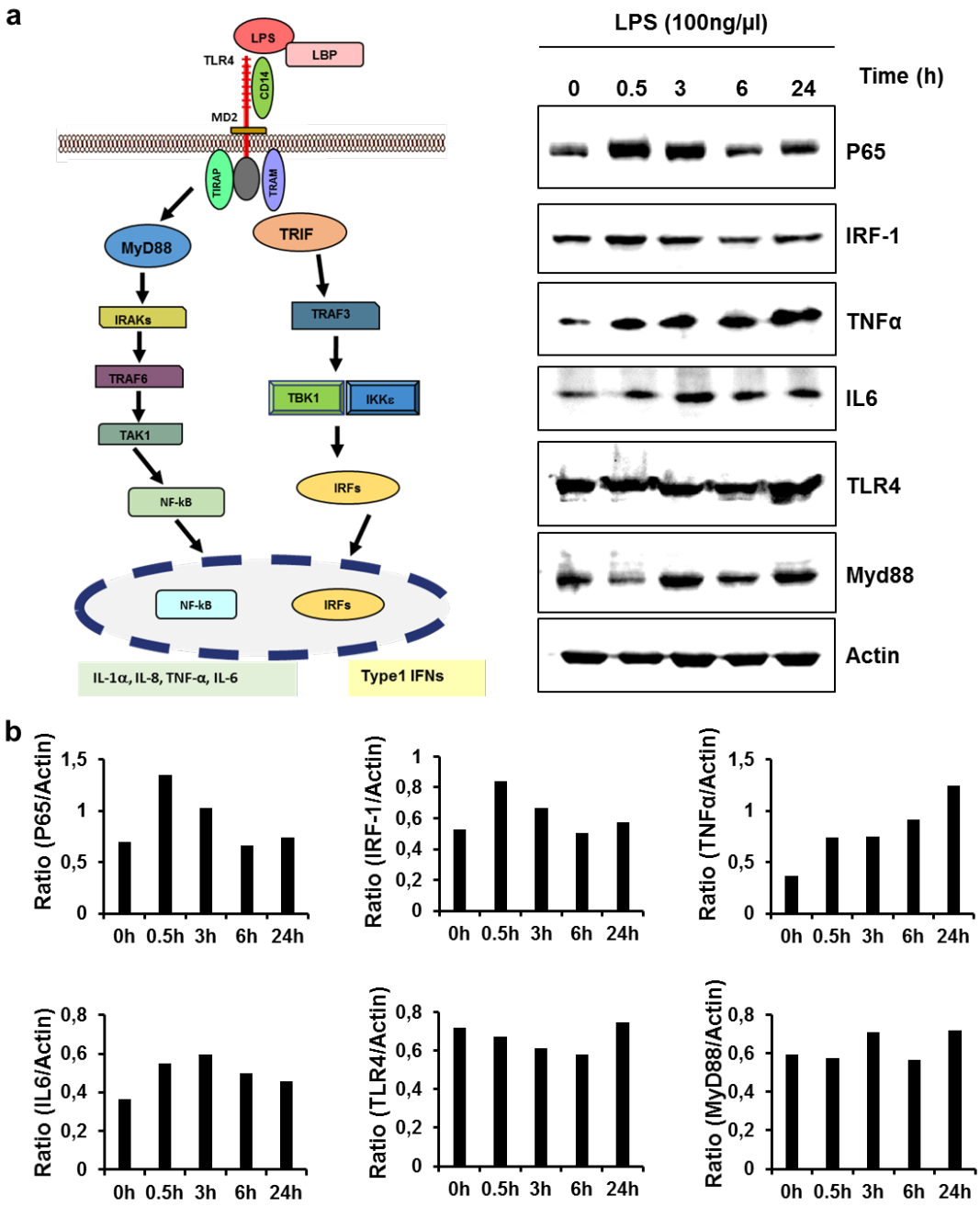


Figure 10. LPS treatment induce TLR4 signaling in a time dependent manner. (a) The representative Western blot analyses depicting activation of TLR4 pathway and its target proteins upon LPS challenge at different time points in MSCs. P65, a component of the heterodimeric NF-κB transcription factor and the IRF-1 transcription factor are swiftly induced at 0.5 h, but down-regulated to basal levels at 6 h after LPS challenge of MSCs. The target genes of NF-κB are induced at 0.5 h and this induction is differentially maintained. The expression of indicated proteins were analyzed in the cell lysates from LPS (100ng/ml) or PBS treated MSCs (n=3). **(b)** The densitometry analysis of respective western blots shown in figure (a).

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2nd Editorial Decision 31 January 2020

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised version of the manuscript:

- Please order the manuscript sections like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Acknowledgements - Author contributions - Conflict of interest - References- Figure legends - Tables and their legends - Expanded View Figure legends

- Please provide a more comprehensive and shortened title of the manuscript with not more than 100 characters (including spaces).

- Please provide the abstract written in present tense throughout.

- In the author contributions, it seems Linda Krug is missing. Please check and declare her contribution.

- 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. Thus, please select 5 of the present Appendix figures as EV figures. 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.

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- Please check that after the above changes all items are called out correctly using the correct nomenclature. Present Supplementary Table 2 seems not to be called out. Please check.

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- Please add scale bars to all magnification boxes shown (e.g. Figs 1C, 3A+B, 6A+D, S3A and S9A), or define their size in the respective figure legend, and indicate more clearly where they originate from (like in Fig. S3). Please try to avoid that the scale bar overlaps with the smaller boxes (as in Fig. S3).

- Several Western blots shown are overcontrasted (e.g. Fig. 6G, alpha-SMA, or Fog. S5B). Please show the blots in the manuscript with similar contrast and brightness as in the source data.

- Thanks a lot for submitting the source data. Please upload the source data files as one file per figure (for main and EV figures). The source data for figures to be shown in the Appendix can be combined into one source data file.

- Regarding data quantification and statistics, can you please check again that, where applicable, the number "n" for how many independent experiments (biological vs. technical replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is specified in the respective figure legends (including the Appendix!).

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

REFEREE REPORTS

Referee #2:

I have now reviewed authors revised manuscript. I would like to congratulate the group on performing all key experiments required to solidly establish their claims. In particular, the potential confounding effect of TLR3 has been extremely well addressed and will make a major impact in the field as siRNA are an emerging therapeutic in the field.

Moreover, the paracrine function of primed MSCs, their sustained immunosuppressive activity, the role of TGFbeta producing macrophages clearly add to the value of this manuscript.

Referee #3:

In the revised version of the manuscript by Munir and colleagues the authors have replied to the reviewer's comments. Thus the manuscript is suitable for publication.

2nd Revision - authors' response 18 February 2020

The authors performed all minor editorial changes.

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- 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.

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- 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.).
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  - 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;
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  - 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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies 5 mice per group were used.
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.	Young male C57 Bl6 mice were randomly selected and allocated to either control or experimental groups. Mice between groups were age-matched.
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.	For wound size analysis, the researchers who performed imaging analysis were blinded to the mice grouping information.
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, described in the corresponding figure legend.
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Is the variance similar between the groups that are being statistically compared?	NA

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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).	Specified in the Materials and Methods section.
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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
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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	Done.
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
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