European Journal of Immunology

Murine macrophage chemokine receptor CCR2 plays a crucial role in macrophage recruitment and regulated inflammation in wound healing

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision

15-Dec-2017

Dear Dr. Boniakowski,

Manuscript ID eji.201747400 entitled "Macrophage chemokine receptor CCR2 plays a crucial role in macrophage recruitment and regulated inflammation in wound healing" which you submitted to the European Journal of Immunology has been reviewed. We are sorry for the delay in the peer review, one of the referees was delayed in submitting the report. The comments of the referees are included at the bottom of this letter.

You will note that the reviewers were split in their enthusiasm. The concern of Reviewer one primarily was with the perceived lack of novelty. We would encourage you to revise with the goal to emphasize precisely where the progress has been made, dealing directly with the issue for this reviewer. Reviewer 2 requested other experiments that were felt to help. In your point by point response, please include responses to all comments and pay particular attention to how you can revise to emphasize where the study has made unique findings, and where it confirms previous work. A revised version of your

manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees™ concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. It is unclear how many experiments you've performed since you've stated only the number of samples/mice. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

On behalf of
Prof. Kenneth Murphy

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Reviewer: 1

Comments to the Author
This manuscript describes that CCR2 is important for wound healing in skin.

I have some difficulty with the following statement which I believe is attempting to establish what has not yet been done:
While it is unknown whether non-classical MoMÎ¦s are recruited from circulation or transition in the wound tissue to Ly6Clo cells from Ly6Chi cells, a clear phenotype shift from proinflammatory to anti-inflammatory has been documented post-injury in many tissues including liver, myocardium, and skeletal muscle[13],[18]"[20].

1) I think that most of these references have shown the switch from classical to non classical Ly6Chi to Ly6Clo monocytes/macrophage. This statement seems to be somewhat misrepresentative. Maybe I am missing the point but reference 13 showed a clear switch. So did the other references.
2) The authors go on to argue that no one has looked in skin however reference 19 above looked at subcutaneous recruitment of monocytes and showed they switched. I am not sure whether the authors are calling this study skeletal muscle but this is not correct.
3) I really think the authors need to come up with a better introduction to establish novelty.
4) I am also failing to understand why the following articles were not considered in the introduction:
CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. Willenborg S1, Lucas T, van Loo G, Knipper JA, Krieg T, Haase I, Brachvogel B, Hammerschmidt M, Nagy A, Ferrara N, Pasparakis M, Eming SA.

In vivo imaging reveals a pioneer wave of monocyte recruitment into mouse skin wounds. Rodero MP1, Licata F1, Poupel L1, Hamon P1, Khosrotehrani K2, Combadiere C1, Boissonnas A1.

In fact, the first article really has done what the authors have claimed has never been done in skin. They do try to dampen the significance of this study in the discussion, but I am unconvinced.

5) The authors could easily examine the role of neutrophils if they think there is an effect on neutrophils in the CCR2-/- mice.
6) I am failing to understand the suggestion that the macrophage recruited into the wound in CCR2-/- mice had less inflammatory cytokines. What is the evidence any macrophage were recruited? This could just be tissue resident macrophage that are already at the site.

Reviewer: 2

Comments to the Author
Boniakowski et al. extend the findings that CCR2+ monocytes are recruited to sites of inflammation for

tissue repair. Their data is novel in that it extends the fields knowledge to the skin. In general, the paper is well-written and the data matches their interpretations. I have only minor comments.

1) Although the authors mention MIP-1/CCL2 being necessary for recruiting LY6c hi monos to sites of inflammation in the introduction and in their model in Figure 1, they did not show that CCL2 is being made at the site of inflammation. It is possible that because monocytes fail to exit the bone marrow in CCR2 deficient animals (Serbina et al. Nature Immunology 2006), that their result would hold true whether or not CCL2 is being made at the cite of inflammation. The final experiment in figure 5 may or may not agree with this interpretation (transfer of CCR2 sufficient monos into CCR2 deficient recipients). It would benefit the paper if the authors were able to show that CCL2 is being made at the site of inflammation. A simple ELISA or qPCR on inflamed vs non-inflamed tissue would suffice.

2) In the final experiment, it would be good to show that the transferred monocytes are indeed recruited to the site of inflammation and that they contain the missing LY6c hi population of monocytes. It has been shown through fate-mapping studies that LY6c hi monocytes are most likely the precursor to LY6c lo monocytes, and that LY6c hi monocytes have a short half-life of less than a day (Yona et al. Immunity 2013). Therefore, are the LY6c hi macrophages found at the site of inflammation constantly being replenished from circulation or are they trafficking and seeding for long-term residency? Essentially my question is, do the transferred cells in experiment 5 live for the entirety of the experiment at the site of inflammation or are they only necessary for a brief time frame? Checking the site of inflammation for seeded monocytes at Day 2 and Day 4 (as previously shown in the figure) would be sufficient.

3) Lastly, Figure 1 would most likely fit better as the last figure as a summary of all the data. (This comment is more for ease of reading, it is merely a subjective opinion and not necessary to change.)

In summary, I believe the authors show that CCR2 plays a role in wound healing in the skin, however, their data would be greatly supported by adding the experiments suggested.

First Revision – authors' response

17-Apr-2018

We greatly appreciate the opportunity to revise and improve our manuscript. We would like to thank the editor and reviewers for the comments regarding our submitted article, manuscript ID eji.201747400: "Macrophage chemokine receptor CCR2 plays a crucial role in macrophage recruitment and regulated inflammation in wound healing." We have made significant revisions to the paper and have added a substantial amount of new data (Figure 4, Supplemental Figures 1 and 2). We have complied with all reviewer requests and thus, our revised manuscript incorporates all of the reviewers' suggestions, experiments, and addresses comments where appropriate. Thus, we would like to submit a revised version of our manuscript as well as a point-by-point response to the reviewers' comments. Thank you for your

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consideration of our revised manuscript. We wish to express our strong and sincere appreciation for your precise comments that have undoubtedly allowed us to significantly improve the quality of our manuscript. The changes in the manuscript are highlighted in red for your ease of review. We feel that the reviewers' suggested experiments/changes have strengthened our manuscript and have enhanced the conclusions of our paper. Thank you for your consideration of our manuscript. Please find our point-by-point response to the reviewers' comments below:

Reviewer: 1

Comments to the Author
This manuscript describes that CCR2 is important for wound healing in skin.

We thank the reviewer for this very detailed and considerate review that has undoubtedly allowed us to improve our paper. We have addressed all concerns with additional experiments and discussion where necessary. We have completely revised the introduction and discussion sections and have added a revised Figure 4 and two new Supplemental Figures to address all concerns detailed below. We have also added significant text to all sections of the paper in an effort to clarify these criticisms and improve the manuscript.

I have some difficulty with the following statement which I believe is attempting to establish what has not yet been done:
"While it is unknown whether non-classical MoMΦs are recruited from circulation or transition in the wound tissue to Ly6Clo cells from Ly6Chi cells, a clear phenotype shift from proinflammatory to anti-inflammatory has been documented post-injury in many tissues including liver, myocardium, and skeletal muscle[13],[18]–[20]."

1) I think that most of these references have shown the switch from classical to non classical Ly6Chi to Ly6Clo monocytes/macrophage. This statement seems to be somewhat misrepresentative. Maybe I am missing the point but reference 13 showed a clear switch. So did the other references.

We thank the reviewer for this comment, and agree that the references included to support this statement identify that there is a transition from Ly6CHi to Ly6CLo cells in the respective tissues. Our statement was not clear, as we were trying to also acknowledge that there is also literature to suggest that Ly6CLo cells may be recruited directly from the circulation, and not derived from Ly6CHi cells(1). We have now significantly expanded this section in the introduction to more clearly state the relevant studies. Further, to differentiate our study from previous work, we have added literature related to resident versus recruited macrophages in tissues. We have now substantially modified our text in the introduction to address these concerns.

2) The authors go on to argue that no one has looked in skin however reference 19 above looked at subcutaneous recruitment of monocytes and showed they switched. I am not sure whether the authors are calling this study skeletal muscle but this is not correct.

We appreciate this comment from the reviewer and fully agree that as written, we have not thoroughly explained how our work differs from the cited manuscripts. The manuscript by Crane et al. (reference 19) used a wound healing model where they inserted a foreign body and then removed the sponge at different time points for cell extraction; whereas in our model we created a full thickness punch biopsy of the dermis/epidermis and then collected the granulation tissue for cell extraction. Additionally, in the Crane paper, they examined F4/80+ cells, which are the tissue resident macrophages. This is a different population of cells than the recruited myeloid cells that we examined. Since the majority of cells that are involved in the inflammatory phase post-injury are recruited from the blood, we chose to focus on this population. Additionally, the F4/80+ resident macrophages populate the tissues prior to birth and are replaced through self-renewal(2,3), making their full characterization in granulation tissue lower yield. Furthermore, in one of our recent publications(4), we found that F4/80+ tissue resident macrophages did not significantly influence the inflammatory profile of the wounds. We have now added this information to our "Introduction" and "Discussion" sections to further clarify the cell populations examined in our study and the differences with regard to previous studies.

3) I really think the authors need to come up with a better introduction to establish novelty.

We completely agree that, as written, our introduction failed to articulate its novelty. We have now significantly restructured our introduction, with specific attention describing how our research is different

from the previous literature on CCR2's role in cutaneous wound healing. We feel that addressing this concern from the reviewer has significantly improved our manuscript and the novelty of our study.

4) I am also failing to understand why the following articles were not considered in the introduction:
CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. Willenborg S1, Lucas T, van Loo G, Knipper JA, Krieg T, Haase I, Brachvogel B, Hammerschmidt M, Nagy A, Ferrara N, Pasparakis M, Eming SA.

In vivo imaging reveals a pioneer wave of monocyte recruitment into mouse skin wounds. Rodero MP1, Licata F1, Poupel L1, Hamon P1, Khosrotehrani K2, Combadiere C1, Boissonnas A1.

In fact, the first article really has done what the authors have claimed has never been done in skin. They do try to dampen the significance of this study in the discussion, but I am unconvinced.

We sincerely appreciate this comment from the reviewer, as we realize that our introduction did not clearly articulate the differences in the experimental methods between these manuscripts and our own work. We have now added the paper by Rodero et al and Willenborg et al to the introduction, with a discussion regarding both similarities and differences. The reviewer is absolutely correct that, like our paper, these papers focus on cutaneous wound healing. However, they differ significantly in the population of cells examined. Both of these papers focus on the F4/80+ population of CD11b+ cells, which is the tissue resident macrophage population. Furthermore, Rodero et al only examined these cells during the first 4 hours post-injury, whereas we wanted to examine wound healing over the first few days, during the inflammatory phase, when the recruited monocyte/ macrophages play a critical role influencing inflammation/healing. The study by Willenborg et al also focused on F4/80+ tissue resident macrophages, and did not begin characterizing the cell expression profile until day 4-14; likely because their paper focused on proangiogenic factors that are important after the initial inflammatory phase has subsided. Thus, our paper is novel in that we characterize the role of CCR2 in the recruited monocyte population (non-F4/80+) specifically during the early inflammatory phase (days 1-4). We realize that our text did not accurately articulate this, and thus we have now clarified this in both the introduction and discussion sections.
5) The authors could easily examine the role of neutrophils if they think there is an effect on neutrophils in the CCR2-/- mice.

We completely agree that neutrophils play an important role in early inflammation, and agree that it is very possible that they have an effect on early inflammation in the CCR2-/- mice. To investigate this, we performed flow cytometry to look at neutrophils within wounds, and compared the neutrophil population between CCR2-/- and CCR2+/+ mice. To do this, we isolated wounds on day 3, and gated in neutrophils (defined as live, lineage-, Ly6G+ cells). We found no difference in the percentage of neutrophils in the CCR2-/- wounds compared with control CCR2+/+ wounds. Thus, our findings suggest that the lack of CCR2 did not affect neutrophil recruitment/presence in wounds. We have now added a figure (Supplemental Figure 2) to illustrate this point.

6) I am failing to understand the suggestion that the macrophage recruited into the wound in CCR2-/- mice had less inflammatory cytokines. What is the evidence any macrophage were recruited? This could just be tissue resident macrophage that are already at the site.

We agree that additional evidence was needed to determine whether the reduced inflammatory was secondary to recruited macrophages. Thus, we performed several additional adoptive transfer experiments to answer this question. For these experiments, macrophages (CD3-, CD11c-, CD19-, Ly6G-, NK1.1-, CD11b+ cells) were isolated from mT/mG mice and adoptively transferred via tail vein injection to a cohort of CCR2-/- and CCR2+/+ mice. These mice were then wounded and in vivo macrophages were analyzed on days 2 and 4 to examine whether our labeled cells tracked to these wounds. We found a significant number of td tomato red cells were recruited to the wounds (new Figure 4D). Additionally, in a recent publication by our group(4), we examined the F4/80+ cell population in wounds and did not find a significant number of F4/80+ cells on days 2-5. This is likely because the full thickness excision concomitantly removed many of the F4/80+ resident macrophages, and left us to interrogate early granulation tissue with fewer F4/80+ cells. The results from our adoptive transfer showing significant recruitment of labeled monocyte/macrophages to the wound, coupled with our previously published findings, suggest that the inflammatory profile we were analyzing was from the recruited monocyte population rather than the tissue resident cells. We have now added a new figure (Figure 4D) to help clarify this point.

Reviewer: 2

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Comments to the Author
Boniakowski et al. extend the findings that CCR2+ monocytes are recruited to sites of inflammation for tissue repair. Their data is novel in that it extends the fields knowledge to the skin. In general, the paper is well-written and the data matches their interpretations. I have only minor comments.

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We appreciate this comment from the reviewer and completely agree that this information regarding CCL2 will improve our study. We have now performed an additional experiment to investigate CCL2 in wound tissue. We have now examined levels of CCL2 on day 2 in both CCR2+/+ and CCR2-/- wounds. We did find CCL2 produced in wounds from both CCR2+/+ and CCR2-/- mice, however we did not find statistically significant differences between the groups (Supplemental Figure 1). Thus, we concluded that CCL2 is produced in the wounds at the site of inflammation and thus, there is still recruitment of monocyte/macrophages to wounds in CCR2-deficient animals.

2) In the final experiment, it would be good to show that the transferred monocytes are indeed recruited to the site of inflammation and that they contain the missing LY6c hi population of monocytes. It has been shown through fate-mapping studies that LY6c hi monocytes are most likely the precursor to LY6c lo monocytes, and that LY6c hi monocytes have a short half-life of less than a day (Yona et al. Immunity 2013). Therefore, are the LY6c hi macrophages found at the site of inflammation constantly being replenished from circulation or are they trafficking and seeding for long-term residency? Essentially my question is, do the transferred cells in experiment 5 live for the entirety of the experiment at the site of inflammation or are they only necessary for a brief time frame? Checking the site of inflammation for seeded monocytes at Day 2 and Day 4 (as previously shown in the figure) would be sufficient.

We thank the reviewer for this comment and completely agree that additional experiments are necessary to show that injected macrophages are actually recruited to the wound. Thus, we performed additional adoptive transfer experiments. For these experiments, macrophages (CD3-, CD11c-, CD19-, Ly6G-, NK1.1-, CD11b+ cells) were isolated from mT/mG mice and adoptively transferred via tail vein injection to a cohort of CCR2-/- and CCR2+/+ mice (see new Figure 4D schematic). These mice were then wounded and in vivo macrophages were analyzed by flow cytometry on days 2 and 4 to examine whether our labeled cells tracked to these wounds. We found a significant number of td tomato red cells were recruited to the wounds on day 2 and that this number increased by day 4 (new Figure 4D). We found that there was a higher percentage of Ly6CHi and Ly6CLo cells recruited to the CCR2-/- on day 2, and that this trend was not significant by day 4, suggesting an earlier cell recruitment in the CCR2-deficient mice. Additionally, inflammatory Ly6CHi cells were higher in the CCR2+/+ to CCR2-/- mice, suggesting this may be responsible for the restoration of normal inflammation and improved healing in the CCR2-deficient mice that received WT cells. This data suggests that by day 4, a significant number of wound macrophages have been recruited from the blood and thus, restoration of normal inflammation is the likely mechanism for the improved wound healing seen in the CCR2-deficient mice that received WT cells.

3) Lastly, Figure 1 would most likely fit better as the last figure as a summary of all the data. (This comment is more for ease of reading, it is merely a subjective opinion and not necessary to change.)

We agree that this figure would fit better as the last figure since it represents a summary of our data, and thus it is now Figure 5 of our paper.

In summary, I believe the authors show that CCR2 plays a role in wound healing in the skin, however, their data would be greatly supported by adding the experiments suggested.

We appreciate the reviewers' positive response and have addressed all points and performed the additional experiments that were suggested. We thank the reviewers for the opportunity to revise and improve our manuscript.

References
1. Olingy CE, San Emeterio CL, Ogle ME, Krieger JR, Bruce AC, Pfau DD, et al. Non-classical monocytes are biased progenitors of wound healing macrophages during soft tissue injury. Sci Rep. 2017;7(1).
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3. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. Immunity. 2013;38(1):79–91.
4. Kimball A, Schaller M, Joshi A, Davis F, denDekker A, Boniakowski A, et al. Ly6C^{Hi} Blood Monocyte/Macrophage Drive Chronic Inflammation and Impair Wound Healing in Diabetes Mellitus. Arterioscler Thromb Vasc Biol [Internet]. 2018 Jan 1; Available from: http://atvb.ahajournals.org/content/early/2018/02/28/ATVBAHA.118.310703.abstract

Second Editorial Decision

29-May-2018

Dear Dr. Gallagher,

It is a pleasure to provisionally accept your manuscript entitled "Macrophage chemokine receptor CCR2 plays a crucial role in macrophage recruitment and regulated inflammation in wound healing" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: https://onlinelibrary.wiley.com/toc/15214141/0/ja). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Britta Engelhardt

Dr. Nadja Bakocevic
Editorial Office

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