European Journal of Immunology

Peer review correspondence

Flagellin-induced expression of CXCL10 mediates direct fungal killing and recruitment of NK cells to the cornea in response to *Candida albicans* infection

Xiaowei Liu, Nan Gao, Chen Dong, Li Zhou, Qing-Sheng Mi, Theodore J. Standiford, and Fu-Shin X. Yu

Correspondence: Dr. Fu-Shin X. Yu, Kresge Eye Institute, Wayne State University School of Medicine, 4717 St. Antoine Blvd, Detroit, MI, 48201, USA

Review Timeline:	Submission date:	20 January 2014
	First Editorial decision:	10 March 2014
	First Revision received:	24 March 2014
	Second Editorial decision:	22 April 2014
	Second Revision received:	29 April 2014
	Accepted:	20 June 2014

Handling Executive Committee member: Prof. Marco Colonna

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 – 10 March 2014

Dear Dr. Yu,

Manuscript ID eji.201444490 entitled "Flagellin-induced Expression of CXCL10 Mediates Direct Fungal Killing and Recruitment of NK Cells to the Cornea in Response to Candida albicans Infection" which you submitted to the European Journal of Immunology has been reviewed. Please accept my sincere apologies for the prolonged delay in the review of your manuscript. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication.

You should also pay close attention to the editorial comments included below. **In particular, please also edit your figure legends to follow Journal standards as outlined in the editorial comments. 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 referee(s) 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 referee(s) 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,
Laura Soto Vazquez

On behalf of
Prof. Marco Colonna

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Reviewer: 1

Comments to the Author
To state (p3 line 22) that there is no topical treatment for candida keratitis is misleading. While not optimal, topical polyenes (amphotericin) can be effective.

Figure 1 legend, n for D and E should be stated.

Part B of figure 2 should be redrawn with the bars having patterns (ie different fills) as it is very difficult to distinguish between them.

Legend of Figure 3 (p29 line 16) refers to bacterial inoculation. Also there should be scale bars on the images.

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Results text p9 line 49 refers to figure 1 when it should be 5. Also the text refers to arrows (line 57) yet there are none on the figure. All images in Figure 5A look “bleached” other than the PBS-IgG, this makes them difficult for the reader to properly interpret. The cfu in these experiments appears to be much higher than other experiments – what is the explanation for this?

Figure 6 A. The images from IgG and NK1.1. antibody treated animals do not appear to be the same magnification. The images in part B need scale bars.

P10 line 51 should be MPO not MOP.

That NK cells are depleted by the antibody should be confirmed.

The data in Fig 7 part A are plotted in an unusual way for a standard antimicrobial assay. They should be plotted a line graph of concentration vs log culturable units. Also the data in part B is from only 2 experiments and therefore cannot be analyzed statistically. Overall CXCL10 does not appear to have particularly good anti-candida activity. Further are the levels reached in the cornea high enough to have any significant effect?

P11 line 51 should be Fig. 8E not 7D

The “golden” appearance of the images in Fig 1.A and 9.A make it difficult to discern differences.

C57 mice typically recover from fungal keratitis quite quickly. There appears to be little recovery in the Soothe treated animals by day 7. Why is this?

Reviewer: 2

Comments to the Author
This manuscript proposes to study the role that the TLR agonist, flagellin plays during Candida albicans (CA) infection of the cornea. The data presented implicate a central role for CXCL10, its receptor CXCR3, and NK cells in resistance to infection and more efficient clearance of CA. The fact that CXCL10 is fungicidal in vitro and is effective when treating animals in vivo is very interesting.
Specific Points

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1. On two occations the difference in CFU, Figs 5 & 6 was less than 2 or 3 fold. While this might be statistically significant, is it biologically meaningful?

2. At the bottom of page 10 the authors refer to a recent report showing that NK cells phagocytize CA and that this might be the reason that these cells are associated with resistance to CA infection. First, they did not demonstrate any phagocytosis by NK cells and thus this seems more appropriate to a point to be made in the discussion. Secondly, it seems that the phagocytosis of CA by NK cells was more involved in the activation of PMNs who are the cells that actually clear CA (ref. 32). This does beg the question as to why when NK cells are neutralized, there are more neutrophils present? Are these cells not well activated and are thus not clearing the infection? Or are they causing tissue damage and thus increasing clinical disease?

3. There is a growing body of literature that implicates deficiency in IL-17 production with susceptibility to CA infection, see recent report in Nat Immunol. 15:143, 2014. Consequently CXCL10 is not the only resistance factor associated with CA infection.

4. Need to be consistent in use of capital letters for chemokine and chemokine receptors. They all should be capitalized.

5. Is the in vivo resistance of CXCL10-treated mice due to direct cytolytic activity or is it related to migration of NK cells and their ability to activate neutrophils?

First Revision – authors' response – 24 March 2014

Responses to the Reviewers' comments

In the manuscript, the revised texts are printed in blue. Because of words limitation, per suggestion of editorial office, we have deleted many detailed methods and refer to our publications used these methods.

Reviewer: 1

Comments to the Author

To state (p3 line 22) that there is no topical treatment for candida keratitis is misleading. While not optimal, topical polyenes (amphotericin) can be effective.

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We added “amphotericin B, a polyene antibiotic with a broad antifungal spectrum, remains the drug of first choice for *CA* keratitis, but controlled clinical trials are lacking and the preparations of amphotericin B as eye drops is extemporaneous [10].” to address the concern.

Figure 1 legend, n for D and E should be stated.

Change was made.

Part B of figure 2 should be redrawn with the bars having patterns (ie different fills) as it is very difficult to distinguish between them.

A color panel B is now used.

Legend of Figure 3 (p29 line 16) refers to bacterial inoculation. Also there should be scale bars on the images.

Change was made accordingly. Scale bars with size were added to the all micrographs.

Results text p9 line 49 refers to figure 1 when it should be 5. Also the text refers to arrows (line 57) yet there are none on the figure. All images in Figure 5A look “bleached” other than the PBS-IgG, this makes them difficult for the reader to properly interpret. The cfu in these experiments appears to be much higher than other experiments – what is the explanation for this?

Arrows were added to the micrograph panels. The images are bleached because of surface irregularity of the infected corneas. This is common and also can serve as an indicator of keratitis. The experiments are in vivo and many factors can determine the outcome of diseases. We usually observed 2000 to 5000 cfu in the controls. The most important in our experiments is whether flagellin induces protection; keratitis will develop in untreated cornea and flagellin pretreatment prevents this from occurring. The results showed in Figure 5 are within the range of what we considered normal. We do however stated in the Method section that “In each experiment, the controls without flagellin pretreatment were expected to have 2000-5000 cfu recovered while flagellin pretreatment eradicated invading pathogens (<10 cuf/cornea) at 1 dpi” to explain the high cfu in this Figure 5. We also mention the factor in the figure legend “Note relatively high recoverable CA in the controls compared to other Figures, and no recoverable cfu in flagellin-pretreated corneas” to state the fact, p24.

Figure 6 A. The images from IgG and NK1.1. antibody treated animals do not appear to be the same magnification. The images in part B need scale bars.

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It was the same magnification. However, because the keratitis was so severe (the eye was closing to or even be perforated, the average clinical score was near to 12, perforation or total destruction of the cornea) that the eye was mostly covered by the eye led. If force the eye led to open, the cornea will be broken. We stated that "The much small size of NK1.1 treated eye was an indicative of the cornea near perforation" to explain the fact, p10.

P10 line 51 should be MPO not MOP.

Change was made.

That NK cells are depleted by the antibody should be confirmed.

We had evidence in the original manuscript (Fig. 6B) but did not describe the results in sufficient detail. We added the following text in Results section (p10) to describe Fig. 6B (please also note a, b c and d was added to panels in Fig. 6B for better description of Fig. 6B).

"Figure 6B showed that the subconjunctival injection of NK1.1 greatly reduced the number of cells expressing Cxcr3 (Fig. 6B-a); the staining intensity of these CXCR3 positive infiltrated cells (6B-b) was also relative lower compared to IgG treated corneas, suggesting the reduction or depletion of NK cells. The NK1.1 treated cornea sections were stained FITC-labeled NK1.1, 4 lightly stained cells were detected (arrows, B-d) in Nk1.1 treated corneas while large number of NK1.1 positive cells were observed in IgG injected controls (B-c). Thus, subconjunctival injection of NK1.1 resulted in depletion of NK cells. Moreover, NK1.1 neutralization also resulted in great thinning of the cornea (ulceration, Fig. 6B-b and -d), consistent with the average clinical score >10 (Fig. 6C), indicating severe keratitis."

The data in Fig 7 part A are plotted in an unusual way for a standard antimicrobial assay. They should be plotted a line graph of concentration vs log culturable units. Also the data in part B is from only 2 experiments and therefore cannot be analyzed statistically. Overall CXCL10 does not appear to have particularly good anti-candida activity. Further are the levels reached in the cornea high enough to have any significant effect?

We presented Fig. 7A in a way matching the way we present our in vivo studies where Y axis represent culturable C. albicans. We agree with the reviewer that "CXCL10 does not appear to have particularly good anti-candida activity". However, because of rapid and great elevation of CXCL10 in flagellin pretreated corneas, the high level CXCL10, albeit low fungicidal activity, may contribute to C. albicans clearance at early stage of infection. To soften our statement regarding the role of CXCL10 in fungal clearance, we added: "Other antimicrobial peptides shown to be involved include CRAMP (human LL-37)

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and β-defensins [15, 39, 40].” P14. We deleted Part B. In a subsequent (waiting to be published) study in which we assessed the effects of CXCL10 on corneal neovascularization caused by C. albicans and suture, we either neutralized or overexpressed CXCL10 and found that fungal burden in C. albicans infected corneas was related to the in vivo levels of CXCL10 in B6 mouse corneas. Moreover, the levels of CXCL10 are also determinant factor for Candida-induced corneal angiogenesis. We included the following figures for reviewer to examine. Taken together, we believe that CXCL10 can reach a level in corneal epithelium high enough to contribute to C. albicans clearance and subsequently to aid in suppression of angiogenesis in a avascular corneas when the pathogenic condition favors (both hem and lymph) angiogenesis. These data should help to answer the question whether leveled CXCL10 reached in the cornea is high enough to have any significant effect. The effects of epithelial expressed CXCL10 on corneal CA keratitis is clear and long lasting.

Figure A. Fungal burden in control CA infected corneas (Cont), and corneas subconjunctivally injected control IgG (IgG), injectged with CXCL10 neutralizing antibodies (αCXCL10), infected with AAV9-GFP, and infected with AAV9-CXCL10). The latter was shown to express CXCL10 in corneal epithelial cells at day 14 of AVV9 injection.

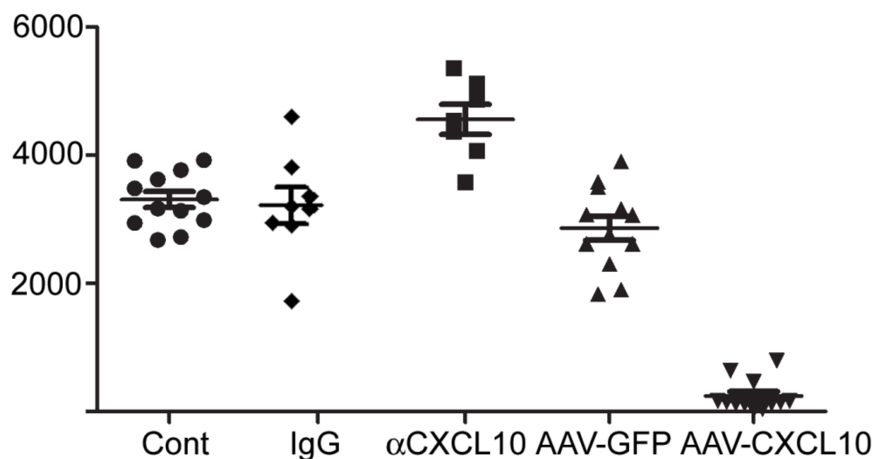
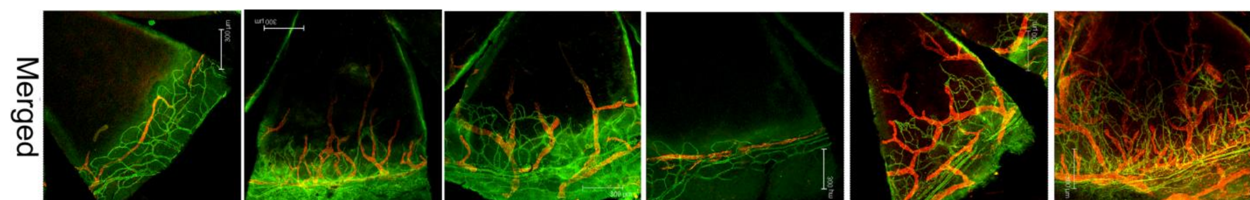


Figure B

Hem- and lymph-angiogenesis (the marks for each condition are the same although the order was changed) at day 7 post CA infection): Green blood vessels and yellow lymph-vessels

Naïve Cont AAV-GFP AAV-CXCL10 IgG CXCL10 neutralizing



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P11 line 51 should be Fig. 8E not 7D

Change was made.

The “golden” appearance of the images in Fig 1.A and 9.A make it difficult to discern differences.

The golden appearance of the images in Figure 1 and 9 were the micrographs taken with slit lamp microscope that is commonly used for Ophthalmologists to perform eye examine. In our lab, we use dissection microscope with a digit camera and slit lamp microscope to photograph diseased corneas. Different fellows have their own preference to use one of two microscopes. Both microscopies are able to show corneal inflammation (keratitis). Although slit lamp microscopy shows less contrast, it avoids the “bleached” effects shown by direction microscope. We have dissection microscope images for Figure 9, but not for Figure 1 (the Rag1KO mice are very expensive). As such we keep the Figure 9 in form of slit lamp microscopy which is more suitable to illustrate corneal hem-angiogenesis (we marked a few in Figure 9 which is taken for to show keratitis; for better illustration of vessels in the cornea, side imaging is needed.) We hope the reviewer understand our choice and considered this concern being addressed.

C57 mice typically recover from fungal keratitis quite quickly. There appears to be little recovery in the Soothe treated animals by day 7. Why is this?

At our hands, C. albicans was rapidly eliminated (by day 3 or early) in C57 cornea; however, the inflammation lingering for a while (usually up to 14 days). At Day 7, there are apparent neovascularization (arrows in Figure 9. Also see above Figure B.

We thanks the reviewer for his/her time spent on careful reading of the manuscript and constructive comments. We trust the reviewer will agree that we have addressed the commends listed above.

Reviewer: 2

Comments to the Author
This manuscript proposes to study the role that the TLR agonist, flagellin plays during Candida albicans (CA) infection of the cornea. The data presented implicate a central role for CXCL10, its receptor CXCR3, and NK cells in resistance to infection and more efficient clearance of CA. The fact that CXCL10 is fungicidal in vitro and is effective when treating animals in vivo is very interesting.
Specific Points

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1. On two occations the difference in CFU, Figs 5 & 6 was less than 2 or 3 fold. While this might be statistically significant, is it biologically meaningful?

We believe the answer is yes. While this manuscript is focused on innate response and Figure 5 showed cfu at day 1 p.i., long term observation revealed that CXCL10 or CXCR3 neutralization caused massive neovascularization while epithelial expression of CXCL10 prevent CA induced corneal neovascularization (Figure B above). We hope to submit a manuscript describe these results within next three month (please also see our response to reviewer 1 regarding Figure 7). Regarding Figure 6, we provided an explanation in the Results section: “Although the increase in fungal burden is moderate (1.37 fold), thinning of the cornea and potential dissemination of *CA* to the anterior chamber suggested the actual number of *CA* in NK depleted eyes might be greater than that recovered from the corneas.” As a matter of fact, the corneal tissues were hard to recover, this was the major reason we did not present CXCL2 expression data—not sufficient cell homogenates. The long term effects (7 days pi) of CXCl10 neutralization were shown in above figure as unpublished data (Fig. B).

2. At the bottom of page 10 the authors refer to a recent report showing that NK cells phagocytize CA and that this might be the reason that these cells are associated with resistance to CA infection. First, they did not demonstrate any phagocytosis by NK cells and thus this seems more appropriate to a point to be made in the discussion. Secondly, it seems that the phagocytosis of CA by NK cells was more involved in the activation of PMNs who are the cells that actually clear CA (ref. 32). This does beg the question as to why when NK cells are neutralized, there are more neutrophils present? Are these cells not well activated and are thus not clearing the infection? Or are they causing tissue damage and thus increasing clinical disease?

We deleted that statement which should be in the discussion and we went through the referred paper again. As we understand, the paper used human neutrophils and NK cells and showed that 1) human NK cells possess antifungal activity through releasing perforin; 2) NK cells need to phagocytize CA to be activated; 3) presence of neutrophils prevent NK cells to contact C. albicans; 4) C albicnas-NK co-culture media (activated NK cells) secreted factors activated PMNs and enhanced their fungicidal activity. We proposed:

“This is consistent with a recent report showing human NK cells are capable of ingesting *CA*, resulting in their activation and of killing these microbes by secreting perforin [43]. Moreover, the activated NK cells may also be indirectly involved in fungal clearance by modulating neutrophil antifungal activity [43]. It is interesting to note that when NK cells are neutralized, there are more neutrophils present and severe tissue damage (Fig. 6), similar to that observed in dextran sodium sulfate-induced colitis in mice [44]. Potential roles of NK cells to regulate neutrophil function are in a context-dependent manner: NK cells are

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activated upon encountering CA and activate neutrophils [43]; when CA are being eliminated by NK cell-released perforin [43] and/or by NK-activated neutrophils, NK cells may induce neutrophil apoptosis [45]." P14

3. There is a growing body of literature that implicates deficiency in IL-17 production with susceptibility to CA infection, see recent report in Nat Immunol. 15:143, 2014. Consequently CXCL10 is not the only resistance factor associated with CA infection.

We agree with the reviewer and added the following to the Discussion section:
"A recent study showed that an unique population of bone marrow neutrophils that produced and responded to interleukin 17 and the autocrine activity of IL-17 induced the production of reactive oxygen species, and increased fungal killing in vitro and in a model of Aspergillus-induced keratitis, suggesting a key role of IL-17 in corneal innate defense [48, 49]. The mechanisms underlying NK recruitment, activation, fungicidal activity, and their interaction with other immune, particularly neutrophils, and residential cells in response to infection are under investigation in our laboratories." P14

3. Need to be consistent in use of capital letters for chemokine and chemokine receptors. They all should be capitalized.

They are all capitalized.

4. Is the in vivo resistance of CXCL10-treated mice due to direct cytolytic activity or is it related to migration of NK cells and their ability to activate neutrophils?

   See response to #2. We do not have evidence to answer these interesting questions and stated that these questions are are under investigation in our laboratories.

We thank the reviewer for thoughtful comments and trust we have fully addressed his/her questions fully.

Second Editorial Decision – 22 April 2014

Dear Dr. Yu,

Thank you for submitting your revised manuscript ID eji.201444490.R1 entitled "Flagellin-induced Expression of CXCL10 Mediates Direct Fungal Killing and Recruitment of NK Cells to the Cornea in Response to Candida albicans Infection" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

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Although the referees have recommended publication, some revisions to your manuscript have been requested. Therefore, I invite you to respond to the comments of the referees and revise your manuscript accordingly.

You should also pay close attention to the editorial comments included below and in the attached file. In particular, your manuscript requires language polishing (see also referee #2 comments). Also please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.

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 referee(s) to ensure the relevance and timeliness of the data.

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

Yours sincerely,
Laura Soto Vazquez

on behalf of
Prof. Marco Colonna

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Reviewer: 1

Comments to the Author
The sentences added to the introduction regarding amphotericin need modification – what is meant by “its poor c.” Also there is an FDA approved polyene drug natamycin that exists as an ophthalmic formulation so does not need to be “extemporaneously” prepared. Perhaps by “extemporaneously” the authors are trying to elude to preparation by a compounding pharmacy.

The requested scale bars for Figure 6 B have yet to be added.

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Several of the photographic slit-lamp images are not great quality as previously stated. The reviewer understands the explanations put forth but suggests that for future studies the authors standardize their methodology between observers so as to get optimal images under the same lighting conditions. There was no need to have filters in light path as there was no fluorescein staining and they do mask some of the detail that could be seen otherwise.

Reviewer: 2

Comments to the Author

I am not convinced that a change of less than two fold in fungal cfu is biologically meaningful. Furthermore, their statement that "the actual number of CA in NK depleted eyes may be greater than recovered", is not persuasive. The authors suggest that there might be some CA in anterior chamber, this could be easily tested by measuring cfu from whole eyes. Why speculate when you can do the experiment?

In addition, the manuscript really needs to be significantly edited for its English usage.

Second Revision – authors' response – 2 April 2014

Reviewer: 1

Comments to the Author

The sentences added to the introduction regarding amphotericin need modification – what is meant by "its poor c." Also there is an FDA approved polyene drug natamycin that exists as an ophthalmic formulation so does not need to be "extemporaneously" prepared. Perhaps by "extemporaneously" the authors are trying to elude to preparation by a compounding pharmacy.

We apologize for the errors mentioned. Apparently, the file uploaded was not the final one we prepared. We now corrected the problems by stating that "Current practice in the treatment of fungal keratitis involves the use of topical antifungal drops such as natamycin and amphotericin B. Topical antifungals can cause toxicity such as punctate keratitis, chemosis, and recurrent corneal epithelial erosions [10]."

The requested scale bars for Figure 6 B have yet to be added.

Scale bar was added to Figure 6B.

Several of the photographic slit-lamp images are not great quality as previously stated. The reviewer understands the explanations put forth but suggests that for future studies the authors standardize their methodology between observers so as to get optimal images under the same lighting conditions. There was no need to have filters in light path as there was no fluorescein staining and they do mask some of the detail that could be seen otherwise.

We thank the reviewer for letting us pass and we have called company for maintenance repair and problems we believe has been corrected. Nevertheless, in each case of photographs presented, there are clinical scores, fungal load, MPO determination, and/or CXCL2 expression. Together, these data give a defined answer to the question we were addressing. The overall quality should not be significantly affected. The point the reviewer made is well taken.

Reviewer: 2

Comments to the Author
I am not convinced that a change of less than two fold in fungal cfu is biologically meaningful. Furthermore, their statement that "the actual number of CA in NK depleted eyes may be greater than recovered", is not persuasive. The authors suggest that there might be some CA in anterior chamber, this could be easily tested by measuring cfu from whole eyes. Why speculate when you can do the experiment?

We changed the statement in the Results part from “Fungal load determination indicated a significantly higher fungal burden in NK cell depleted corneas (Fig. 6D) albeit the increase was moderate (1.37 fold)” to “Plate counting revealed a small increase in fungal burden in NK cell depleted corneas (1.37 fold) (Fig. 6D)” We also deleted the questioned statement “Although the increase in fungal burden is moderate (1.37 fold), thinning of the cornea and potential dissemination of CA to the anterior chamber suggested the actual number of CA in NK depleted eyes might be greater than that recovered from the corneas” from Discussion section of the manuscript.

In addition, the manuscript really needs to be significantly edited for its English usage.

We adapted the version from the editorial office and the corresponding author had thoroughly read the manuscript and his daughter, Jessica Yu, (J.D. Ohio State) has proof read it again.
Regarding naming genes, mRNA, and proteins, we following the rules listed by Genetics and Molecular Biology as the following:

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General nomenclature rules (applicable to mouse, rat, and chicken):
• Full gene names are not in italics and Greek symbols are NEVER used
o eg: insulin-like growth factor 1
• Gene symbols
o Greek symbols are never used
o hyphens are almost never used
o gene symbols are italicized, first letter upper case all the rest lower case
 eg: Igf1 (italicized)
• Proteins designations
o same as the gene symbol, but not italicized and all upper case
 eg: IGF1
• mRNA and cDNA use the gene symbol and formatting conventions
o eg: "... levels of Igf1 (italicized) mRNA increased when..."

Third Editorial Decision – 12 May 2014

Dear Dr. Yu,

It is a pleasure to provisionally accept your manuscript entitled "Flagellin-induced Expression of CXCL10 Mediates Direct Fungal Killing and Recruitment of NK Cells to the Cornea in Response to Candida albicans Infection" 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: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). 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,
Laura Soto Vazquez

European Journal of Immunology

Peer review correspondence

on behalf of

Prof. Marco Colonna

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