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CORRESPONDENCE

Does CXCR3 chemokine receptor expression by CD8⁺ T cells affect their moving towards or only their binding to virus-infected monocytes? [version 1; peer review: 1 approved with reservations]

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Abstract

This correspondence concerns a recent publication in *Immunity* by Hickman *et al.*¹ who analyzed the effect of *Cxcr3* knockout on migration of CD8⁺ T cells towards and within vaccinia virus-infected mouse ears. They found that *Cxcr3* knockout had no effect on CD8⁺ T cell migration into the infected ears, a relatively mild effect on virus clearance, and an effect on the contact of CD8⁺ T cells with virus-infected cells. Curiously, despite having these basically sound and interesting data, Hickman *et al.* exaggerated the effect on virus clearance ("dramatically impaired virus clearance") and focused their conclusions on assumed differences in migration towards infected cells ("CXCR3 chemokine receptor enables local CD8⁺ T cell migration") rather than on better proven differences in binding to infected cells. I believe that from the data presented by Hickman *et al.* on the effect of *Cxcr3* knockout a migration effect independent from the binding effect cannot be concluded beyond discussion. The fact that CXCR3 is a chemokine receptor, and that most researchers consequently expect a chemokine-gradient-dependent migration effect of the *Cxcr3* knockout mutation, increases the risk of misleading readers when approached through the Hickman *et al.* narrative. The here-initiated discussion of their article may help to avoid such a misleading.

Keywords

CXCR3, T cell, CD8, virus, monocyte, migration, binding, intravital multiphoton microscopy

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1

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Any reports and responses or comments on the article can be found at the end of the article.

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Hickman *et al.*¹ established a vaccinia virus (VV) mouse ear-infection model, and investigated the importance of CXCR3 expression by CD8⁺ T cells for their ability to locate and bind to infected cells, and to help eradicate the virus. The receptor CXCR3 can bind the chemokines CXCL9 and CXCL10, of which genes in this mouse ear model were found to be abundantly expressed by virus-infected tissue¹. Using a number of techniques, Hickman *et al.* assayed the migration of CD8⁺ T cells into the infected ears, including into dense areas of infected monocytes, and to the draining lymph nodes. Among the techniques used were intravital multiphoton microscopy (MPM) experiments, which allowed them beautifully to follow the migration of individual CD8⁺ T cells. Recipient mice included wildtype (wt; albino C57BL/6), *CXCR3*^{-/-} and *CD8*^{-/-} genotypes, while CD8⁺ lymphocytes used for grafting were isolated from CFP OT-I and *CXCR3*^{-/-} DsRed OT-I transgenic mice. The recombinant vaccinia viruses used were VV-NP-eGFP and VV-NP-S-eGFP, of which only the latter contains the CD8⁺ T cell determinant peptide sequence SIINFEKL which can be recognized in the MHC context of this system by the T cell receptor transgenically expressed in OT-I mice. Hickman *et al.* investigated a number but not all possible combinations of these mice, cells and viruses, and their different effects on virus replication and CD8⁺ T cell migration.

Findings by Hickman *et al.* were that (1) viral clearance by *Cxcr3*^{-/-} CD8⁺ T cells was somewhat impaired at intermediate times of infection, (2) migration of *Cxcr3*^{+/+} and *Cxcr3*^{-/-} CD8⁺ T cells into infected ears and also to draining lymph nodes was similarly efficient, (3) both *Cxcr3*^{+/+} and *Cxcr3*^{-/-} CD8⁺ T cells could be found in dense areas of VV-infected monocytes, although the relative abundance was about 1.4-fold higher for *Cxcr3*^{+/+} CD8⁺ T cells (see their Figure 5D), (4) *Cxcr3*^{-/-} CD8⁺ T cells were more motile than *Cxcr3*^{+/+} CD8⁺ T cells in dense areas of infected monocytes (see their Figure 5F), (5) *Cxcr3*^{+/+} CD8⁺ T cells probably could bind better to virus-infected cells than found for *Cxcr3*^{-/-} CD8⁺ T cells (see their Figures 5H and 5I), and (6) could also kill these cells more efficiently (see their Figure 6).

Together with the high-quality pictures and videos, the above listed data, most of which appear solid, contained enough substance for publication. For reasons that are not supported by the data, however, in their narrative Hickman *et al.* exaggerated the effect of the *Cxcr3* knockout on viral clearance, and speculated as a major model of explanation that *Cxcr3* knockout reduced penetration of CD8⁺ cells into infected cell areas. Here I expand on the two main issues, I and II, followed by a direct listing of the eight questions/issues as I placed them on the *Immunity* site (the first two therein overlap with the below items I and II). I moved the discussion to *F1000Research* to improve the visibility of what I hope should be a valuable public discussion.

(I) Upon comparison of the development of VV-infection in ears of wt and *Cxcr3*^{-/-} mice, Hickman *et al.* obtained results that they presented in their Figure 3F and summarized as “Likewise, we saw no overt changes in morbidity after epicutaneous VV infection; however, *Cxcr3* deficiency interfered with viral clearance at intermediate times of infection (days 5–7)”. This statement is correct in stressing the modesty of the effect, but neglects that at day 5 post infection (p.i.) the virus titer is more than four-fold higher in wt

than in *Cxcr3*^{-/-} mice. This fact should have been either noted and discussed or the data re-evaluated in case of an error. This issue would probably not have remained unnoticed if the main narrative had properly stressed that the *Cxcr3*^{-/-} knockout effect on virus clearance was rather modest and restricted to days 5–7 p.i. Instead, however, the Summary section of the article says “*Cxcr3*^{-/-} mice exhibited dramatically impaired CD8⁺-T-cell-dependent virus clearance”, and this interpretation may have prevented co-authors, editors and reviewers from taking a thorough look at the day 5 p.i. result.

(II) Whether the Figure 3F issue will seriously affect our understanding of CXCR3 function is questionable, but that might well be the case with another issue. By proper quantification, Hickman *et al.* show that the *Cxcr3*^{-/-} CD8⁺ T cells are more motile and less abundant than *Cxcr3*^{+/+} CD8⁺ T cells in dense areas of infected monocytes (see their Figures 5D and 5F), which may be explained by differences in binding to the virus-infected cells (see their Figures 5H and 5I). However, without support by proper evidence and at least without proper quantification, the authors portray as their major model that the *Cxcr3*^{-/-} CD8⁺ T cells have relative difficulties to enter dense areas of infected monocytes. The evidence should come from data presented in Figure 5C and movie S6, but the data is finally drawn together in a rather subjective and non-quantitative interpretation by the authors “Overall, however, whereas high numbers of wt T cells entered fields of virus-infected cells, *Cxcr3*^{-/-} T cells hesitated at the perimeter of heavily infected areas of the tissue (Figure 5C; Movie S6).” Their Figure 5D shows that the percentages of *Cxcr3*^{+/+} CD8⁺ T and *Cxcr3*^{-/-} CD8⁺ T cells are on average around 44% and 32%, respectively, and I wonder, even if the relatively small differences in presence levels could in part be explained by differences in penetration efficiencies, how these penetration efficiencies could reliably be judged without proper quantification. The proper assay for quantifying the penetration efficiencies would probably be the MPM technique shown in Figure 5E, but if I interpret the Figure 5E results correctly, the *Cxcr3*^{-/-} CD8⁺ T cells have no apparent deficit in penetrating the virus-infected region, they only more readily leave. The lower presence of the *Cxcr3*^{-/-} CD8⁺ T cells compared to the *Cxcr3*^{+/+} CD8⁺ T cells might be entirely explained by the lesser binding to the virus-infected cells, causing faster migration within this region and easier leaving of the region. It seems to me that, because CXCR3 is a chemokine receptor, Hickman *et al.* forcefully concentrate on a model different from binding. In my opinion, they should have focused on trying to find reasons for the differences in binding efficiencies, rather than assume without proper evidence that *Cxcr3*^{-/-} CD8⁺ T cells have difficulties infiltrating regions of infected cells. Hickman *et al.* expressed their belief in the reduced penetration model in their title “CXCR3 chemokine receptor enables local CD8(+) T cell migration for the destruction of virus-infected cells”, in their description of the Figure 5D result “Although wt and *Cxcr3*^{-/-} T cells were equally distributed outside of infected areas of the dermis, a higher percentage of wt T cells than of *Cxcr3*^{-/-} T cells penetrated virus-infected areas (Figure 5D)”, and at several other sites in the article.

In summary, Hickman *et al.* produced good and interesting data, but they harmed their own article by an exaggerated and misleading narrative. I would like to invite Hickman *et al.* to address the above

concerns, as well as a few other issues as they were already placed on the *Immunity* site:

1. The major conclusion of the authors regarding the importance of CXCR3 for clearance of vaccinia virus from infected mouse ears appears to be summarized in their statement: “Likewise, we saw no overt changes in morbidity after epicutaneous VV infection; however, *Cxcr3* deficiency interfered with viral clearance at intermediate times of infection (days 5–7)”. I wonder how this conclusion agrees with the day 5 result depicted in Figure 3F, which says that the virus-titer in wt mice is about four-fold higher. This observation is unlikely to be within chance variation, given the small SEM values and the fact that “All experiments were repeated at least three times with $n = 2-5$ mice/group”. Probably the $p < 0.0001$ value depicted in the figure refers to this measurement.

2. The authors conclude that *Cxcr3*^{-/-} CD8⁺ cells show reduced penetration of dense areas of infected monocytes compared to wt CD8⁺ cells. However, the only parameter that appears to be measured reliably is the number of cells present, and if I interpret Figure 5E correctly, the *Cxcr3*^{-/-} cells are not impaired in their ability to penetrate the infected area but only leave more easily (and then often come back again). From Figure 5C and movie S6, I can’t follow the interpretation by the authors that “Overall, however, whereas high numbers of wt T cells entered fields of virus-infected cells, *Cxcr3*^{-/-} T cells hesitated at the perimeter of heavily infected areas of the tissue (Figure 5C; Movie S6).”

3. The authors conclude that “*Cxcr3*^{-/-} CD8⁺ T Cells activate and home normally”. Whereas most of their experimental data were obtained at day 5 p.i., for the “normal homing” conclusion in regard to draining lymph nodes they looked at day 2 p.i. (Figure 4). As far as I understand, for “homing” some degree of specificity should be shown. For the presence of transferred cells in infected ears the authors provide evidence of active homing in Figure S1, because, as they show, the transferred cells do not traffic to uninfected ears. I can see little evidence that the transferred cells detectable in the draining lymph node already at day 2 after infection are there because of active homing? Also in regard to their Figure 1B findings, day 2 p.i. may not be an appropriate time point to examine specific homing.

4. The authors write “Together, these data show that CXCR3 is not required for SIINFEKL-expressing VV-induced activation of OT-I CD8⁺ T cells *in vivo*”, which most readers will interpret as that

the SIINFEKL peptide was relevant. This is potentially misleading as the authors do appear not to have analyzed the effect of the SIINFEKL-epitope on the activation of *Cxcr3*^{-/-} OT-I CD8⁺ cells.

5. It might be easier to interpret the findings if data measuring the outcome of the infection in *CD8*^{-/-} hosts in the context of the different cell transfers were included. The report seems to concentrate on measurements at day 5 after infection. Did, for example, the mice survive?

6. This is a study about clearance of virus from the mouse ear. Figure S3B shows that about 50% of the infected cells are CD45-negative, and consist mostly of keratinocytes as the authors explain. The virus infections of monocytes and keratinocytes should probably be understood as communicating barrels. Then why is it that for most experiments the authors chose not to show any results for the CD45-negative cells, although they systematically show virus titers and numbers of infected monocytes per ear? I feel this is an exaggeration of the principle to avoid unnecessary complexity in scientific stories.

7. In Figure 4B the authors show that two days after infection the endogenous CD8⁺ cells in the draining lymph nodes do not express detectable IFN γ levels. But, apparently, at this day the inflammation had hardly started (see Figure 2C). Nevertheless, in other experiments measuring at day 5 p.i., at which the inflammation is pronounced, the authors seem to use the Figure 4B result to define all IFN γ ⁺ CD45⁺ cells as transferred CD8⁺ T cells (see Figures 6B-D and the main text discussion of these results). With regard to IFN γ expression by endogenous cells, it would be interesting to have further justification why the data of a non-inflamed situation (day 2 p.i.) were extrapolated to an inflamed situation (day 5 p.i.).

8. As a small point, it is unclear what is shown by the green color in Figure 4D, where green stains CD69 as well as virus infected cells.

Competing interests

The authors declared no competing interests.

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Joanna R Groom

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Without changing the main findings of the article "CXCR3 Chemokine Receptor Enables Local CD8+ T cell Migration for the Destruction of Virus-Infected Cells" (Hickman *et al.* Immunity 2015), the author wishes to clarify some discussion points.

Firstly, the author claims the Hickman *et al.*, "exaggerated the effect of CXCR3 knockout on viral clearance". The data being referred by the author is Figure 3E,F, which demonstrates an increase in infected inflammatory monocytes/ear at days 5-7 in CXCR3^{-/-} OT-I transferred animals and ear viral titres respectively. Indeed there is a disconnected in the Day 5 data, where the CXCR3^{-/-} OT-I transferred animals have a lower titer, but higher infected monocytes compared to WT animals. This data should be re-evaluated by Hickman *et al.*, for this discrepancy. However, the author fails to recognise or mention any data provided in Figure 6 E-J, which revisits the reduced viral titre in CXCR3^{-/-} OT-I transferred animals. This data provides significant evidence of reduced clearance of infected monocytes and viral titer. All this data, appears to be generated on Day 5 of infection, thus conclusions of Hickman *et al.* are valid. The author should comment on the data presented in Figure 6 prior to acceptance of this critique.

The second, and major area of clarification refers to the discussion of whether CXCR3^{-/-} is required for entry into areas of high infection or the binding of infected cells. The images and supplementary movies, show the clear accumulation of CXCR3^{-/-} OT-I cells at the perimeter of highly infected tissues, which has led Hickman *et al.* to highlight the role for CXCR3-dependent migration into these areas. One issue complicating the interpretation of this data is the phenotype in the CXCR3^{-/-} animals that have large, high density regions of infected cells. These areas are missing in WT or co-transferred (both genotypes) OT-I animals, due to the clearance of viral infected monocytes. Therefore, the accumulation of OT-I cells at the periphery of these heavily infected areas of tissue can not be equally assessed, and the data presented using co-transferred genotypes is somewhat less compelling. Experiments that transfer WT OT-I cells into animals following establishment of infection could help answer this question, but as the data stands, there is not a clear indication that the accumulation of CXCR3^{-/-} OT-I cells is due to the infection or

CXCR3-dependent migration. Hickman *et al.*, observe that although reduced, CXCR3^{-/-} OT-I cells do areas of high infection and have compelling data (Figure 5 E-I) indicating that once there, these cells are more mobile, and have reduced contacts with infected tissue. These experiments indicate a mechanism for CXCR3 beyond entering the site of high infection. Hickman *et al.*, conclude that "these data show that relative to WT cells CXCR3^{-/-} CD8⁺ T cells exhibit decreased presence in virus-infected regions and shorter contacts with infected cells", however the author is not satisfied with the discussion of the major phenotype within infected tissue – the reduced binding of CXCR3^{-/-} OT-I cells to infected monocytes. This critique highlights the need for following studies that primarily focus on the mechanism of CXCR3 within infected viral tissues; migration and penetration into high viral areas versus the binding of infected cells – or both. The study from Hickman *et al.*, describes data where both mechanisms are likely to play a role and they present this data openly.

Hickman *et al.*, have published an exhaustive study investigating the role for CXCR3 in the clearance of skin Vaccinia Virus infection by CD8⁺ cells. Overall this author provides an evaluation of the Hickman *et al.*, paper that may enrich discussions on the mechanism of CXCR3, but does not alter the results or interpretation of the manuscript.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 16 Nov 2015

Johannes M. Dijkstra, Fujita Health University, Dengaku-gakubo, Japan

Dear Dr. Groom,

Thank you for your review, it is highly appreciated.

I am glad that you agree with me that Hickman *et al.* should re-evaluate their Fig. 3F. After I will have received all reviewer reports, I will rewrite my correspondence and incorporate your request to discuss the Fig. 3F issue in regard to other day 5 findings presented by Hickman and co-workers. The simplest explanation would be that an error occurred during the construction of Fig. 3F, but it is up to Hickman and co-workers to elaborate on that.

In regard to viral clearance the Hickman *et al.* study is about a time-window effect (days 5-7), whereas they found that the times of total clearance appeared to be similar in the different backgrounds. Whether a restricted time-window effect can be considered "dramatically impaired viral clearance" may be a matter of taste, but because of their writing style it took me a while to realize that their study was only about a time-window effect. I can only assume that co-authors and reviewers had the same problem, because as soon as one does focus on days 5-7, one immediately sees the discrepancy of the Fig. 3F day 5 result. I will not change that part of my criticism, because I think that it is fair.

As for the discussion on whether CXCR3 is important for entering the areas of infected monocytes or for binding to the infected cells. You appear to agree with me that both

mechanisms could be (partially) responsible, and that the evidence should have come from a direct comparison between CXCR3-/- OT-I cells and WT OT-I cells. In regard to the reduced penetration model you state "the data presented using co-transferred genotypes is somewhat less compelling" whereas for the reduced binding model you state that the authors "have compelling data (Figure 5 E-I) indicating that once there, these cells (note by me: CXCR3-/- OT-I cells) are more mobile, and have reduced contacts with infected tissue". So we largely agree on that matter, although I may feel more strongly than you that for the reduced penetration model Hickman *et al.* did not present any convincing evidence. The compelling part of their reduced contact model derives from the fact that they properly quantified the times of contacts and speeds of migration through the infected cell areas, and the "less compelling" part from their reduced penetration model derives from the fact that they did not quantify it.

Nevertheless, Hickman *et al.* chose to highlight the penetration model as the major conclusion in their title, which says that CXCR3 enables migration. By all means I think that this is wrong from logic point of view, as they should have only highlighted the model for which they provided evidence and not the model for which they did not provide solid evidence. I appreciate that you add some nuance to the debate, but I probably will not change my text on this matter.

I fully agree with your statement "This critique highlights the need for following studies that primarily focus on the mechanism of CXCR3 within infected viral tissues; migration and penetration into high viral areas versus the binding of infected cells – or both." The important part of discussions like the current one is to help sharpen the questions for future research, and I thank you again for your contribution.

Sincerely,

Johannes Dijkstra

Competing Interests: I declare that I have no competing interests that affected my response to the Reviewer.

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