Dusp5 negatively regulates IL-33 mediated eosinophil survival and function

Derek A Holmes, Jung-Hua Yeh, Donghong Yan, Min Xu and Andrew C Chan

Corresponding author: Andrew Chan, Genentech, Inc.

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Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 24 April 2014

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and I am afraid that the overall recommendation is not very positive.

As you can see the referees find the topic interesting. However, they also find that the advance provided by the present set of findings is not sufficient to consider publication here. While referee #1 finds that the analysis should be extended to human eosinophils, both referee #2 and 3 also indicate that further work is needed to show that loss of Dusp5 in eosinophils is the cause of the observed immune response phenotype and that one can't exclude that NK cells might contribute to the phenotype. The referees also raise several technical concerns as well. Given these comments from good experts in the field and as we require strong support from referees to move forward with a paper, I am afraid that we can't offer to consider publication here.

I am very sorry that I can't be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.
REFEREE REPORTS

Referee #1:

In the paper "Dusp5 negatively regulates IL-33-mediated eosinophil survival and function" by Holmes et al, the authors suggest that the DUSP5 protein is a negative regulator of IL-33-mediated eosinophil survival and activation. They use in vivo model of parasitic infection in WT and DUSP5 KO mice and expression array analysis of BM-derived eosinophils (BMDE) to support their finding and provide mechanistic insight into this process by demonstrating increased ERK1/2 signaling and elevated level of anti-apoptotic molecules in the lack of Dusp5. These findings are important and valuable in terms of understanding the mechanism of eosinophil activation and the potential to design new therapies for regulating eosinophil function and survival. However several major concerns need to be addressed in order to strengthen the conclusions of this work and to elevate its significance. Overall, we think that they are premature in their conclusions and sloppy in the experimental part.

1. The authors base their conclusions solely on the experiments in mice. Relevance of these conclusions to human eosinophils needs to be addressed by demonstrating expression of Dusp5 in human eosinophils and their response to IL-33 signaling. Also it would be informative to assess the level of expression of Dusp5 in various human immune cells, including T-cells and eosinophils.

2. Expression array analysis of IL-33 response was conducted in BMDE, which is an artificial way to grow eosinophils in vitro. The key findings from this experiment need to be verified in tissue and/or peripheral blood eosinophils. The analysis should be also performed on resting eosinophils to establish how similar or different they are prior to IL-33 stimulation.

Other suggestions/concerns:
1. Elevated expression of Dusp5 (Fig.1A) is only shown on the RNA level and no statistics is provided. Can you verify it on the protein level? In fact Fig. E1D suggests that the level of Dusp5 should be tested following induction of cells rather than in the resting conditions.
2. The rationale for using Rag2 KO is not clear. Other cells such as ILC2 which are the prominent source of IL-5 are still present in this mouse. Have you attempted to verify the phenotype in the regular KO mouse? If different phenotypes are observed this would suggest that T-cells are important for regulating eosinophil survival.
3. For all the figures more clarity in labeling will be helpful. For instance, it is unclear whether Fig 7F is a graphical display of the results from Fig 7E or a separate experiment.
4. In Fig.7A the authors demonstrate by Western blot increased nuclear level of phospho-Erk and decreased level of cytoplasmic phospho-Erk in Dusp5KO cells. This is surprising because if Dusp5 is not dephosphorylating Erk, then both fractions should potentially be increased. Combined total phospho-Erk level (nuclear + cytoplasmic) should be higher in Dusp5KO (due to loss of DUSP5's phosphatase activity), but it does not seem so.
5. In this regards controls for purity of nuclear and cytoplasmic fractions should be performed for all relevant experiments. Also, the 0 timepoint for U0126 stimulation is missing in Fig 7E but is present in the graph in 7F. Moreover, the beta-actin loading control needs to be present in Fig 7E because it is referenced in 7F.
6. Control lanes in Western blot experiments in Fig E9C and Fig 5D are overexposed and seem to be unequal. This needs to be corrected in order to properly interpret the results. For Fig 5B, the pixel intensity needs to be normalized to loading control. For Fig E1D loading control as well as control for efficient activation in DuspKO cells is missing.
7. Bouffi et al reports IL33 activates murine eosinophils via transcriptional responses including Dusp5 (JI 2013); this data should discussed in the context of the presented findings.

Referee #2:

The family of dual specificity phosphatases (DUSPs) regulates activation of cytoplasmic or nuclear functions of diverse MAP kinases. Some DUSP members have wide tissue distribution while others are expressed at high levels in some tissues or cell types. The authors found that DUSP5 mRNA is expressed at particular high levels in NK cells and eosinophils. Eosinophils belong to the innate
branch of the immune system playing a major role in immunity to helminths and allergic diseases. Thus, the authors decided to investigate the effect of DUSP5 gene deletion on eosinophil development, response to helminthes and signaling of cytokine known to control eosinophil activity.

DUSP5-/- showed no significant alteration in the number of eosinophils, neutrophils, monocytes or NK cells and other cells of the immune system. However, in DUSP5-/- mice deprived also of T and B cells (Rag2-/-) to distinguish between the effects of eosinophils from adaptive immunity, they found a higher percentage of eosinophils two weeks after infection by N. brasiliensis in bone marrow, spleen, blood, bronchoalveolar lavage fluid. As a consequence, DUSP5-/- mice cleared more efficiently parasite burden following infection with N. brasiliensis. However, the affect of DUSP5 deficiency with an intact adaptive arm of immunity on N. brasiliensis infection was not investigated. The data suggested that during infection, DUSP5 deficiency does not affect proliferation of eosinophil precursors. However it prolongs their survival.

IL-33 and IL-5 positively regulate survival and effector functions of eosinophils by activating several signaling pathways, including MAPKs and are crucial in controlling helminth infection. Thus, the authors asked whether DUSP had an effect on IL-33/IL-5 signaling. It was found that DUSP5 mRNA in eosinophils was substantially increased upon IL-33 but not by IL-5 stimulation and present data supporting a role for DUSP5 on IL-33-mediated eosinophil survival. Microarray analysis showed that DUSP5-deficient BM-derived eosinophils secreted significantly greater amounts of IL-13, GM-CSF and IL-1 proteins. Moreover, initial mechanistic studies link the axis IL-33, nuclear ERK1.2 DUSP5 to the tuning of BCL-XL expression, suggesting that in addition to regulate cellular activation and cytokine production in response to IL-33, DUSP5 has a pro-survival action on eosinophils. Together, these data are consistent with a negative regulatory role of DUSP5 in IL-33, but not IL-5, mediated eosinophil activation and survival via expression augmented expression of BCL-XL.

The work is well presented; the experiments are well designed and technically sound. The correlative conclusions are consistent with data presented.

I have only one major reservation about the biological significance of this work, namely the limitation of studying the effect of DUSP5 deficiency in absence of T and B cells. As the authors admit in the discussion, IL-33 has broad effects on many cell types beyond eosinophils and the greater ability of DUSP5-/- Rag-/- mice to clear N. brasiliensis may be due to additional cellular components.

Minor point
DUSP5 mRNA in eosinophils is substantially increased upon IL-33 stimulation. Also, DUSP5 mRNA is expressed at particular high levels in NK cells and eosinophils. However, the authors should present data on differences of protein levels as well.

Referee #3:

The well-written manuscript provides convincing evidence for a novel role for the dual specificity phosphatase, DUSP5 in regulating IL-33 signaling in eosinophils, thereby modulating the activation and survival of those cells.

The impact of DUSP5 in IL-33 signaling in eosinophil is evident, what is less clear is the role of this phosphatase during the defense to parasite infection. The authors have a body of data suggesting that DUSP5 gene expression is increased during parasite loading but neither the DUSP5 protein content is shown, nor is the role of NK cells discussed, while evidence suggest that DUSP5 is equally if not more upregulated in those cells. While the authors nicely excluded an involvement of T and B-cells in there system, by crossing the DUSP5-deficient mice on a RagKO background, NK cells are still present and might contribute to the immune response especially. The authors should conduct experiments to address this. Also, it is very disappointing that many statistical analysis with p values were conducted on just 2 experiments. Last, but not least western blots lacking loading controls such as in figure 7 E are quite hard to interpret.
This reviewer feels that the manuscript is quite interesting and novel, but the data need some rework.

I would like to thank you and the Reviewers for previously considering our manuscript entitled "Dusp5 negatively regulates IL-33 mediated eosinophil survival and function" (EMBOJ-2014-88453) for publication at the EMBO Journal. We have analyzed the comments from the three Reviewers in great detail.

We were pleased that the Reviewers appreciate the biological significance and novelty of our work. Our study describes a novel mechanism by which IL-33 regulates eosinophil survival through a MEK/BCL-XL dependent pathway. These studies provide important mechanistic information about how allergic mediators modulate eosinophil function and function. Given the genetic associations described for IL-33 and its cognate receptor IL-1RL1 for the development of human asthma combined with the ongoing clinical phase programs targeting IL-33 and IL-1RL1 in human asthma, our mechanistic insights will likely provide a greater understanding of IL-33 causation of human asthma.

As you point out, all three reviewers raised a major issue of whether NK cells, which also express DUSP5, may be contributing to the phenotypes of eosinophilia and increased worm clearance observed in our Dusp5-/- mice. We have completed additional experiments to address this issue: we have analyzed the effects of Dusp5-deficiency on NK and ILC2 cells, beyond our previous analysis of other innate cell types and find no alterations in NK or ILC2 cells in a homeostatic state or following N. brassiliensis infection. In addition, we depleted NK cells using an anti-Asialo GM1 antibody and find that NK cell depletion does not affect any of phenotypes we report here. Hence, our collective data reaffirm that the phenotypes observed are likely to be IL-33 and eosinophil dependent.

We have also completed a large body of additional experiments to address each and every issue or question raised. Given the enthusiasm of the reviewer's comments of the significance and novelty of our findings, I am hoping that you will reconsider our revised manuscript. I am attaching a "Reply to Reviewers" with a point-by-point reply to each of the Reviewer's comments and a revised manuscript with yellow highlighted text where there has been major change. I believe that our revised manuscript addresses all issues raised and would like to thank the Reviewers for their constructive suggestions.

I can re-submit our revised manuscript through the EMBO submission system if you permit. Thank you again for your time and consideration.

Thanks for sending me the point-by-point response and the revised version. I have now had a time to take a look at everything. I appreciate the added changes and I would be willing to send the revised version back to the original referees.

However, I should also add that I would strong support from the referees to move forward with the paper for publication here and it is this issue that is a little bit hard to anticipate at this stage. You can go ahead and submit the paper via the online system and I will make sure that it gets assigned to me.

Reply to Reviewers

We thank all three reviewers for their comments and in the revised manuscript provide additional data and/or the requested clarification to support our working model of how DUSP5 regulates
eosinophil survival and activation during Th2 immune responses. We appreciate that Reviewers 1 and 3 underscore the biological significance of this work. Reviewer 1 notes that these findings are important and valuable in terms of understanding the mechanism of eosinophil activation and the potential to design new therapies for regulating eosinophil function and survival. Reviewer 3 notes the novelty of the observations and comments that this well-written manuscript provides convincing evidence for a novel role for the dual specificity phosphatase, DUSP5, in regulating IL-33 signaling in eosinophils, thereby modulating the activation and survival of these cells. We also thank Reviewer 2 for his/her summary that our work is well presented, the experiments are well designed and technically sound. To address Reviewer 2’s comments, we also provide additional experimental data to address his/her concerns and provide our rationale of why we believe that our studies provide important biological and mechanistic insights into the regulation of eosinophils in Th2-mediated diseases.

The ongoing Phase 3 clinical trials of anti-IL-5 and anti-IL-5R have established a clear role for IL-5 in allergic asthma through modulation of eosinophil survival and function. While Phase 1 studies of anti-IL-33R are just emerging, the single nucleotide polymorphisms (SNP) in IL-33 and its cognate receptor IL-1RL1 as genetic risk factors for the development of eosinophilia and asthma exacerbations provide a clear genetic basis for the role of the IL-33/IL-1RL1 signaling pathway in human asthma (Gudjartsson et al., Nat Genetics 41:342-347, 2009; Torgerson et al., Na Genetics, 43:887-892, 2011). Our study here adds important mechanistic insights of how IL-33 modulates eosinophil function and survival through a novel MEK/BCL-XL dependent pathway. These insights may provide the basis for potential novel targets for small molecule discovery for the treatment of human asthma.

Point-by-point reply to Reviewers (Authors’s response in green):

REVIEWER 1:

In the paper "Dusp5 negatively regulates IL-33-mediated eosinophil survival and function" by Holmes et al, the authors suggest that the DUSP5 protein is a negative regulator of IL-33-mediated eosinophil survival and activation. They use in vivo model of parasitic infection in WT and DUSP5 KO mice and expression array analysis of BM-derived eosinophils (BMDE) to support their finding and provide mechanistic insight into this process by demonstrating increased ERK1/2 signaling and elevated level of anti-apoptotic molecules in the lack of Dusp5. These findings are important and valuable in terms of understanding the mechanism of eosinophil activation and the potential to design new therapies for regulating eosinophil function and survival. However several major concerns need to be addressed in order to strengthen the conclusions of this work and to elevate its significance. Overall, we think that they are premature in their conclusions and sloppy in the experimental part.

We provide all of the requested data and/or clarification in a point-by-point manner below to address each of the Reviewer’s concerns. Our revised study should address the entirety of the Reviewer’s comments. The major additions are highlighted in yellow in the revised manuscript text.

Major comments:

1. The authors base their conclusions solely on the experiments in mice. Relevance of these conclusions to human eosinophils needs to be addressed by demonstrating expression of Dusp5 in human eosinophils and their response to IL-33 signaling. Also it would be informative to assess the level of expression of Dusp5 in various human immune cells, including T-cells and eosinophils.

We agree and appreciate the reviewer’s concern that not all physiological findings in mice directly translate into humans, especially in the case of eosinophils (Lee et al., J Allergy Clin Immunol., 130:572-584, 2012). We have isolated innate and adaptive human cell populations by FACS sorting from the blood of human donors. Human eosinophils also express Dusp5 mRNA (Figure E13A) and its level of expression is increased ~4-fold following treatment with recombinant human IL-33 (Figure E13B). Of note, IL-5 regulation of Dusp5 differs between humans and mice. While mouse eosinophils do not upregulate Dusp5 mRNA following treatment with IL-5, we observe a 3-fold increase in Dusp5 mRNA expression in human eosinophils following IL-5 treatment (data not
shown), the latter being consistent with data reported by Temple and colleagues (Temple et al, A J Resp Cell Mol Biol, 25;425, 2001). We have added this data into the Discussion (pages 20-21).

Additional investigation of human Dusp5 levels following therapeutic intervention with anti-IL-5, anti-IL-5R and/or anti-IL33R Abs will provide further insight into the specific and/or overlapping roles of IL-5 and IL-33 in Dusp5 regulation of human eosinophil functions.

2. Expression array analysis of IL-33 response was conducted in BMDE, which is an artificial way to grow eosinophils in vitro. The key findings from this experiment need to be verified in tissue and/or peripheral blood eosinophils. The analysis should be also performed on resting eosinophils to establish how similar or different they are prior to IL-33 stimulation.

As Reviewer 1 points out, the field has utilized in vitro expansion of eosinophils from murine bone marrow (BMDEos) as a standard methodology given the paucity of primary cells for experimental analysis. The field has demonstrated over the years that this experimental system recapitulates expression of transcription factors required for eosinophil development, expression of eosinophil granular proteins, and response to chemotactic signals (Sturm et al., Eur J Immunol, 43(8): 2217-28, 2013) and has served as a reliable model for eosinophilopoiesis (Ben Baruch-Morgenstern et al., Nat Immunol, 15;36-44, 2014). While this method provides greater amounts of eosinophils for in vitro analysis, potential differences cannot be discounted.

To address the reviewer’s concerns, we chose to elicit eosinophils in the peritoneum with recombinant IL-33 to characterize the in vivo effect of IL-33 on Dusp5/-/- eosinophils. As described in Figure 4D, daily administration of IL-33 by intraperitoneal (IP) injection increases peritoneal eosinophils, which is further augmented in Dusp5/-/- mice. Using the same protocol of daily IL-33 IP injections, we purified eosinophils by FACS sorting on day 7 and analyzed cytokine expression profiles by RT-PCR. We chose to focus our analysis on IL-4 and IL-13, since both IL-4 and IL-13 mRNAs and secretion were enhanced in IL-33-treated Dusp5/-/- BMDEos compared to Dusp5+/+ BMDEos (Figure 6C). Similar to our observation in BMDEos, IL-33 elicited freshly isolated peritoneal eosinophils from Dusp5/-/-mice expressed higher levels of IL-4 and IL-13 compared to IL-33 elicited Dusp5+/+ peritoneal eosinophils (Figure 6B). As a control, TNF- level from in vitro expanded BMDEos or freshly isolated peritoneal eosinophils were also analyzed. The results were also comparable between cells isolated from Dusp5+/+ or Dusp5/-/- mice (Figure 6C). To illustrate this direct comparison, we replaced the graphs depicting IL-1 and GM-CSF release from BMDEos from the original manuscript (which also demonstrated increased levels IL-1 and IL-18 from Dusp5/-/- BMDEos) with graphs depicting IL-4, IL-5 and TNF- in the revised manuscript. Thus, based on this additional data now described on page 14 and Figures 6B and 6C, we confirm that the studies utilizing BMDEos are recapitulated in freshly isolated peritoneal eosinophils.

Minor comments:

1. Elevated expression of Dusp5 (Fig 1A) is only shown on the RNA level and no statistics is provided. Can you verify it on the protein level? In fact, Fig. E1D suggests that the level of Dusp5 should be tested following induction of cells rather than in the resting conditions.

We have added the statistical analysis demonstrating that Dusp5 mRNA expression is more abundant in CD4+ T cells, eosinophils and NK cells compared to other cell populations (Figure 1A). The reagents available to analyze expression of DUSP5 protein are quite limited in quality. The original commercial anti-DUSP5 antisera used has deteriorated in quality. We have tested a number of additional commercial anti-DUSP5 antibodies, but none of these antibodies provide high quality performance. We generated a polyclonal antisera against a DUSP5 peptide now described in Materials and Methods on page 26. Using this polyclonal antisera, we confirmed that IL-33 induces DUSP5 protein levels (Figure E8B), consistent with the increased Dusp5 mRNA levels following IL-33 treatment (Figure E8A). We observed a specific band at the appropriate molecular weight in IL-33 treated Dusp5+/+ eosinophils, while this band was not observed in Dusp5/-/- eosinophils under identical parallel treatment conditions. The relative low abundance of DUSP5 protein in unstimulated cells, despite detectable at the mRNA level, remains below the level of detection using our anti-DUSP5 antisera. These data appear as a new figure E8B and described in the text on page 12.

2. The rationale for using Rag2 KO is not clear. Other cells such as ILC2 which are the prominent source of IL-5 are still present in this mouse. Have you attempted to verify the phenotype in the
regular KO mouse? If different phenotypes are observed this would suggest that T-cells are important for regulating eosinophil survival.

As described on pages 7 and 8, Dusp5-deficient mice have altered thymic development (Figure E2A) and hyperactive CD4+ T cell proliferation following TCR engagement (Figure E3A). These data are consistent with those reported by Warren Leonard and colleagues using overexpression of a DUSP5 transgene under the control of a H2-Kb promoter and Ig heavy chain enhancer (Kovanen et al, J Biol Chem, 283:17362, 2008). Therefore, to remove the contributions of hyperactive Dusp5-deficient T cells, we crossed Dusp5-/- mice onto a Rag2-/- background. Rag2-/- mice are able to clear Nippostrongylus brasiliensis though with delayed kinetics, highlighting the contributions of both adaptive and innate immune cells to parasite clearance. In addition, while we observe increased eosinophil survival and activation in Dusp5-/-Rag2-/- mice, these phenotypes are also observed in Dusp5-/-Rag2+/+ mice (Figures E5A-C).

We have also characterized additional immune populations in Nippostrongylus infected Dusp5+/+Rag2-/- and Dusp5-/-Rag2-/- mice to determine if other cell types are altered. We observe no differences in neutrophil, NK cell, ILC2 and monocyte numbers in the spleen (Figures E4A-C), mesenteric lymph nodes or BALF (data not shown) between Dusp5+/+Rag2-/- and Dusp5-/-Rag2-/- mice infected with Nippostrongylus brasiliensis. These data are discussed on pages 8 and 9.

We also provide, in the revised manuscript, data demonstrating that depletion of NK cells, which also express high levels of Dusp5 mRNA (Figure 1A), using an anti-Asialo GM1 antibody does not alter the increased worm burden or eosinophilia observed in Dusp5-/-Rag2-/- mice. These data are discussed on page 9 and depicted in Figures E4D and E4E. Hence, NK cells do not play a requisite role in the phenotypes of eosinophilia or lesser worm burden observed in Dusp5-/-Rag2-/- mice. Our phenotype is also consistent with observation that mice with chronic eosinophilia (IL-5 transgenic mice) have accelerated helminth expulsion (Dent et al., Infect Immun., 67:989-993, 1999).

Therefore, the collective body of data including expression analysis, lack of effects on NK and ILC2 cell numbers, NK cell depletion experiments and the observation that eosinophil survival and activation is augmented in Dusp5-/- mice, supports our working model that eosinophils are likely the cellular population responsible for the enhanced anti-helminth response. We have retained a sentence in the Discussion (page 20) that we cannot rule that other cell populations may not also play additional contributory roles to this phenotype.

3. For all the figures more clarity in labeling will be helpful. For instance, it is unclear whether Fig 7F is a graphical display of the results from Fig 7E or a separate experiment.

We agree with the reviewer that our labeling of figures should be more clear. In the revised manuscript, we have added clarifying text to the each of the figure legends:

Figure 1C-F: We added text stating that these graphs are from the same experiment, and are representative of 3 independent experiments (page 38).

Figure 3A-B: We clarified that the graphical data in 3B is from the FACS analysis in 3A and multiple other experiments (page 40).

Figure 3C-D: We clarified that the graphical data in 3D is from the FACS analysis in 3C and other experiments (page 41). We also clarified that chimeric mice were infected with Nippostrongylus brasiliensis (page 41).

Figure 5A: we quantified expression of BCL-2 and BCL-XL relative to ACTIN protein levels. These ratios are provided in the form of fold-change below each lane of the Western blot (page 43).

Figure 5B: BCL-2 and BCL-XL pixel intensity is quantified from the sum of 4 independent Western blots (page 43).
Figures 6E and 6F: We clarified that the graphical data on the right represent the values from the FACS histograms on the left and from a total of three independent experiments (page 45).

Figure 7A and 7B: We clarified that the graphical data on the right represent quantitation of immunoblots shown on the left (page 46).

Figure 7D-E: We clarified that 7E is the graphical data from the immunoblot shown in 7D (page 47).

Figure E7: We clarified that data from E7B-D, 2D and 3C-D were from the same experiment and data representative of multiple experiments (page 52).

4. In Fig.7A the authors demonstrate by Western blot increased nuclear level of phospho-Erk and decreased level of cytoplasmic phospho-Erk in Dusp5KO cells. This is surprising because if Dusp5 is not dephosphorylating Erk, then both fractions should potentially be increased. Combined total phospho-Erk level (nuclear + cytoplasmic) should be higher in Dusp5KO (due to loss of DUSP5’s phosphatase activity), but it does not seem so. We share this concern of Reviewer 1. We have performed these experiments multiple times and all experimental results are consistent. The Keyes group has demonstrated that DUSP5 is a nuclear phosphatase and can serve as a nuclear anchor for ERK1 and ERK2 through a putative phosphatase-independent nuclear anchoring function (Mandl et al., Mol Cell Biol., 25:1830-45, 2005). We do not fully understand the regulation of cytoplasmic vs nuclear functions of DUSP5 in Erk regulation and this is an area of additional investigation that we are pursuing using forms of DUSP5 that localize within the nucleus or cytoplasm, but beyond the scope of these studies.

We have performed the experiment suggested by the Reviewer to analyze total cell pERK1/2 using RIPA buffer. These new data presented in Figure 7A and discussed on page 15 are precisely what we and Reviewer 1 predicted. There is increased pERK1/2 in Dusp5-/- BMDEos following addition of IL-33. These data are also consistent with the observation that ERK-responsive genes are further upregulated in IL-33 stimulated Dusp5-/- BMDEos (Table E2).

Given that additional work will be needed beyond the scope of the present studies to better understand the nuclear vs cytoplasmic distributions of DUSP5 and substrates, we have replaced Figure 7A with the total cell pERK1/2 experiment.

5. In this regards controls for purity of nuclear and cytoplasmic fractions should be performed for all relevant experiments. Also, the 0 timepoint for U0126 stimulation is missing in Fig 7E but is present in the graph in 7F. Moreover, the beta-actin loading control needs to be present in Fig 7E because it is referenced in 7F.

To address the concerns of the reviewer with regard to fractionation purity, we analyzed relative purity of the nuclear and cytoplasmic fractions by immunoblotting for HSP90 as a cytoplasmic marker and HDAC2 as a nuclear marker. These data are presented in Figure E12 and described in the text in Materials and methods on page 25. Representative data in Figure E12 indicate >95% purity of each fraction.

With regard to the U0126 experiments, we added the requested actin blot as the bottom panel of Figure 7D. Figure 7D also contains all time points including timepoint 0 for the U0126 cells. In addition, quantitation of BCL-XL and BCL-2 relative to actin protein levels assessed by immunoblotting in Figure 7D are quantitated in graphical view in Figure 7E.

6. Control lanes in Western blot experiments in Fig E9C and Fig 5D are overexposed and seem to be unequal. This needs to be corrected in order to properly interpret the results. For Fig 5B, the pixel intensity needs to be normalized to loading control. For Fig E1D loading control as well as control for efficient activation in DuspKO cells is missing. We have provided blots from a shorter immunoblot exposure for Figures 5D and E9C. Reviewer 1 is
correct that lanes 1 and 4 in Figure E9D have less actin- this is due to the ongoing apoptosis of cells cultured in the absence of survival cytokines for 3 days (vs cells treated with IL-5 in lanes 2 and 4 and IL-33 in lanes 3 and 6). While the unstimulated cells have reduced actin compared to IL-5 or IL-33 stimulated cells (lanes 1 vs 2 or 3 OR lanes 4 vs 5 or 6), there is equivalent loading when comparing unstimulated, IL-5 treated or IL-33 treated Dusp5+/+ vs Dusp5−/− eosinophils (lanes 1 vs 4, lanes 2 vs 5 or lanes 3 vs 6).

Regarding the reviewer’s concern for Figure 5B, the relative pixel intensity is the cumulative intensity of Bcl-xl and Bcl-2 over 4 independent experiments. While these were not normalized to actin control, we provide additional quantitation of Bcl-xl or Bcl-2 levels normalized to actin of Figure 5A in Figure E9C. We have also provided the normalized quantitation of Bcl-XL and Bcl-2 to actin below the representative immunoblot in Figure 5A.

We have added an actin immunoblot as a loading control for Figure E1A.

7. Bouffi et al reports IL33 activates murine eosinophils via transcriptional responses including Dusp5 (JI 2013); this data should discussed in the context of the presented findings. Bouffi et al. have reported an increase in Dusp5 mRNA expression in IL-33, but not IL-4, stimulated BMDEos. We have added this data into our discussion (page 18) and have cited it in references as recommended by Reviewer #1.

REVIEWER #2

The family of dual specificity phosphatases (DUSPs) regulates activation of cytoplasmic or nuclear functions of diverse MAP kinases. Some DUSP members have wide tissue distribution while others are expressed at high levels in some tissues or cell types. The authors found that DUSP5 mRNA is expressed at particular high levels in NK cells and eosinophils. Eosinophils belong to the innate branch of the immune system playing a major role in immunity to helminths and allergic diseases. Thus, the authors decided to investigate the effect of DUSP5 gene deletion on eosinophil development, response to helminthes and signaling of cytokine known to control eosinophil activity.

DUSP5−/− showed no significant alteration in the number of eosinophils, neutrophils, monocytes or NK cells and other cells of the immune system. However, in DUSP5−/− mice deprived also of T and B cells (Rag2−/−) to distinguish between the effects of eosinophils from adaptive immunity, they found a higher percentage of eosinophils two weeks after infection by N. brasiliensis in bone marrow, spleen, blood, bronchoalveolar lavage fluid. As a consequence, DUSP5−/− mice cleared more efficiently parasite burden following infection with N. brasiliensis. However, the affect of DUSP5 deficiency with an intact adaptive arm of immunity on N. brasiliensis infection was not investigated.

The data suggested that during infection, DUSP5 deficiency does not affect proliferation of eosinophil precursors. However it extends their survival.

IL-33 and IL-5 positively regulate survival and effector functions of eosinophils by activating several signaling pathways, including MAPKs and are crucial in controlling helminth infection. Thus, the authors asked whether DUSP had an effect on IL-33/IL-5 signaling. It was found that DUSP5 mRNA in eosinophils was substantially increased upon IL-33 but not by IL-5 stimulation and present data supporting a role for DUSP5 in IL-33-mediated eosinophil survival. Microarray analysis showed that DUSP5-deficient BM-derived eosinophils secreted significantly greater amounts of IL-13, GM-CSF and IL-1 proteins. Moreover, initial mechanistic studies link the axis IL-33, nuclear ERK1,2 DUSP5 to the tuning of BCL-XL expression, suggesting that in addition to regulate cellular activation and cytokine production in response to IL-33, DUSP5 has a pro-survival action on eosinophils. Together, these data are consistent with a negative regulatory role of DUSP5 in IL-33, but not IL-5, mediated eosinophil activation and survival via expression augmented expression of BCL-XL.

The work is well presented; the experiments are well designed and technically sound. The correlative conclusions are consistent with data presented.

We thank the reviewer for his/her remarks regarding the experimental rigor and interpretation of our
findings. We address Reviewer 2’s comments below and provide additional experimental data to clarify our findings and to address these concerns.

Major comments:

1. I have only one major reservation about the biological significance of this work, namely the limitation of studying the effect of DUSP5 deficiency in absence of T and B cells. As the authors admit in the discussion, IL-33 has broad effects on many cell types beyond eosinophils and the greater ability of DUSP5−/− Rag2−/− mice to clear N. brasiliensis may be due to additional cellular components.

We appreciate Reviewer #2’s reservation about other cell types involved in the observed phenotype in Dusp5−/−Rag2−/− mice. First, we agree that DUSP5 likely plays a multitude of biological roles in many cell types. Our studies presented in Figures E2 and E3 confirm and extend the findings of Leonard and colleagues that DUSP5 has an inhibitory role on thymic development and naïve CD4+ T cell proliferation (Kovanen et al, J Biol Chem 283:17362, 2008). Our primary focus for this manuscript is to define the mechanistic basis by which DUSP5 regulates eosinophil survival and function. To accomplish this, we took a reductionist approach by analyzing Dusp5−/−Rag2−/− mice to remove the potential confounding contributions of hyperactive Dusp5−/− T cells. We provide additional data in the revised manuscript to assess additional non-eosinophil cell types that may contribute to the phenotype we report here (see below).

In this study, we utilized the N brasiliensis experimental system to investigate how DUSP5 regulates eosinophil functions. As mentioned above, the majority of experiments utilized DUSP5 deficient mice on a RAG2 deficient background so that we would not confound the biological results with the underlying effects of a hyperactive T cell phenotype. However, we have also analyzed the effects of DUSP5 deficiency on a RAG2-sufficient background. These data are presented in Figures E5A-C in the original manuscript demonstrating that the primary endpoint of increased eosinophils following N brasiliensis infection also occurs in DUSP5 deficient RAG2-sufficient mice.

With regard to other cell types, in addition to the data presented in the original manuscript that neutrophils and monocytes are not altered with DUSP5-deficiency following N brasiliensis infection (Figures E4A and E4B), we provide additional data in the revised manuscript that natural killer (NK) cells and ILC2 cells are also not altered in DUSP5-deficient mice under homeostatic conditions (data not shown) or following N brasiliensis infection (Figure E4C).

Finally, we formally analyzed the contributions of NK cells, which express comparably high levels of DUSP5 as eosinophils (Figure 1A), following N brasiliensis infection. These data are discussed on page 9 and presented in Figures E4D and E4E of the revised manuscript. The eosinophilia and greater ability to clear N brasiliensis observed in DUSP5-deficient mice were both retained even when NK cells were depleted with treatment with anti-Asialo GM1 antibody. Hence, while we cannot exclude that other cell types may still contribute to the phenotypes observed, our collective data strongly supports a DUSP5- and eosinophil-dependent mechanism for the phenotypes observed. We have retained a sentence in the Discussion (page 20) that we cannot exclude all other cell types that may contribute to the phenotypes observed.

Minor comments:

1. DUSP5 mRNA in eosinophils is substantially increased upon IL-33 stimulation. Also, DUSP5 mRNA is expressed at particular high levels in NK cells and eosinophils. However, the authors should present data on differences of protein levels as well.

There are no high quality anti-DUSP5 Abs commercially available. We had previously used a commercially available anti-DUSP5 antisera whose quality has declined over time. We have tested all additional available commercial antisera without any success. In the revised manuscript, we generated a new anti-DUSP5 antisera described on page 26 and provide a protein immunoblot analysis of DUSP5 levels in mouse eosinophils following treatment of IL-33. These data are presented in Figure E8B. The quality of this new antisera is still not ideal, but we can detect an ~42kDa protein consistent with DUSP5 that is absent in the Dusp5-deficient cells. The lack of
detectable DUSP5 protein in unstimulated eosinophils and NK cells despite measurable mRNA levels likely reflects the limitation of our anti-DUSP5 Ab reagent. We will pursue generation of improved reagents, but we believe that our conclusions presented in this study are well supported despite this limitation.

Reviewer #3

The well-written manuscript provides convincing evidence for a novel role for the dual specificity phosphatase, DUSP5 in regulating IL-33 signaling in eosinophils, thereby modulating the activation and survival of those cells.
We thank the Reviewer for underscoring the novelty and significance of our studies. We provide additional data in the revised manuscript and a point-by-point reply to address the Reviewer’s concerns.

Major comments:

1. The impact of DUSP5 in IL-33 signaling in eosinophil is evident, what is less clear is the role of this phosphatase during the defense to parasite infection. The authors have a body of data suggesting that DUSP5 gene expression is increased during parasite loading but neither the DUSP5 protein content is shown, nor is the role of NK cells discussed, while evidence suggest that DUSP5 is equally if not more upregulated in those cells. While the authors nicely excluded an involvement of T and B-cells in there system, by crossing the DUSP5-deficient mice on a RagKO background, NK cells are still present and might contribute to the immune response especially. The authors should conduct experiments to address this.

Reviewer #3 raises an important issue whether other cell types might contribute to the observed phenotypes.

To assess the effects of DUSP5 on other cell types that may contribute to the phenotypes observed, we investigated where DUSP5 deficiency would affect other cell types beyond the increased numbers of eosinophils and single positive thymocytes observed in our study. Numbers of neutrophils, monocytes, NK and ILC2 cells were NOT altered in Dusp5-/- mice under homeostatic conditions (data not shown) or following N brasiliensis infection (Figures E4A, E4B and E4C). Since DUSP5 mRNA is also highly expressed in NK cells, we further investigated the contributions of NK cells. We analyzed whether the phenotypes of increased worm burden and eosinophilia observed in Dusp5-/-Rag2-/- mice were retained following NK cell depletion with anti-asialo GM1 antibodies. Both phenotypes of increased worm burden and eosinophilia were retained in NK cell depleted Dusp5-/-Rag2-/- mice when compared to NK cell depleted Dusp5+/+Rag2-/- mice. These data are presented in Figures E4D and E4E and discussed on page 9. Thus, our collective body of data suggests that the phenotypes observed are primarily due to the effects of DUSP5 on eosinophils. This notwithstanding, we retained a sentence in the Discussion (page 20) that we cannot rule out other potential contributory cell types.

Minor comments:

1. Also, it is very disappointing that many statistical analysis with p values were conducted on just 2 experiments.
We have addressed this concern with additional experiments in the few cases where 2 experiments were conducted. As expected, the additional experiments continue to support our working model. These are updated in the revised manuscript.

2. Last, but not least western blots lacking loading controls such as in figure 7 E are quite hard to interpret.
In the revised manuscript, we show the actin loading controls which we had performed as we normalized for quantitation purposes (but had not included in the original manuscript). We also have
added graphs in the revised manuscript to depict the quantification normalized for actin or other loading controls. These include:

Figure 5A- We provide the quantitation of Bel-XL and Bel-2 protein levels normalized for actin at the bottom of the figure. In addition, we provide graphical depiction of this data in Figure E9C.

Figure 7D (previously Fig. 7E)- we have provided the appropriate actin blot in the new Figure 7D as well as quantitation of BCL-XL and BCL-2 normalized for actin in the corresponding Fig. 7E.

Figure E1D- We have added the actin blot at the bottom of this figure.

2nd Editorial Decision 07 August 2014

Thank you for submitting your revised manuscript to The EMBO Journal, which is a resubmission of MS 88453. I asked the original referees #1 and 3 to re-review the paper. I have now heard back from both of them and I am afraid that there are still outstanding issues that prevent publication here.

As you can see below, while referee #1 is satisfied with the revised version, referee #3 still has major concerns with it. I would like to say that I am not in complete agreement with some of the remaining issues raised by referee #3. I also see that many of the issues can be resolved with better presentation of the findings and some re-organization of the figures. However, the key issue here is the lack of a Dusp5 Antibody to demonstrate that Dusp5 is indeed expressed in the relevant cells. In agreement with the referee, we also find that the quality of figure E8B to show that Dusp5 is upregulated in response to IL-33 fairly poor.

I have also sought further external advice on this issue from an independent advisor who is also a good expert in the field. I have now heard back from the advisor and s/he is in agreement with the concerns raised regarding the Dusp5 Ab and finds that this would have to be resolved in order to consider publication here. As it is not clear how this can be achieved, I am afraid that I see no other chance but to reject the manuscript.

I am very sorry that I can't be more positive on this occasion.

REFEREE REPORTS

Referee #1:
My queries have been adequately addressed.

Referee #3:
This is a resubmitted manuscript and while a lot of work seems to have been added based on a first reading of the rebuttal letter, the resulting manuscript is quite deceiving.

For example, the authors spent 1/2 of a page discussing a comment made by reviewer #1 concerning their finding of increased nuclear and cytoplasmic ERK in absence of DUSP5 (old Fig 7A &B) and even seem to agree with the reviewer. In contrast, throughout the revised manuscript they keep discussing their original findings of "increased nuclear ERK1/2 activation" (in particular in the abstract), despite having added a new figure 7A that only contains ERK from whole cell extracts, but not from nuclear fractions.

Other example. In response to a concern of reviewer #3, the authors claim to have incorporated additional data into figures that were originally generated from only 2 experiments, but in absence of a descriptive list (which is not provided in the rebuttal), it is hard to tell which figure was upgraded. All figures in the resubmitted manuscript (except first time submitted figures) are carbon copies of the original manuscript.
Furthermore, many scientifically sound experiments that were suggested by the reviewers are either only added as supplementary material in the Expanded View section (not present in the hard copy manuscript) or expedited as "data not shown", which is against the journal's policy. From the author guidelines of EMBO J:

"The EMBO Journal does not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures."

Last but not least, to base an entire work on the absence of critical reagents or their low quality as the authors infer in response to concerns of reviewer #1 & 2 is certainly not speaking for the scientific relevance of this work and thus has no place in a high quality standard journal such as EMBO J.

At best this is a bad revision, at worst it is a deceptive revision.......

Correspondence 03 September 2014

Thank you again for all of your time and effort in evaluating our manuscript.

We have successfully utilized a new anti-DUSP5 antibody to demonstrate that DUSP5 protein is upregulated in eosinophils following IL-33, but not IL-5 stimulation.

We have also made changes in the text and figures as suggested by Reviewer 3 and have provide a more detailed reply to Reviewer 3’s comments and suggestions in the attached “Reply-to-Reviewer 3” document.

I am hopeful that the new Western blots address the major concern of Reviewer 3. As the Reviewers 1-3 previously noted the biological significance and novelty of our study, we re-emphasize the novelty of the MEK/BCL-XL mechanism described here by which IL-33 mediates eosinophil survival. Given the genetic associations described for IL-33 and its receptor for the development of human asthma, our mechanistic insights will likely provide a greater understanding of IL-33 causation of human asthma.

I look forward to hearing about how we could forward our revised manuscript for publication in EMBO. Thanks.

2nd Revision - authors’ response 03 October 2014

Reply to Reviewer #3

We thank Reviewer 3 for pointing out our oversights. We provide a point-by-point reply below to his/her comments. Our comments are represented in green in italicized font.

This is a resubmitted manuscript and while a lot of work seems to have been added based on a first reading of the rebuttal letter, the resulting manuscript is quite deceiving. For example, the authors spent 1/2 of a page discussing a comment made by reviewer #1 concerning their finding of increased nuclear and cytoplasmic ERK in absence of DUSP5 (old Fig 7A &B) and even seem to agree with the reviewer. In contrast, throughout the revised manuscript they keep discussing their original findings of "increased nuclear ERK1/2 activation" (in particular in the abstract), despite having added a new figure 7A that only contains ERK from whole cell extracts, but not from nuclear fractions.

We apologize for this oversight as it was not meant to be deceiving. Reviewer 3 is correct that the revised manuscript shows total cellular ERK 1/2 activation (Figure 7A). While we had accurately described these findings on page 15 of the Results section, we forgot to change the text in the
abstract (p 2) and later appearing text (pages 16, 17 and 18). These oversights are corrected in the manuscript in yellow to reflect total cellular ERK activation.

Other example. In response to a concern of reviewer #3, the authors claim to have incorporated additional data into figures that were originally generated from only 2 experiments, but in absence of a descriptive list (which is not provided in the rebuttal), it is hard to tell which figure was upgraded. All figures in the resubmitted manuscript (except first time submitted figures) are carbon copies of the original manuscript.

In each case, we performed additional experiments, but did not change the original figures since they were representative of at least 3 independent experiments. At the Reviewer’s request, we have changed Figures 2D, 3D and 4E to show each mouse data point from all triplicated independent experiments.

For figures 6D, 6F, E2C and E7D, since each experiment has a slightly different dynamic range, we provide below for the Reviewer’s analysis each of the 3 independent experiments. The left figure in each case represents the figure shown in the manuscript. For figures 6D and E2C, the dynamic ranges are sufficiently close enough between experiments that we also provide a compilation analysis of all three independent experiments. ** \( P < 0.01 \) and *** \( P < 0.001 \)
Furthermore, many scientifically sound experiments that were suggested by the reviewers are either only added as supplementary material in the Expanded View section (not present in the hard copy manuscript) or expedited as "data not shown", which is against the journal's policy.

From the author guidelines of EMBO J:
"The EMBO Journal does not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures."

We apologize for this oversight.
1. Page 8. We have added the peripheral blood and inguinal lymph node analysis in the revised Table E1 and Figure E2C, respectively.
2. Page 9. We have added the induction of eosinophils in N. brasiliensis infected mice following depletion of NK cells in a new Figure E4F.
3. Pages 13-14. We added the protein expression of pro-apoptotic proteins to Figures E9C and E9D.
4. Pages 14-15. We have added the IL-5-mediated secretion of IL-4, IL-13 and TNF-alpha from bone marrow derived eosinophils in a new Figure E10C.
5. Page 15. We added the p38 and JNK activation in a new Figure E11B.
In each case, the addition is highlighted in yellow.

Last but not least, to base an entire work on the absence of critical reagents or their low quality as
the authors infer in response to concerns of reviewer #1 & 2 is certainly not speaking for the scientific relevance of this work and thus has no place in a high quality standard journal such as EMBO J.

To address the reviewers concerns regarding expression of DUSP5 in eosinophils, we have successfully identified a new commercially available anti-DUSP5 monoclonal antibody (mAb) that provides technically suitable analysis of endogenous murine DUSP5. In the new Figure 4A, we demonstrate that DUSP5 protein is expressed in unstimulated BM derived eosinophils and expression is induced following IL-33. These new data are described on page 12 in yellow-highlighted text. Description of this anti-DUSP5 mAb is on page 26 of Methods.

Accepted 14 October 2014

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the advisor who I also had involved on the previous version to take a look at the revision. The main issue was the lack of a Dusp5 Ab to show that eosinophils express Dusp5 protein. I have now heard back from the advisor and as you can see below, s/he finds that you have satisfactorily addressed this issue as well as the other remaining points. Given this, I am therefore pleased to proceed with the acceptance of the paper for publication here.

ADVICE

The authors have found a novel, commercially available anti-DUSP5 antibody that they convincingly used in Fig 1E to validate the lack of protein in CD4 T cells of their knock-out and in Figure 4 to demonstrate that eosinophils increase the expression of Dusp5 upon IL-33 stimulation. Accordingly I consider that the revised manuscript should be published.