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The NF-κB1 transcription factor prevents the intrathymic development of CD8 T cells with memory properties

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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 25 March 2011

Thank you for submitting your manuscript to the EMBO Journal. Two referees have now seen your manuscript and their comments are provided below.

As you can see both referees find the analysis interesting, but they also raise significant concerns with the analysis. Both referees raise similar issues namely that further data is needed to address if the development of the innate-like CD8+ T cells is due to an indirect role of IL-4 as recent papers have suggested. This is an issue that has to be fully sorted out with the inclusion of additional data in order for further consideration here. If you can address the concerns raised in full then we are willing to consider a revised version. I don't know if you have data on hand to address these issues, but I would like to ask you to carefully consider your options and let me know as soon as possible if you prefer to take the paper elsewhere at this stage or if you would like to revise the manuscript along the lines as indicated by the referees. Given the uncertainty of the experimental outcome, I am sure that you can also understand that I at this stage can make no commitment to the paper. In case you would like to submit a revised manuscript, I can extend the revise deadline to 6 months. I should also point out that is EMBO Journal policy to allow a single round of revision only and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

I thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2:

This manuscript examines the development of a CD8 cell population with effector/memory characteristics in mice deficient in NF-kB1. Data over the last 5 years has shown an increased proportion of such cells in a number of mouse strains having mutations in molecules involved in T cell signaling and transcription, including Itk, Id3, Cbf, KLF2 and SLP76. However, recent data indicates that the development of these cells in many, if not all, of these strains is not cell-autonomous but is secondary to increased IL-4 produced by hematopoietically selected memory-type CD4 cells that express the transcription factor PLZF. This paper adds data on an additional strain that provides insight into the regulation of thymic-derived memory CD8 cells. While the paper is interesting and the experiments presented for the most part well done, the paper does little to address some of the most recent and critical data regarding development of these memory-like CD8 cells. The authors argue that that innate CD8 cells in the Nfkb1-/- mice have distinct features (as do several of the strains). However, given the recent findings on cell autonomy by several groups, additional experiments need to be shown/performed to address the current issues in this area.
Specific comments:

The authors present data that the memory CD8 cells in the Nfkb1-/- mice are very similar to those in Itk-/- mice, being selected on hematopoietic cells using both MHC Class Ib and Ia. They also argue that these cells differ from those found in Itk-deficient mice for several reasons, including that the Nfkb1-/- CD8 cells have high levels of CD5, are not dependent on IL-15, and do not express NK1.1. The authors argue that the Nfkb1-/- mice are also distinct in having low numbers of NKT cells. While some of these differences are notable, others are not so clear and the authors may have misrepresented the literature (see recent review by Hogquist and colleagues). Most notably, Itk-/- mice also have low numbers of iNKT cells. This should be corrected in the paper. (The levels of NK1.1 expressed on CD8 cells are also quite low in some of the publications.)
The authors discuss the IL-4 dependency by the innate/memory-like T cells that develop in other deficient strains, but claim that the innate/memory-like T cells that develop in the Nfkb1-/- mouse differ and cannot transmit the innate phenotype to bystander WT thymocytes in a mixed bone marrow chimera. This could result from the phenotype in the Nfkb1-/- mouse being less prominent than some of the other strains. However, given that this IL-4 dependent, non-CD8+ cell-intrinsic mechanism is consistent across many different genetic strains that give rise to expanded populations of innate/memory-like CD8 cells, the authors need to show the mixed bone marrow chimera data in this manuscript. The authors also need to show the status of CD4 TCRαβ cells and γδ cells and whether an increased population of PLZF+ CD4+ cells is present in the Nfkb1-/- mice (as well as cytokine production by CD4 cells). It would also be of interest to see these stains in the Nfkb1-/-Il15-/- mice. Potentially, these experiments (along with the IL-15 data) would further set this group of innate/memory-like T cells apart from others and may help further our mechanistic understanding of how these cell types are regulated, which would be required for consideration in EMBO.

Additional comments:
It is difficult to evaluate the reduction in CD8+ cells in the Nfkb1-/-KbDb-/- mice. Graphs of both percentages and cell numbers should be shown.

The authors examine dependency of selection on MHC Class Ia and present findings that suggest that the thymically-derived memory CD8 cells are dependent on both MHC Class Ia and Ib presented on hematopoietic cells. Evaluation of thymic transfers using B2m-deficient mice and thymic lobes should be included to more fully evaluate requirements for MHC Class I selection on hematopoietic cells.

The authors argue that these phenotypes may be secondary to reduced negative selection in the Nfkb1-/- mice. However, their interpretation of some of the negative selection data, particularly in terms of TCR levels is certainly not clear to this reader. The authors should show a negative control for T3.70 staining, preferably staining of a non-transgenic mouse, not an isotype control. The authors should clarify the statement, "Finally, the finding that nfkb1-/- CD44hi but not CD44lo CD8SP thymocytes express lower TCRb levels provides a key link between impaired negative selection, reduced TCR expression and the development of CD8 memory-like thymocytes..." TCR levels go up upon selection. I am not certain what the authors are trying to convey. If the authors think negative selection is at the heart of the issue, it would be useful to show another negative selection system, even some type of in vitro culture system.

In summary, while the experiments are for the most part well-done, the paper does not address the most recent data demonstrating that these thymic-derived cells are not cell-autonomous in multiple other strains. While I don't like to penalize people for recent publications, the newer papers and insight were published 8-9 months ago and the authors state that they have unpublished results relevant to this issue. These results and others need to be shown for consideration in EMBO. As it stands, this paper does not address the current data and issues in the field.

Referee #3:

The manuscript by Gugasyan et. al. aims to address the role of NF-κB1 in conventional versus innate-like T cell development. The authors find that an innate-like CD8+ T cell populations develops in the absence of NF-κB1. This population is characterized by high expression of CD44, CD122, and Eomesodermin. Additionally, this innate-like population produces IFNγ upon ex vivo stimulation with PMA and ionomycin, and the development of these innate-like T cells appears to be cell intrinsic. The authors also find that this innate-like population of T cells does not depend on IL-15 for development and that this population is somewhat dependent on MHC class Ia for expression; although in KbDb-/- nfκb1-/- mice, there is a small population of CD8+ T cells that develop independently of MHC class Ia. Further, there appears to be some defect in negative selection upon absence of NF-κB due to a decrease in Sirpα+ dendritic cells that promote negative selection.

This paper helps to further elucidate the molecules contributing to the development of innate-like T cells. However, previous groups have discerned that the development of these innate-like CD8+ T cells expressing Eomesodermin are actually due, at least in part, to a population of T cells (both αβ and γδ) expressing PLZF and producing high amounts of IL-4. In this manuscript, the authors have not addressed whether the indirect role of IL-4 is also contributing to the development of innate-like CD8+ T cells. This is a major flaw in the manuscript, and experiments determining whether or not the NF-κB1 deficient cells are not dependent on IL-4 for their innate-like development need to be performed.

Additionally, there are a few minor problems for the authors to address:

1) The authors claim that there is a decrease in the CD4:CD8 ratio in mice deficient in NF-κB. Is this decrease significant?
2) It is unclear the number of experiments performed that tested the Vβ repertoire of the CD8 SP population. Additionally, do the Eomesodermin+ CD8 SP thymocytes also have a diverse Vβ repertoire?
3) In Figure 6, when the authors look at the various DC populations, are those differences significant?
4) In Figure 7D, it is unclear which lymph nodes are being shown. The methods state that the mesenteric lymph nodes were examined while the figure legend states that resident lymph nodes were examined. This issue should be clarified.
5) In Figure 2C, the authors should provide absolute numbers of cells.

1st Revision - authors' response 16 September 2011

Referee #2:

This manuscript examines the development of a CD8 cell population with effector/memory characteristics in mice deficient in NF-kB1. Data over the last 5 years has shown an increased proportion of such cells in a number of mouse strains having mutations in molecules involved in T cell signaling and transcription, including Itk, Id3, Cbf, KLF2 and SLP76. However, recent data indicates that the development of these cells in many, if not all, of these strains is not cell-autonomous but is secondary to increased IL-4 produced by hematopoietically selected memory-type CD4 cells that express the transcription factor PLZF. This paper adds data on an additional strain that provides insight into the regulation of thymic-derived memory CD8 cells. While the paper is interesting and the experiments presented for the most part well done, the paper does little to address some of the most recent and critical data regarding development of these memory-like CD8 cells. The authors argue that that innate CD8 cells in the Nfkb1-/- mice have distinct features (as do several of the strains). However, given the recent findings on cell autonomy by several groups, additional experiments need to be shown/performed to address the current issues in this area.

Response: Although our reported findings on the development of CD8 SP thymocytes with memory characteristics in NF-kB1 deficient mice share similarities with phenotypes reported in other mouse strains, we now provide additional data clearly establishing that, unlike the other mutant strains reported to date, the development of this phenotype in NF-kB1 deficient mice does not depend on the expansion of a thymic population of IL-4 producing hematopoietic $CD4^+$ cells that express the transcription factor PLZF. In response to the experiments proposed by Reviewer #2, we have now included critical data showing a). Mice lacking NF-kB1 do not display an expanded population of TCR ab^+ or gd^+ $CD4^+$ thymocytes expressing PLZF; b). That the IL-4 expression profile of $CD4^+$ thymocytes in wild-type and nfkb1-/- mice are equivalent; and c). That in mixed bone marrow chimera wild-type CD8SP thymocytes that develop amongst neighbouring NF-kB1 deficient thymocytes, do not acquire a memory-phenotype. Not only do these key experiments settle the issue that there is no role for a PLZF+ thymocyte bystander effect in the development of this phenotype by NF-kB1 deficient mice, but it also highlights the involvement of a novel NF-kB1 regulated pathway in the control of CD8 memory cell properties.

The authors present data that the memory CD8 cells in the Nfkb1-/- mice are very similar to those in Itk-/- mice, being selected on hematopoietic cells using both MHC Class Ib and Ia. They also argue that these cells differ from those found in Itk-deficient mice for several reasons, including that the Nfkb1-/- CD8 cells have high levels of CD5, are not dependent on IL-15, and do not express NK1.1. The authors argue that the Nfkb1-/- mice are also distinct in having low numbers of NKT cells. While some of these differences are notable, others are not so clear and the authors may have misrepresented the literature (see recent review by Hogquist and colleagues). Most notably, Itk-/- mice also have low numbers of iNKT cells. This should be corrected in the paper. (The levels of NK1.1 expressed on CD8 cells are also quite low in some of the publications.)

Response: It was not our intention to misrepresent the literature, and we thank Reviewer #2 for pointing out our mistake. In response to this valid point, we have removed the comment stating the absence of NF-kB1 and ITK have different impacts on the sizes of the NKT cell populations. With the inclusion of the new data, space constraints have instead directed us to focus our attention on highlighting the major differences between these strains. These include the finding that $nfkb1^{-/-}$ CD8SP cells acquire memory characteristics independently of IL-15 and that the IL-4 producing $PLZF^+$ thymocyte population in the NF-kB1 deficient mutant is very similar to that of wild-type mice (Discussion; p18).

The authors discuss the IL-4 dependency by the innate/memory-like T cells that develop in other deficient strains, but claim that the innate/memory-like T cells that develop in the Nfkb1-/- mouse differ and cannot transmit the innate phenotype to bystander WT thymocytes in a mixed bone marrow chimera. This could result from the phenotype in the Nfkb1-/- mouse being less prominent than some of the other strains. However, given that this IL-4 dependent, non-CD8+ cell-intrinsic mechanism is consistent across many different genetic strains that give rise to expanded populations of innate/memory-like CD8 cells, the authors need to show the mixed bone marrow chimera data in this manuscript.

Response: In response to this key issue, we have undertaken an extensive analysis of the potential dependency of the nfkb1-/- CD8 innate/memory on IL-4 by examining intact $nfkb1^{-/-}$ mice and

mixed bone marrow chimeras (Figure 2 and SFigure 2). In the mixed chimera studies, we established mice engrafted with equal (50% nfkb1$^{-/-}$ (Ly5.2^{+}): 50% wt (Ly5.1^{+})) and unequal (85% nfkb1$^{-/-}$ (Ly5.2^{+}): 15% wt (Ly5.1^{+})) ratios of wild type and nfkb1-/- bone marrow. Regardless of the chimera combination, the wild-type CD8SP thymocytes that develop in this chimeric environment, resemble conventional naïve T cells and not memory-like cells. Even when nfkb1$^{-/-}$ thymocytes are in the majority (85%), a memory phenotype was not observed in the wild-type CD8SP thymocyte population. Consistent with this finding, we did not observe an expanded population of IL-4 producing CD4+ thymocytes in the BM chimeras. As indicated earlier in this rebuttal letter, like the BM chimeras, thymocyte subsets in nfkb1$^{-/-}$ mice do not display a pronounced expansion of IL-4 producing PLZF+ thymocytes. To reinforce this point, we show that the proportion of IL-4 producing NKT cells in the thymus of wild type and nfkb1-/- mice is equivalent (Figure 2C,D).

The authors also need to show the status of CD4 TCRαβ cells and γδ cells and whether an increased population of PLZF+ CD4+ cells is present in the Nfkb1-/- mice (as well as cytokine production by CD4 cells).
Response: We already had data showing the number of *TCRαβ*$^{+}$CD4SP thymocytes (Fig 1B) and TCRb expression on wt and nfkb1$^{-/-}$ CD4SP thymocytes (Fig 6C). We have now included data that examines PLZF expression in wild-type and nfkb1$^{-/-}$ thymocyte subsets (Figure 2A) which show that there is not an increase in the size of the PLZF^{+}CD4^{+} T cell population within the nfkb1$^{-/-}$ thymus. Furthermore, we find that both wt and nfkb1$^{-/-}$ CD4^{+} thymocytes produced negligible levels of IL-4 and IFN-g following stimulation. As a control, we show equivalent levels of IL-4 are produced by wt and nfkb1$^{-/-}$ NKT cells (Figure 2C,D). We also provide additional data showing a comparable distribution of *TCRgd*$^{+}$ thymocytes in wt and nfkb1$^{-/-}$ mice. Importantly, the number of *TCRgd*$^{+}$CD4SP cells is not elevated in nfkb1$^{-/-}$ thymi (Figure 2B), a phenotype that is clearly different from the marked increase of this population in ITK null mice. Collectively, the new experiments suggested by reviewer #2 provide important evidence that the generation of CD8 memory-like cells in the thymus of nfkb1-/- mice appears to be independent of IL-4 producing CD4 cells that express PLZF.

It would also be of interest to see these stains in the Nfkb1-/- Il15-/- mice. Potentially, these experiments (along with the IL-15 data) would further set this group of innate/memory-like T cells apart from others and may help further our mechanistic understanding of how these cell types are regulated, which would be required for consideration in EMBO.
Response: While we have performed all the stains in the nfkb1$^{-/-}$ mice as well as in the mixed BM chimeras that were suggested by the reviewer, we were unable to perform a similar analysis in the nfkb1$^{-/-}$Il15$^{-/-}$ mice. The reason for not doing this analysis is because this mouse strain no longer exists. The studies we show in the paper using nfkb1$^{-/-}$ Il15$^{-/-}$ mice were done while the Gerondakis laboratory was still at the Walter and Eliza Hall Institute. This strain was not transferred to the Burnet Institute, where we now reside and no longer exists at the WEHI. Because we did not have access to sperm freezing at the time we were unable to resurrect the strain by this means. Both the nfkb1$^{-/-}$ and Il15$^{-/-}$ mice are poor breeders and based on our past experience, any attempt to re-derive the nfkb1$^{-/-}$Il15$^{-/-}$ mice would have taken a minimum of 12 months. This problem has been further compounded by the logistics of having to temporally relocate our mice to a smaller facility because of the impending construction of a new building directly above our institutional animal facility. As a result of limited space, we simply do not have enough space to breed a new strain. We hope that the reviewers are sympathetic to our situation. While agreeing that further analysis of nfkb1$^{-/-}$ Il15$^{-/-}$ mice may prove interesting, we believe our finding that memory-like nfkb1$^{-/-}$ CD8SP cells do not require IL-15 and don't depend on the IL-4 producing population of PLZF^{+} cells are the key pieces of data that point to the absence of any role for these cytokines in the generation of the memory phenotype in NF-kB1 deficient mice.

Additional comments:
It is difficult to evaluate the reduction in CD8+ cells in the Nfkb1-/-KbDb-/- mice. Graphs of both percentages and cell numbers should be shown.
Response: We have now inserted bar graphs of percentages and absolute numbers for CD8SP and CD8^{+}CD24^{lo} cells for each strain used in this study (Figure 4D). Our results are consistent with our previous interpretation. Changes to the text (page 11 & 12) include the analysis of absolute cell numbers.

The authors examine dependency of selection on MHC Class Ia and present findings that suggest that the thymically-derived memory CD8 cells are dependent on both MHC Class Ia and Ib presented on hematopoietic cells. Evaluation of thymic transfers using B2m-deficient mice and thymic lobes should be included to more fully evaluate requirements for MHC Class I selection on hematopoietic cells.

Response: We agree that the use of B2m-deficient mice would have provided a more comprehensive analysis of the requirements for selection. However, for the reasons outlined above, we were not in a position to import B2m deficient mice into our facility to undertake these experiments, nor were these mice available through our external collaborators. Again, we apologize for this situation. In response to this point raised by the reviewer, we have inserted a caveat in the discussion that a requirement of memory-like cells for MHC class Ia or Ib molecules needs to be confirmed by studying the development of nfkb1$^{-/-}$ CD8SP cells on a B2m-deficient background (p. 19)

The authors argue that these phenotypes may be secondary to reduced negative selection in the Nfkb1-/- mice. However, their interpretation of some of the negative selection data, particularly in terms of TCR levels is certainly not clear to this reader.

Response: We agree that the wording of this section in the previous manuscript was not as clear as it could have been, so we have attempted to improve our explanation of this connection in this revised version. In effect we propose that a clue to how memory-like CD8^{+} cells in the thymus of nfkb1$^{-/-}$ mice may be generated emerges from the finding that the majority of nfkb1$^{-/-}$ CD44^{hi}CD8SP thymocytes are CD5^{hi}TCR^{lo}, indicating that during selection these cells received a strong TCR signal. With negative selection normally deleting the vast majority of DP thymocytes with high TCR affinity, our findings raise a potential scenario whereby impaired negative selection in nfkb1$^{-/-}$ mice might permit a pool of thymocytes with higher affinity TCRs to be targeted by hematopoietic cells during positive selection. Whilst acknowledging that it remains to be shown that a defect in negative selection is indeed required for the development of the memory phenotype, given we have eliminated the involvement of bystander PLZF thymocytes, we felt based on the available data we have generated that this is a plausible model that unites these two changes seen in the NF-kB1 deficient mice.

The authors should show a negative control for T3.70 staining, preferably staining of a non-transgenic mouse, not an isotype control.

Response: For the purposes of a staining control, C57BL/6 (wt) thymocytes were originally stained with the T3.70 clonotype Ab in each experiment. This data has now been inserted into Figure 6A and 6B to distinguish positive and negative staining with the T3.70 Ab.

The authors should clarify the statement, "Finally, the finding that nfkb1-/- CD44hi but not CD44lo CD8SP thymocytes express lower TCRb levels provides a key link between impaired negative selection, reduced TCR expression and the development of CD8 memory-like thymocytes..." TCR levels go up upon selection. I am not certain what the authors are trying to convey. If the authors think negative selection is at the heart of the issue, it would be useful to show another negative selection system, even some type of in vitro culture system.

Response: We agree with Reviewer #2 that the sentence quoted above lacks clarity. This sentence has now been omitted. As suggested by reviewer #2, we have inserted new data examining negative selection in vitro by stimulating wt and nfkb1$^{-/-}$ thymocytes with plate-bound anti-CD3 Ab (Figure 6E, F). This new data has reveals that the loss of NF-kB1 in thymocytes does not alter the levels of DP thymocyte cell death in this culture model and instead suggests that the reduced efficiency of negative selection may not be intrinsic to the thymocytes. With NF-kB1 regulating other aspect of thymic function, including the size of the thymic CD8Sirpa^{hi} DC population, that is known to be important in promoting negative selection, the anti-CD3 Ab experiments help to clarify the mechanistic basis of the negative selection defect.

In summary, while the experiments are for the most part well-done, the paper does not address the most recent data demonstrating that these thymic-derived cells are not cell-autonomous in multiple other strains. While I don't like to penalize people for recent publications, the newer papers and insight were published 8-9 months ago and the authors state that they have unpublished results relevant to this issue. These results and others need to be shown for consideration in EMBO. As it stands, this paper does not address the current data and issues in the field.

Response: Based on the thoughtful suggestions of Reviewer #2, the additional experiments we have now performed and included in this revised manuscript do in our opinion address certain shortcomings in the original data and relevant issues in the field.

Referee #3:

The manuscript by Gugasyan et. al. aims to address the role of NF-κB1 in conventional versus innate-like T cell development. The authors find that an innate-like CD8+ T cell populations develops in the absence of NF-κB1. This population is characterized by high expression of CD44, CD122, and Eomesodermin. Additionally, this innate-like population produces IFNγ upon ex vivo stimulation with PMA and ionomycin, and the development of these innate-like T cells appears to be cell intrinsic. The authors also find that this innate-like population of T cells does not depend on IL-15 for development and that this population is somewhat dependent on MHC class Ia for expression; although in KbDb-/- nfκb1-/- mice, there is a small population of CD8+ T cells that develop independently of MHC class Ia. Further, there appears to be some defect in negative selection upon absence of NF-κB due to a decrease in Sirpα+ dendritic cells that promote negative selection.

This paper helps to further elucidate the molecules contributing to the development of innate-like T cells. However, previous groups have discerned that the development of these innate-like CD8+ T cells expressing Eomesodermin are actually due, at least in part, to a population of T cells (both αβ and γδ) expressing PLZF and producing high amounts of IL-4. In this manuscript, the authors have not addressed whether the indirect role of IL-4 is also contributing to the development of innate-like CD8+ T cells. This is a major flaw in the manuscript, and experiments determining whether or not the NF-κB1 deficient cells are not dependent on IL-4 for their innate-like development need to be performed.

Response: We would like to thank Reviewer #3 for their constructive review of the manuscript. We agree that the issue of a role PLZF positive cells may play in the memory phenotype of NF-kB1 deficient mice needed to be dealt with, and we are confident that the new experiments unambiguously address this issue.

We have addressed the role of IL-4-producing $PLZF^+$ thymic T cells in NF-kB1-deficient mice by showing that, 1). Mice lacking NF-kB1 do not have an expanded population of TCR ab^+ or gd^+ $CD4^+$ thymocytes expressing PLZF (Figure 2A, B); 2). We observe equivalent and low levels of IL-4 producing $CD4^+$ thymocytes in both wild-type and NF-kB1 deficient mice (Figure 2D); 3). Our mixed bone marrow chimera studies demonstrate that wild-type CD8SP thymocytes that develop amongst neighbouring NF-kB1-deficient thymocytes do not acquire a memory-phenotype (Figure 2E & SFigure2). Collectively, our data demonstrates that the acquisition of memory-like CD8 cells in the thymus of $nfkb1^{-/-}$ mice does not depend on a marked expansion of IL-4 producing $PLZF^+$ cells. We believe this new data highlights a novel and important role for NF-kB1 in CD8 T cell development that sets it apart from the studies reported for the other mutant strains.

Additionally, there are a few minor problems for the authors to addresss:

1) The authors claim that there is a decrease in the CD4:CD8 ratio in mice deficient in NF-κB. Is this decrease significant?

Response: We have increased the number of mice analysed to N=5 per genotype and tested statistical significance by performing the unpaired two-tailed t-test which confirms significance at P=0.05. The data in the results is presented (p. 6) as; (*wt* 3.04 ± 0.29 versus $nfkb1^{-/-}$ 2.09 ± 0.19; n=5 per genotype; *P=0.05*).

2) It is unclear the number of experiments performed that tested the Vβ repertoire of the CD8 SP population. Additionally, do the Eomesodermin+ CD8 SP thymocytes also have a diverse Vβ repertoire?

Response: The Vb repertoire staining of CD8SP cells was performed in 2 independent experiments (p.26). We have inserted new data showing that memory-like $nfkb1^{-/-}$ CD8SP cells co-express cell surface CD44 and intracellular Eomes (Figure 1G and p.8). This data combined with the bar graphs showing the Vb repertoire for $nfkb1^{-/-}$ $CD44^{hi}$CD8SP cells by inference demonstrate that Eomesodermin+ CD8 SP thymocytes also have a diverse Vβ repertoire.

3) In Figure 6, when the authors look at the various DC populations, are those differences significant?
Response: In this experiment, we analysed the number of thymic DCs from 4-pooled thymuses per genotype, and performed the experiment twice. A total of 8 mice were used to examine thymic DC subsets from wt and nfkb1$^{-/-}$ mice, which represents a significant number of mice used for the study. Rather than perform statistics on two experiments, we have inserted the data from the second experiment (Figure 7 now). Collectively, these data reveal a ~68% reduction in nfkb1$^{-/-}$ Sirpa^{hi} DCs, a ~40% decrease in Sirpa^{lo} DCs and ~45% reduction in nfkb1$^{-/-}$ conventional DCs. The most pronounced reduction is observed for circulating Sirpa^{hi} DCs, which are have a role in negative selection.

4) In Figure 7D, it is unclear which lymph nodes are being shown. The methods state that the mesenteric lymph nodes were examined while the figure legend states that resident lymph nodes were examined. This issue should be clarified.
Response: In the legend to Figure 8 (p.31), we have now changed this to mesenteric lymph nodes.

5) In Figure 2C, the authors should provide absolute numbers of cells.
Response: We have now inserted bar graphs to show the absolute number of CD8SP cells and CD8^{+}CD24^{lo} cells for each genotype (Figure 4).

2nd Editorial Decision 07 October 2011

Thank you for submitting your revised manuscript to the EMBO Journal. Your manuscript has now been seen by the original two referees and their comments are provided below.

As you can see both referees find that the introduced changes have strengthen the manuscript and both are supportive of publication here. However, there are a few minor issues that have to be sorted out before we can proceed with publication here. I would therefore like to ask you respond to the remaining concerns in a last round of revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2:

This manuscript is substantially stronger with the addition of new data on PLZF-expressing cells, IL-4 production and mixed bone marrow chimeras, which all support that NF-kB1 acts in suppressing innate CD8 cell development by a unique mechanism. However, before this paper can be considered for publication, the figures need to be improved, particularly the new figure 2, which was almost unreadable. The flow plots and all fonts need to be larger. The authors should also temper their conclusion that the phenotypes are not secondary to IL-4, solely because they have not blocked IL-4 genetically or via antibodies. Finally, the authors argue that innate phenotypes are due to hematopoietic selection, rather than selection solely on MHC Class Ib. I believe the Urdahl paper also presents the same arguments as does Broussard et al, which strongly makes this case. These findings should be cited for this issue.

Referee #3:

The manuscript by Gugasyan et. al. aims to address the role of NF-κB1 in conventional versus innate-like T cell development. The authors find that an innate-like CD8+ T cell populations develops in the absence of NF-κB1. This population is characterized by high expression of CD44, CD122, and Eomesodermin. Additionally, this innate-like population produces IFNγ upon ex vivo stimulation with PMA and ionomycin, and the development of these innate-like T cells appears to be cell intrinsic. The authors also find that this innate-like population of T cells does not depend on IL-15 for development and that this population is somewhat dependent on MHC class Ia for expression; although in KbDb-/- nfκb1-/- mice, there is a small population of CD8+ T cells that develop independently of MHC class Ia. Further, there appears to be some defect in negative selection upon absence of NF-κB due to a decrease in Sirpα+ dendritic cells that promote negative selection.

This paper helps to further elucidate the signaling pathways involved in the development of innate-like T cells. As it stands, the data presented represent an important contribution to the field.

Nonetheless, previous studies have indicated that the development of these Eomesodermin-positive innate-like CD8+ T cells is actually due a population of T cells (both αβ and γδ) expressing PLZF and producing high amounts of IL-4. While the authors of the current manuscript have attempted to address this possibility in their system, the data presented in this manuscript do not definitively demonstrate that the development of innate-like CD8+ T cells deficient in NF-κB1 is independent of IL-4. The authors would need to either cross the nfκb1-/- mice to CD124-/- similarly to the Hogquist group, or use an anti-IL-4 antibody to deplete IL-4 in vivo similarly to the Koretzky group, to discern the involvement of IL-4 in their system. Therefore, the authors should revise their conclusions to be less emphatic concerning the involvement of IL-4 in the development of nfκb1-/- innate-like CD8+ T cells.

Additionally, there are a few minor problems for the authors to address:

1) The authors claim that NK1.1 is a characteristic marker of innate-like CD8 T cells without citing a reference.
2) In figure 1B, the authors provide cell numbers for one set of experiments. Although the data is convincing, it is curious that not all the experiments were combined.
3) In figure 2E it may be helpful to the reader to add that the gating is on CD45.1+ CD8 SP cells so that the reader knows immediately that they are examining the WT population.
4) The title of Figure 4 should be rewritten. The data does not indicate that the development of nfκb1-/- innate-like CD8 T cells develop independently of MHC class Ia and Ib.

2nd Revision - authors' response 01 November 2011

Referee #2

This manuscript is substantially stronger with the addition of new data on PLZFexpressing cells, IL-4 production and mixed bone marrow chimeras, which all support that NF-kB1 acts in suppressing innate CD8 cell development by a unique mechanism. However, before this paper can be considered for publication, the figures need to be improved, particularly the new figure 2, which was almost unreadable. The flow plots and all fonts need to be larger. The authors should also temper their conclusion that the phenotypes are not secondary to IL-4, solely because they have not blocked IL-4 genetically or via antibodies. Finally, the authors argue that innate phenotypes are due to hematopoietic selection, rather than selection solely on MHC Class Ib. I believe the Urdahl paper also presents the same arguments as does Broussard et al, which strongly makes this case. These findings should be cited for this issue.
Response: We thank Reviewer #2 for his/her positive feedback in reviewing our manuscript. In response to the reviewer's comments we have now improved the size of the flow plots and all fonts in Figure 2. As requested, we have tempered the assertion that the development of memory CD8 cells is not due to IL-4, and we have inserted the references to the work by Urdahl et al and Broussard et al.

Referee #3

The manuscript by Gugasyan et. al. aims to address the role of NF-kB1 in conventional versus innate-like T cell development. The authors find that an innate-like CD8+ T cell populations develops in the absence of NF-kB1. This population is characterized by high expression of CD44, CD122, and Eomesodermin. Additionally, this innate-like population produces IFNg upon ex vivo stimulation with PMA and ionomycin, and the development of these innatelike T cells appears to be cell intrinsic. The authors also find that this innate-like population of T cells does not depend on IL-15 for development and that this population is somewhat dependent on MHC class Ia for expression; although in KbDb-/- nfkb1-/- mice, there is a small population of CD8+ T cells that develop independently of MHC class Ia. Further, there appears to be some defect in negative selection upon absence of NF-kB1 due to a decrease in Sirpa+ dendritic cells that promote negative selection.
This paper helps to further elucidate the signaling pathways involved in the development of innate-like T cells. As it stands, the data presented represent an important contribution to the field.
Response: We thank Reviewer #3 for his/her positive feedback in reviewing our manuscript.

Nonetheless, previous studies have indicated that the development of these Eomesodermin-positive innate-like CD8+ T cells is actually due a population of T cells (both αβ and γδ) expressing PLZF and producing high amounts of IL-4. While the authors of the current manuscript have attempted to address this possibility in their system, the data presented in this manuscript do not definitively demonstrate that the development of innate-like CD8+ T cells deficient in NF-κB1 is independent of IL-4. The authors would need to either cross the nfκb1-/- mice to CD124-/- similarly to the Hogquist group, or use an anti-IL-4 antibody to deplete IL-4 in vivo similarly to the Koretzky group, to discern the involvement of IL-4 in their system. Therefore, the authors should revise their conclusions to be less emphatic concerning the involvement of IL-4 in the development of nfκb1-/- innate-like CD8+ T cells.
Response: We have now clarified the text relating to the potential involvement of IL-4. The experiments performed in response to the Reviewer's comments have demonstrated that the development of memory-like CD8 T cells within the NF-κB1-deficient thymus is "not due to an expanded population of IL-4 producing PLZF+ thymocytes". We clarify this result in the Discussion by explaining that "a potential role for IL-4 cannot be unequivocally ruled out since IL-4 has not been eliminated in these studies".

Additionally, there are a few minor problems for the authors to address:
1) The authors claim that NK1.1 is a characteristic marker of innate-like CD8 T cells without citing a reference.
Response: We have now inserted a reference to indicate that NK1.1 is associated with innate-like CD8 T cells.

2) In figure 1B, the authors provide cell numbers for one set of experiments. Although the data is convincing, it is curious that not all the experiments were combined.
Response: These data were presented as one representative cohort of mice for reasons of clarity. As shown in Figures 3B and 4D, the analysis of different cohorts displayed the same phenotype.

3) In figure 2E it may be helpful to the reader to add that the gating is on CD45.1+ CD8 SP cells so that the reader knows immediately that they are examining the WT population.
Response: In the legend, we have highlighted the population of wt Ly5.1+ cells in bold to indicate that this is the population being analysed in the histograms.

4) The title of Figure 4 should be rewritten. The data does not indicate that the development of nfkb1-/- innate-like CD8 T cells develop independently of MHC class Ia and Ib.
Response: We have corrected this to "Loss of NF-κB1 promotes memory marker acquisition independently of MHC class Ia" Further to the original issue raised by Referee #3 in relation to the diverse Vβ repertoire (Figure 1E). We have since considered the data to be best represented as reflecting the relative frequency of each Vβ within the separate CD44hi and CD44lo populations of CD8SP cells and have modified this figure accordingly. The data still support the conclusion that the nfκb1-/- CD44hi CD8SP thymocytes utilize a diverse Vβ TCR repertoire, but the clarity of this figure is improved.

