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Am80-GCSF synergizes myeloid expansion and differentiation to generate functional neutrophils that reduce neutropenia-associated infection and mortality

Lin Li, Xiaotian Qi, Weili Sun,Hisham Abdel-Azim, Siyue Lou, Hong Zhu,Nemani V. Prasadarao, Alice Zhou, Hiroyuki Shimada, Koichi Shudo, Yong-Mi Kim, Sajad Khazal, Qiaojun He, David Warburton, and Lingtao Wu

Corresponding author: Lingtao Wu, CHLA/USC

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Editor: Céine Carret

1st Editorial Decision 19 May 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study relevant and of interest. However, they raise substantial concerns on your work, which should be convincingly addressed in a major revision of the present manuscript.

You will see that referees 1 and 2 are particularly concerned about the technical aspect of the work and would like to see the data strengthened, especially regarding the first half of the manuscript. Referees 1 and 3 highlight the relevance of the second part of the study (the in vivo data), but referee 3 suggests increasing the clinical aspect of the work by providing a titration experiment, which we agree would improve the study.

Given that all of them find the message novel and interesting we would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and that acceptance of the manuscript would entail a second round of review.

I should remind you that it is EMBO Molecular Medicine policy to allow a single round of major revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. I realize that

addressing the referee comments in full would involve a lot of additional experimental (and grammatical/spelling) work and I am uncertain whether you will be able (or willing) to return a revised manuscript within the 3 months deadline and I would also understand your decision if you chose to rather seek rapid publication elsewhere at this stage.

Should you decide to revise your article for EMBO Molecular Medicine, revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Please make sure to format your article according to our guidelines and provide all the requested editorial amendments as listed below.

I look forward to seeing a revised form of your manuscript as soon as possible.

Should you find that the requested revisions are not feasible within the constraints outlined here and choose, therefore, to submit your paper elsewhere, we would welcome a message to this effect.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

See my comments to the authors below

Referee #1 (Remarks):

Neutrophils are key players in infection defense. G-CSF is therefore used since >20 years to shorten the period of neutropenia after chemotherapy. The authors demonstrate the surprising finding that G-CSF alone is able to induce the development of neutrophils from precursors in vitro and in experimental animals in vivo, yet these cells are functionally immature by a number of measures and thus are unable to protect mice from experimental bacterial infections. The authors then show, that stimulating the retinoic acid system with the artificial trigger Am80 is able to induce neutrophil maturation alone, yet in insufficient numbers. However, when provided in combination with G-CSF, Am80 is able to produce sufficiently high numbers of fully mature neutrophils in vivo, that are protective.
While the main finding is very interesting, the manuscript suffers from several technical flaws, data that are inconsistent with the provided interpretation or not convincing and, not the least, very complicated writing with partly poor English. Overall this serves to significantly dampen the enthusiasm of this reviewer.

Key issues are listed below:

Major:
1) Generally the image quality is very poor (e.g. Fig. 1A ii, 2D v, 4D/E, 6A/B and supplementals). This does not allow to distinguish morphological differences in the nuclear shapes of cells, which are used by the authors to highlight inefficient maturation of the cells.
2) In Fig. 1B the collection of measured gene regulations is not clear and seems to vary at will (e.g. CD66c/b on day 1, CD66c and CD11b on day 2 and CD66c and CD18 on day 6). There is no rationale provided, for why this was chosen. Generally, the differences in gene regulation are also often very small (e.g. CD18 (day 6): Am80 ~0.7, Am80+GCSF ~ 1.2. Highly significant by statistics, yet very small in effect).
3) The interpretation of the data in Fig. 1B is not supported by the figure. Example 1: "We found that compared to Am80 in the early and late differentiation induction stages, Am80-GCSF induced significantly higher expression of RA-target genes that regulated growth inhibition and granulocytic differentiation (Fig. 1B; day 1 vs. day 2 vs. day 6), including RAR 2 (Alvarez et al, 2007; Soprano et al, 2004), C/EBP (Lekstrom-Himes, 2001; Park et al, 1999), CD11b (Park et al, 1999), CD66 (Park et al, 1999), and CD18 (Bush et al, 2003)." Data show: Am80 values higher in RAR 2, C/EBP, CD66c and CD11b (day 2). Example 2: "GCSF only induced higher expression of RAR 2 and C/EBP at day 6 than did Am80". Data show, that also CD66c and CD18 are higher.
4) Fig. 1C: Why was "CD66" measured and not a more specific version such as CD66b, that was also measured in the qPCR data?

5) Why were normal neutrophils from peripheral blood tested in Fig. 1D? This does not fit to any of the other data of the MS, which work with cell lines or CD34+ precursors.
6) Fig. 3E: the interpretation "among CCIN mice groups, both low and medium doses of Am80 sustained significantly higher numbers of neutrophils than did GCSF in PB" is not supported by the data. Data on low dose show: G-CSF: 22*10^4/ml, Am80: 22*10^4/ml. This is identical. Also the notion "Am80-GCSF were similar to Am80 in PB but comparable to GCSF in BM" is not at all supported by the shown values.
7) Fig. 6D: "Compared to GCSF mice, neutrophil production in Am80-GCSF mice was not associated with significant loss of body weight". The data show a loss from ~20g to below 15 g. This is >25%, so very significant. Typically, a loss of >20% requires to terminate the experiment. Also in the same experiment: why is there weight loss at all in the control experiment, when there is neither CPA treatment nor infection?

Minor:
1) Fig. 2E, day 6, CD11b: X-Axis is strange
2) Fig. 3J, 16h PB: Y-axis labelling missing
3) Fig. 4C, 3hr: differences between G-CSF and A+G very small, even if measured significant.

Referee #2 (Comments on Novelty/Model System):

This is probably the best available model, short of a clinical trial in humans.

Referee #2 (Remarks):

The authors propose that the combination of a retinoic acid agonst, Am80, and recombinant human G-CSF promote a more functional proliferative and differentiation program, which may be useful in chemotherapy-induced neutropenia. This is a data-rich manuscript.

Shortcomings center on the work is done mostly in mouse, not humans, and the investigators do not consider that there may be nuances in RA/GCSF signaling between species.

While pharmacologic dissection of granulopoiesis is shown to be informative, it does not reach the same level of rigor as RNAi silencing or gene targeting.

The authors refer to GCSF and its relationship to myeloid malignancies - the precise contribution to leukemia in severe congenital neutropenia, severe aplastic anemia, and breast cancer are either controversial or specific to the underlying disease and need for chronic administration of GCSF. It will be very difficult to demonstrate that the addition of Am80 would mitigate this risk, as the mouse studies fail to model this phenomenon.

Figure 7 should include G-CSF as a separate stimulus for granulopoiesis.

Referee #3 (Remarks):

The paper by Li et al "Am8-GCSF synergizes...." is of great interest, as it suggests a solution to an important clinical problem, neutropenia associated infection following chemotherapy.

It uses a combination of human and murine studies, but the most compelling data, protection and survival from lethal infection is in the mouse.

The key data in the paper is Figure 5, which documents the marked survival advantage of the combination compared to either drug alone. This is impressive at the doses used. However the doses used are difficult to extrapolate across species, as affinity of receptor, metabolism and other

variables necessarily differ.

So a key experiment, which would improve the paper, and enhance its clinical relevance, is to titrate the doses of AM80 and GCSF. This is relevant as it would help define a 'therapeutic window'. Compliance of all drugs is variable and GCSF causes a lot of bone pain, hence compliance is variable, and so dosing is also.

The lowest doses of AM80 and GCSF which synergize would be of significant clinical relevance.

1st Revision - authors' response 19 August 2016

Reviewer 1's comments and our responses

1. "Generally the image quality is very poor (e.g. Fig. 1A ii, 2D v, 4D/E, 6A/B and supplementals)."

Response

We apologize for those low-quality images. We have now provided high-resolution images that show a clear contrast between nucleus and cytoplasm, which allow distinguishing the degrees of neutrophil morphological differentiation between more mature vs. less mature neutrophils.

2. "In Fig. 1B the collection of measured gene regulations is not clear and seems to vary at will (e.g. CD66c/b on day 1, CD66c and CD11b on day 2 and CD66c and CD18 on day 6). There is no rationale provided, for why this was chosen."

Response

We regret for not providing sufficient information and clear interpretation before. Because Am80 promoted granulocytic differentiation by selectively activating RARα to alter transcription of RA-target genes, we investigated RARα-dependent gene expression modulated by Am80-GCSF in generating functional neutrophils. We found that 6 of 12 different RARα target genes that play key roles in granulocytic differentiation were dynamically modulated by Am80-GCSF at different differentiation induction stages, including tumour suppressor $RAR\beta_2$, terminal granulocytic differentiation regulator *C/EBPε*, as well as neutrophil innate immunity regulators *CD66c, CD66b, CD11b,* and *CD18*. Interestingly, whereas both $RAR\beta_2$ and *C/EBPε* were consistently induced in all differentiation induction stages, they were associated with different transcriptional inductions of innate immunity regulators, i.e., *CD66c* throughout all time, *CD66b* in the early stage, *CD11b* in the middle stage, and *CD18* in the late stage, showing that Am80-GCSF may mediate a course of neutrophil differentiation-associated innate immunity development. In the revised manuscript, we have now provided rationale and interpretation about such transcriptional inductions of those innate immunity regulators at different differentiation induction stages (Results: page 5, lines 12 to page 6, lines 1 to 3).

3. "The interpretation of the data in Fig. 1B is not supported by the figure. Example 1: "We found that compared to Am80 in the early and late differentiation induction stages, Am80-GCSF induced significantly higher expression of RA-target genes that regulated growth inhibition and granulocytic differentiation (Fig. 1B; day 1 vs. day 2 vs. day 6), including RARβ2 (Alvarez et al, 2007; Soprano et al, 2004), C/EBPε (Lekstrom-Himes, 2001; Park et al, 1999), CD11b (Park et al, 1999), CD66 (Park et al, 1999), and CD18 (Bush et al, 2003)." Data show: Am80 values higher in RARβ2, C/EBPε, CD66c and CD11b (day 2). Example 2: "GCSF only induced higher expression of RARβ2 and C/EBPε at day 6 than did Am80". Data show, that also CD66c and CD18 are higher."……"Generally, the differences in gene

regulation are also often very small (e.g. CD18 (day 6): Am80 ~0.7, Am80+GCSF ~ 1.2. Highly significant by statistics, yet very small in effect)."

Response

We agree with the reviewer that our data showed that: a) Am80-GCSF promoted significantly higher expression of target genes than did Am80 in the early and late differentiation induction stages, while Am80 inducing higher expressions in the middle stage; and b) although both GCSF and Am80-GCSF are highly significant by statistics in promoting transcriptions of *RARβ*$_2$, *C/EBPε*, *CD66c*, and *CD18* than did Am80 in the late differentiation induction stage, such effects were relatively small. We apologize for any confusion that may have introduced in the previous version and have now clarified data presentation in the revised manuscript (Results: page 6, lines 3 to 9).

4. "Fig. 1C: Why was "CD66" measured and not a more specific version such as CD66b, that was also measured in the qPCR data?"

Response

We regret for not providing necessary rationale before. It is known that either CD66a, CD66b, CD66c, or CD66d can independently transmit signals in neutrophils, whereas co-expression of different CD66 subunits with CD18 surface markers are associated with the critical development of CR3-dependent neutrophil innate immunity.[1-3] Since Am80-GCSF induced significantly higher transcriptions of *CD66c*, *CD66b*, and *CD18* than did Am80 in both early and late differentiation induction stages, we chose to examine the corresponding protein levels of CD66-CD18 modulated by Am80-GCSF. We have now highlighted this point in the revised manuscript (Results; page 6, lines 9 to 14).

5. "Why were normal neutrophils from peripheral blood tested in Fig. 1D? This does not fit to any of the other data of the MS, which work with cell lines or CD34+ precursors."

Response

In the previous version, data in both Fig. 1D and 1E were derived from normal peripheral blood (PB) specimens. We regret for not clearly emphasizing the use of these normal PB neutrophils before. Whereas NB4 cell line was used in a few parallel tests (Appendix-1 Figs. S1A, B; S2), normal primary human hematopoietic specimens were used in the studies (Fig. 1, Appendix-1 Fig. S1C, D), including both normal primary human hematopoietic CD34+ precursors derived from umbilical cord blood and normal PB specimens collected from normal human donors. By using these normal specimens, we determined that neutrophils induced by Am80-GCSF from normal primary PB mononuclear cells gained CR3-dependent innate immunity, similar to those in normal primary human PB neutrophils or neutrophils induced from CD34+ cells. We have now highlighted the use of these normal specimens in the revised manuscript. Moreover, we have provided new data derived from normal PB specimens, designated as new Fig. 1D. Together, the newly arranged Fig. 1D-F data showed that neutrophils induced by Am80-GCSF from normal primary human PB mononuclear cells display effective innate immunity, mimicking bactericidal activities observed in normal primary human PB neutrophils. All of these changes have now been included in the revised manuscript (Results: page 6, lines 12 to 1 from the bottom).

6. "Fig. 3E: the interpretation "among CCIN mice groups, both low and medium doses of Am80 sustained significantly higher numbers of neutrophils than did GCSF in PB" is not supported by the data. Data on low dose show: G-CSF: 22*10^4/ml, Am80: 22*10^4/ml. This is identical. Also the notion "Am80-GCSF were similar to Am80 in PB but comparable to GCSF in BM" is not at all supported by the shown values."

Response

We apologize for any confusion that may have introduced in the previous version. The interpretations for Fig. 3E data were focusing on the "numbers of neutrophils" (see "neutrophils" section) but not the total numbers of cells (see "Total cells" section). We have now clarified data presentation in the revised manuscript where this original Fig. 3 has been arranged as Fig. 4 because of an added new Fig. 3 (Results: page 10, lines 10 to 15).

Moreover, in order to highlight our focus on recovering "numbers of neutrophils," we have now deleted the statistical significance markers that were previously presented in original Fig. 3E and 3I under the section of "neutrophils, (%)".

7. "Fig. 6D: "Compared to GCSF mice, neutrophil production in Am80-GCSF mice was not associated with significant loss of body weight". The data show a loss from ~20g to below 15 g. This is >25%, so very significant. Typically, a loss of >20% requires to terminate the experiment. Also in the same experiment: why is there weight loss at all in the control experiment, when there is neither CPA treatment nor infection?"

Response

A) We agree with the reviewer that a loss of >20% body weight could be one of moribund signs for euthanizing a mouse. However, we found that significant loss of body weight by bacterial infection in GCSF mice was not always immediately associated with other clinical moribund signs. Therefore, to evaluate infection-induced mortality in this study, mice were considered moribund when at least two of following clinical signs were observed: impaired ambulation, inability to remain upright, decreased or labored breathing, or no response to external stimuli, as described before.[4] We have now highlighted this standard in the revised manuscript (Materials and Methods: page 24, 2nd paragraph, lines 3 to 6).

B) In this study, control mice were not injected with CPA but were subjected to bacterial infection in parallel to all other test groups' mice. On the other hand, all mice in four different test groups, including vehicle, GCSF, Am80, and Am80-GCSF, were subjected to both CPA injection and bacterial infection. Thus, control mice did encounter loss of body weight under perpetual systemic intravenous bacterial infection. The design for control and test groups in different mouse models has now been provided in Appendix-1 Table S3 as well as stated in the related figure legends. Furthermore, the design for with or without CPA injection of mice has also been emphasized in the revised manuscript (Results: page 9, 2nd paragraph, line 1 from the bottom to page 10, lines 1 to 2).

8. "Fig. 2E, day 6, CD11b: X-Axis is strange."

Response

We apologize for this error and have now made correction in the figure. Because we have included new drug titration data that are designated as new Fig. 3, this original Fig. 2E has now been rearranged as Fig. 2B in the revised manuscript.

9. "Fig. 3J, 16h PB: Y-axis labelling missing."

Response

Y-axis of 3 hr (section i) and 16 hr (section ii) in Fig. 3J share the same legend "bacteria (10^3/ml)". To avoid confusion, we have now added a legend to Y-axis of 16 hr (section ii). Because we have

now provided new drug titration data that are designated as new Fig. 3, this original Fig. 3J has now been arranged as Fig. 4J in the revised manuscript.

10. "Fig. 4C, 3hr: differences between G-CSF and A+G very small, even if measured significant."

Response

We agree with the reviewer's comments. Although bacterial killing induced by Am80-GCSF after 3 hr of bacterial infection was significant compared to GCSF mice ($P < 0.01$), the numbers of killed bacteria by Am80-GCSF mice were relatively small. However, after 16 hr of infection, the difference had increased markedly ($P < 0.001$). This result suggests that neutrophils induced by Am80-GCSF are capable of continuously killing bacteria in a longer period of transient infection. This original Fig. 4C has now been arranged as Fig. 5C due to an added new Fig. 3.

11. "very complicated writing with partly poor English."

Response

We apologize for those language problems. We have now made corrections in the revised manuscript by using simpler and shorter sentences with close attention to syntax.

Reviewer 2's comments and our responses

1. "Shortcomings center on the work is done mostly in mouse, not humans, and the investigators do not consider that there may be nuances in RA/GCSF signaling between species."

Response

We thank the reviewer for pointing this out. To enhance clinical relevance of this study by providing a 'therapeutic window' reference for the future clinical study of Am80-GCSF treatment of cancer chemotherapy-induced neutropenia (CCIN), we have now titrated the dose ranges of Am80 when combined with GCSF in primary acute myeloid leukemia (AML) patient specimens. We have now defined that several dose combinations of Am80-GCSF effectively induce functional neutrophils while suppressing leukemic growth, likely through mediating an altered transcription of RA signaling molecules in AML patient specimens. We have now presented these new data in a new Fig. 3 in the revised manuscript (see details in the Response to reviewer 3's comments).

2. "While pharmacologic dissection of granulopoiesis is shown to be informative, it does not reach the same level of rigor as RNAi silencing or gene targeting."

Response

One of the findings derived from this study has revealed a differential course of proliferation vs. differentiation in primary human specimens, as shown by: a) in normal primary human hematopoietic precursors, Am80-GCSF synergizes active proliferation with effective granulocytic differentiation to generate significantly larger amount of functional neutrophils than does Am80; and b) Am80-GCSF produces functional neutrophils while inhibiting malignant growth in primary human AML specimens. These results raise a question: How can Am80-GCSF modulate such differential processes in normal vs. malignant cells to coordinate innate immunity development with neutrophil production? We agree with the reviewer that in order to determine the mechanisms of such differentially synergized regulatory processes by Am80-GCSF, a future rigor study is needed to define an array of transcription factors that coordinate with RARα at distinct developmental

stages, through RNAi silencing and/or gene targeting in normal and malignant cells in the presence or absence of Am80-GCSF, respectively. We have now thoroughly discussed this needed future study in the revised manuscript (Discussion: page 16, 2nd paragraph, lines 1 to 3 to page 18, lines 1 to 2).

3. "The authors refer to GCSF and its relationship to myeloid malignancies - the precise contribution to leukemia in severe congenital neutropenia, severe aplastic anemia, and breast cancer are either controversial or specific to the underlying disease and need for chronic administration of GCSF. It will be very difficult to demonstrate that the addition of Am80 would mitigate this risk, as the mouse studies fail to model this phenomenon."

Response

We agree with the reviewer that our mouse models have the limitations, which mainly mimic CCIN-associated infection and mortality rather than GCSF-induced possible myeloid overexpansion. However, compared to GCSF *in vivo*, Am80-GCSF induces sufficient numbers of functional neutrophils while preventing myeloid overexpansion (Figs. 5-7). Also, Am80-GCSF can induce functional neutrophils while inhibiting leukemic growth in cultured AML specimens (Fig. 3). Therefore, further clinical studies of Am80-GCSF for CCIN treatment may be critical to evaluate directly whether Am80-GCSF combination mitigates the risk of myeloid malignance in human.

4. "Figure 7 should include G-CSF as a separate stimulus for granulopoiesis."

Response

We thank the reviewer for pointing this out and have now included a GCSF section related to transcription of RA target genes in the figure. Due to the addition of a new Fig. 3, the original Fig. 7 has now been designated as Fig. 8 in the revised manuscript.

Reviewer 3's Comments and our responses

"The key data in the paper is Figure 5, which documents the marked survival advantage of the combination compared to either drug alone. This is impressive at the doses used. However the doses used are difficult to extrapolate across species, as affinity of receptor, metabolism and other variables necessarily differ. So a key experiment, which would improve the paper, and enhance its clinical relevance, is to titrate the doses of AM80 and GCSF. This is relevant as it would help define a 'therapeutic window'."

Response

We thank the reviewer for calling our attention to this important issue. Clinical GCSF doses have been recognized worldwide in the past over 2 decades. Also, the medium human plasma concentration-dose of GCSF (25 ng/ml) in mediating granulocytic differentiation of different human hematopoietic precursors *in vitro* has been well established.[5-7] Thus, it is important to identify the dose ranges of Am80 when combined with GCSF in mediating neutrophil differentiation to develop innate immunity against infection. Moreover, systematic review and meta-analysis of 5,256 patients show that giving GCSF to AML patients post-chemotherapy does not affect overall survival or infectious rate,[8] whereas GCSF may induce myeloid malignancy in neutropenic patients.[9-12] Therefore, by testing different doses of Am80 when combined with GCSF for their effect on generating functional neutrophils while suppressing malignant growth in AML specimens, the defined dose ranges could have potential to serve as a reference baseline for an add-on therapy to GCSF in the future clinical study of Am80-GCSF for CCIN treatment. We have now titrated the combination of 25 ng/ml GCSF with different Am80 doses ranged from low to high human plasma

concentrations converted from clinical usages, including 20, 50, 100, and 150 nM Am80. These different combinations were evaluated for their *in vitro* effects on generating functional neutrophils while suppressing leukemic growth in primary AML patient specimens. The resultant data have now been provided in a new Fig. 3, whereas some other changes have been made in original Fig. 2, correspondingly. All these revisions are now presented in the revised manuscript (Results: page 7, 2nd paragraph, lines 3 to page 9, lines 1 to 10).

References

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2nd Editorial Decision 01 September 2016

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please address referee 1 concerns: we strongly recommend that you provide new light microscopy figures as suggested, rewrite according to this referees recommendations and have a native english speaker thoroughly go through the text to improve the english quality.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your article.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

1) I am still not convinced by the image quality. The authors have simply run their original images through some kind of image restoration/contrast enhancement. The original images are the same and other than stated by the authors, running an image restoration algorithm on the same data does not improve their resolution. It is required to obtain images of typical H&E stains, where a nucleus is dark blue/violet against a light blue cytoplasm. Taking color photos through a good 63x NA 1.4 oil lens would provide perfectly resolved, hematology textbook quality images. This criticism applies to all provided light microscopy images.

Referee #1 (Remarks):

Major:
1) I am still not convinced by the image quality. The authors have simply run their original images through some kind of image restoration/contrast enhancement. The original images are the same and other than stated by the authors, running an image restoration algorithm on the same data does not improve their resolution. It is required to obtain images of typical H&E stains, where a nucleus is dark blue/violet against a light blue cytoplasm. Taking color photos through a good 63x NA 1.4 oil lens would provide perfectly resolved, hematology textbook quality images. This criticism applies to all provided light microscopy images.
2) Authors state in their revision that "the numbers of killed bacteria by Am80-GCSF mice were relatively small". I am not sure they understand their own data correctly. The Y-axis of this graph shows "Bacteria (10^4/ml)" and the graph shows ~ 0.8*10^4/ml for A+G and ~1.4*10^4/ml for G-CSF. Control is 5.9*10^4/ml. So, within 3 h neutrophils have killed 88 or 76% of all bacteria. This is not really a little, this is almost all of the bacteria. What is small, is the difference between A+G and G-CSF. This is what I referred to.
3) The English is still very poor. Also in the (helpful) newly added text. It should be strongly improved.

Referee #3 (Comments on Novelty/Model System):

My queries from first review have been answered, and so I think it is publishable

2nd Revision - authors' response 18 September 2016

Editor's recommendations and our responses

1. "have a native english speaker thoroughly go through the text to improve the english quality."

Response

We apologize for any existing language problems. The native English speakers, both Dr. David Warburton (a co-author of this manuscript and influential scientist/leader in Regenerative Medicine) and Dr. Martin Broome with expertise in Medical Biology, have now thoroughly reviewed the text, and edited the manuscript to improve the English quality.

Reviewer 1's comments and our responses

1. "I am still not convinced by the image quality. The authors have simply run their original images through some kind of image restoration/contrast enhancement. The original images are the same and other than stated by the authors, running an image restoration algorithm on the same data does not improve their resolution. It is required to obtain images of typical H&E stains, where a nucleus is dark blue/violet against a light blue cytoplasm. Taking color photos through a good 63x NA 1.4 oil lens would provide perfectly resolved, hematology textbook quality images. This criticism applies to all provided light microscopy images."

Response

We agree with the reviewer that the quality of the images could be better. However, we respectfully disagree with the reviewer's comments on the staining method. We believe that Giemsa stain is a better stain than H&E stain for the purpose of this study.

Giemsa stain, also called differential stain, is specific for the phosphate groups of DNA and thus distinctively blots the regions of DNA. It is a classic blood film stain for peripheral blood (PB) smears and bone marrow (BM) specimens. To date, Giemsa stain has been widely applied as a standard method for evaluating neutrophil morphologic differentiation through assessing the degrees of neutrophil nuclear segmentation (Gallagher et al, 1979; Ding et al, 2013). This was why, as with many other groups, we used Giemsa stain of PB and BM cells for determining changes in neutrophil nuclear segmentation.

We have now provided new light microscopy images derived from Giemsa stain. We did our best to capture new images with better quality to clearly distinguish nucleus and cytoplasm. We believe that these new images allow for evaluating the degrees of neutrophil morphologic differentiation reflected by neutrophil nuclear segmentation in more mature vs. less mature neutrophils.

2. "Authors state in their revision that "the numbers of killed bacteria by Am80-GCSF mice were relatively small". I am not sure they understand their own data correctly. The Y-axis of this graph shows "Bacteria (10^4/ml)" and the graph shows ~ 0.8*10^4/ml for A+G and ~1.4*10^4/ml for G-CSF. Control is 5.9*10^4/ml. So, within 3 h neutrophils have killed 88 or 76% of all bacteria. This is not really a little, this is almost all of the bacteria. What is small, is the difference between A+G and G-CSF. This is what I referred to."

Response

We agree with the reviewer. Our data (Fig. 5C) showed that, compared to GCSF mice, bacterial killing in Am80-GCSF mice significantly increased more after 16 hr of infection ($P < 0.001$) than bacterial killing after 3 hr of infection ($P < 0.01$).

3. "The English is still very poor. Also in the (helpful) newly added text. It should be strongly improved."

Response

Please see our response to Editor's recommendations #1.

References

Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F, and Gallo R (1979) Characterization of the Continuous, Differentiating Myeloid Cell Line (HL-60) From a Patient With Acute Promyelocytic Leukemia. *Blood,* **54**(3): 713-733

Ding W, Shimada H, Li L, Mittal R, Zhang X, Shudo K, He Q, Prasadarao NV, Wu L (2013) Retinoid agonist Am80-enhanced neutrophil bactericidal activity arising from granulopoiesis in vitro and in a neutropenic mouse model. *Blood* **121**(6)**:** 996-1007

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Lingtao Wu
Journal Submitted to:	EMBO Molecular Medicine
Manuscript Number:	EMM-2016-06434

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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?	The sample sizes chosen to ensure adequate effect sizes for both in vitro and in vivo experiments were based on our pilot experiments and/or earlier publications.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Please see 1.a.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Inclusion criteria: Normal C57BL6/J mice (female, 16-22 gram, 6-8 week old) were purchased from Jackson Laboratory. Criteria for exclusion of mouse from sample sets during the experiments: obviously wounded mouse, although very unlikely but could possibly result from fighting/biting among the mice housed in the same cage, that would possibly impact mouse's physiological response to infection and infection-related survival. There was no any occurrence of sample exclusion in our animal works. These criteria were pre-established.
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.	Mice were randomly divided into different test groups. For in vitro experiments, the same numbers of cells were plated at day 0 for different test groups.
For animal studies, include a statement about randomization even if no randomization was used.	Before assessing normal baseline of neutrophil numbers in mice with vetscan counting as well as before any experiment procedure, mice were randomly divided into different groups.
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.	Investigators were aware of groups when performing the treatments. However, when performing the tests, samples were labeled with consecutive numbers, by which group identity was only revealed after analyses.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Immediately after randomly grouping the mice, each of mice were further specifically marked by physically punching ears and/or with permanent staining on tails. For survival and survival related analyses, group identity was monitored with these physical markers on each of mice. Samples from mice for bacterial killing, neutrophil recovery, and morphologic differentiation analyses were labeled with consecutive numbers. Group identity was only revealed after tests.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The significance of the data was appropriately evaluated with either Student's unpaired two-tailed t test or log-rank test. Please see Statistical Analysis section under Materials and Methods for further details.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

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).	Please see the sections "Flow cytometric analysis" and " Antibody neutralization of neutrophil ROS production and bacterial killing", respectively, under Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Please see the information of NB4 cell line presented in the legends of Sup-Fig. S1A, Appendix-1.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Normal C57BL6/J mice (female, 16-22 gram, 6-8 week old) were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were housed in CHLA Institutional animal facilities with an air-conditioned environment at 25°C on a 12 hours light-dark schedule and had access to food and water freely.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal studies were performed according to the guidelines of protocols approved by Children's Hospital Los Angeles (CHLA) Institutional Animal Care and Use Committee.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	The ARRIVE guidelines were considered and followed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Children's Hospital Los Angeles (CHLA) Institutional Review Board.
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.	Protocol for use of primary human specimens was approved by Children's Hospital Los Angeles (CHLA) Institutional Review Board, and the study was conducted in accordance with the Declaration of Helsinki.
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.	More detail information for human specimens used in this study can be provided upon request to corresponding author.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	NA
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).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Human CCIN model (Appendix-1 Fig. S3) described by other groups' studies (Crawford et al, 1991; Trillet-Lenoir et al, 1993) was referenced in this study.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA