Peripheral Natural Killer Cell Maturation Depends On The Transcription Factor Aiolos

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<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>14 January 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>20 February 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>29 July 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>27 August 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>01 September 2014</td>
</tr>
<tr>
<td>Accepted</td>
<td>15 September 2014</td>
</tr>
</tbody>
</table>

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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 20 February 2014

Thank you for submitting your manuscript to The EMBO journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the analysis interesting, but they also raise significant issues with the paper that should be resolved for publication here. Referee #3 raises a number of inconsistencies that should sorted out and both referees #1 and 2 would like to see more mechanistic insight into the observed phenotype, Aiolos target genes and how Aiolos is regulated. Should you be able to address the concerns raised then I would like to invite you to submit a revised version of the manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision 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://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS
Referee #1:

In this paper by the group of Stephen Nutt, a well-known investigator in the field of immunopoiesis, the authors analyze the role of Aiolos in NK cell development.

Aiolos is a member of the Ikaros family of transcription factor which are important regulators of immune system development. Aiolos expression pattern reveal a striking restriction to the lymphoid lineage, i.e. B cells, T cells and NK cells. It has been reported that Aiolos deficiency in mice leads to severe defects in the B cell compartment, while the T compartment does not seem to be affected. Although Aiolos deficient mice have been engineered more than 15 years ago, the present study is the first to analyze their NK cell compartment and thus is of great interest.

Using gene and protein expression analysis Holmes, Thong & coll, first report that Aiolos expression is relatively stable along NK cell development. Flow cytometric analysis of the different NK cell development stages then reveal that despite a relatively unimpaired NK cell numbers, Aiolos deficiency leads to a cell-intrinsic severe block in the transition from immature to mature NK cells. Interestingly, despite this stringent phenotype, follow-up ex vivo analyses of NK cells functions from those mice reveal only mild defects in their abilities to secrete cytokines or produce cytotoxic molecules. In vivo, the authors report that MCMV virus clearance is slightly delayed, whereas clearance of NK-sensitive tumors is significantly enhanced. Mechanistically, the authors report that Aiolos does not seem to be regulated or regulate the expression of the transcription factors T-bet, Eomes and Blimp-1, that are critically involved in the transition from immature to mature stages. Based on these results, the authors conclude that while Aiolos by itself does not play a role in the early steps of NK cell differentiation, it critically acts in peripheral NK cell finally through a novel regulatory circuit, i.e. independent of the others known regulators of this process.

Overall, the general conclusion of this study is well supported by the presented data and is definitely of interest, although it could benefit from answering the following points:

1. Mechanistic insights regarding this differentiation block: spontaneous death of immature cells ? lack of survival of mature cells ? impaired proliferation ?

2. The authors describe the appearance of an 'aberrant' CD27+ KLRG1+ population in Aiolos deficient mice, with low expression of CD11b and Ly49C but high expression of cKit and CD51. Considering that KLRG1 expression is upregulated on proliferating NK cells, the use of this marker in phenotypic analyses of NK cell development might blur the actual description of the different NK cells differentiation stages. Indeed, another possibility is that this KLRG1+ phenotype just results from an NK-penia driven homeostatic proliferation of normal immature NK cells (CD122+ NK1.1+ DX5+ CD27+ ckit+ CD11b-/lo NKG2+ Ly49D/H+ Ly49C-).

3. As the authors point out a few times, although not really precisely described to date, NK cell subpopulations are known to have distinct functional patterns in terms of cytokine secretion, cytotoxic potential and trafficking. Considering the very significant different repartition of the various cell subpopulations between Aiolos knock out mice and wild type mice, and the fact that all functional assays are done on total NK cells, it is difficult to interpret the functional differences. So, in order to carefully study the potential role of Aiolos in NK cell functions, experiments using sorted cell populations would have to be performed. As an example, in Fig 4 in vitro experiments reveal a modest, if any, increase in IL-15 driven proliferation, and argue that this can stem from the known enhanced proliferative capacity of CD27+ cells. Moreover, in Fig3D, the author show that CD122 seems to be enhanced on Aiolos KO spleen cells compared to WT cells thus suggesting that IL-15 sensitivity can be different between the 2 cell populations. It would be interesting to know if CD122 expression is really enhanced as a consequence of Aiolos deficiency, or if a normal differential expression between immature and mature subpopulations might explains this results considering the absence of mature cells in Aiolos KO mice.

4. Figure 4 legend's indicates that the cytotoxic activity is tested against RMA-S-Rae1 cells (MHC-I deficient AND NKG2D triggering) and not RMA-S cells. As opposed to the author's statement in the manuscript, NK cell cytotoxic activity in this assay therefore does not only results from a lack of MHC-I expression.
5. Throughout the manuscript, the authors should present the stages of NK cell maturation by using the most usual way (CD27 and CD11b histogram plot) rather than CD27 and KLRG1.

Referee #2:

The authors investigate the role of the transcription factor Aiolos for NK cell development and function. Expression of Aiolos was induced at the pre-pro NK cell stage and Aiolos remained expressed during differentiation down the NK lineage. Mice genetically deficient for Ikzf3, the gene encoding Aiolos, had normal numbers of NK cells in spleen and bone marrow. Ikzf3-deficient NK cells remained CD27+ and did not mature into the CD27- KLRG1+ state. Ikzf3-deficient NK cells produced less IFN-γ and had lower levels of GzmB in comparison to wildtype NK. However, Ikzf-deficient NK cells were as effective as their wildtype controls in killing target cells when stimulated with IL-15 alone or in combination with IL-21. Aiolos expression was independent of T-bet or Blimp1 and T-bet, Eomes and Blimp1 expression was normal in Ikzf3-deficient mice. Mice genetically lacking Ikzf3 were more susceptible to MCMV and showed reduced rejection of RMA-S and B16 tumors.

General comments

This is an interesting manuscript investigating the role of the transcription factor Aiolos for differentiation and function of NK cells which has been largely ignored in the past. While the experiments seem carefully designed, the data does not provide much mechanistic insights into how Aiolos controls differentiation and function of NK cells. A revised manuscript addressing the specific points below may be suitable for publication in EMBO J.

Specific Comments

1. In general, the observed phenotype is not mechanistically explained. What is regulated by Aiolos and what regulates Aiolos? No direct target genes are presented. Transcriptome analysis of NK cells from wildtype and Ikzf3 knockout mice could help to better understand the phenotype and raise enthusiasm.

2. There is a tendency to higher absolute NK cell numbers in the spleen of Ikzf3-deficient mice (Figure 3E) which is in contrast to lower proliferation in vitro in response to IL-15 (Figure 5A,B). How can this be explained?

3. Ikzf3-deficient NK cells did not show reduced cytotoxicity (Figure 5C,D) but tumor rejection in vivo was significantly impaired (Figure 7B-E). What is the mechanism of tumor rejection if not cytotoxicity?

4. The increased susceptibility to MCMV has to be further investigated. NK cell function (IFN-γ production, NK population expansion, cytotoxicity, migration/recruitment) needs to be analyzed. The reduced virus control in salivary glands is puzzling because virus control in salivary glands is believed to be CD4 T cell-dependent. Could Ikzf3 deficiency in CD4 T cells contribute to the observed phenotype?

Referee #3:

To the Authors:

The authors describe how the transcription factor Aiolos regulates maturation and function of peripheral NK cells. The role of Aiolos in NK cell development and maturation has not been previously studied, thus the data are novel. However, many of the findings appear contradictory and confusing. The lack of consistency throughout the manuscript is concerning.

There are at least 3 pieces of data that are not consistent. In particular data in figure 4 suggests differences in cytokine production and cytotoxicity between WT and KO NK cells, whereas figure 5
then shows normal killing and cytokine production. Furthermore, it is confusing why Aiolos-deficient mice are more susceptible to virus but more resistant to tumors. The explanation in the discussion is not convincing because it is known that cytotoxicity via activating Ly49H receptor and perforin/granzyme release that mediates resistance against MCMV. Take away the Ly49H receptor or effector molecules, and mice are extremely susceptible to MCMV infection. Lastly, Aiolos/-/- NK cells mediate the same degree of cytotoxicity against missing-self tumors in vitro in figure 5, but kill missing-self tumors better in vivo in figure 7. Why is this so? The manuscript contains many findings that lack consistency.

Perhaps the discrepancy between virus and tumor can be explained by differences in adaptive immunity between WT and Aiolos/-/- mice. Are T and B cell responses defective in Aiolos/-/- mice during virus and tumor challenge? In the viral challenge model, the differences in viral titer between the two groups of mice at day 10 and 18 can be explained by differences in CD8+ T cell responses. Have these been measured? The late influences (beyond the first couple of days) of Aiolos-deficiency can also be ruled out by crossing mice to a RAG-deficient background.

The clonal-like expansion of a specialized subset of NK cells expressing the Ly49H receptor is responsible for protection against virus. Is this Ly49H NK cell expansion defective in Aiolos/-/- mice? This could be another explanation for the higher viral titers.

It is surprising that decreased overall expression of IFNγ and granzyme B in Aiolos/-/- mice leads to increased tumor control of RMA/s and B16, especially due to the fact that the in vitro killing of RMA/s RAE1b is similar between WT and KO. Could this be due to enhanced infiltration of immature NK cells in these tumors? Or loss of inhibition by downregulation of KLRG1? Characterization of the tumor-infiltrating NK cells would greatly strengthen this paper.

Minor:

The discussion section uses CD27 and CD11b to describe the maturation stages of NK cells. However, throughout the manuscript, the authors make no use of these markers together to examine NK cell maturation. As this group first described use of these markers in combination, it would be beneficial to show some of this staining. It is unclear why this group has chosen to substitute KLRG1 for CD11b in all of their phenotyping data. Why was this done? Are these 2 markers interchangeable? CD27 should be shown versus CD11b in the panels of these figures to reach consensus with the majority of published literature on NK cell maturation.

In the final paragraph of their discussion, the authors talk about how less mature NK cells (CD27hi) have a higher proliferative potential during IL-15 in vitro stimulation or during lymphopenia, and how this might explain the greater protection against tumor challenge. This does not make sense. Why would the tumor microenvironment mimic IL-15 in vitro or lymphopenia? Presumably more mature NK cells possessing higher cytotoxicity would favor tumor clearance. Is there evidence that CD27+KLRG1- NK cells are more protective than CD27-KLRG1+ NK cells against tumors in vitro or in vivo?

Figures 1 and 2 should be combined. In figure 1, although the authors state that the cell sorting strategy will be explained in a supposed future publication, it would be beneficial to include the representative gating strategy in order to fully understand and interpret the RNA seq data.

The numbering of Figure 3 doesn't make sequential sense. Figure 3E should be relabeled 3B.
Referee #1:

In this paper by the group of Stephen Nutt, a well-known investigator in the field of immunopoiesis, the authors analyze the role of Aiolos in NK cell development. … Although Aiolos deficient mice have been engineered more than 15 years ago, the present study is the first to analyze their NK cell compartment and thus is of great interest. … Overall, the general conclusion of this study is well supported by the presented data and is definitely of interest, although it could benefit from answering the following points:

1. Mechanistic insights regarding this differentiation block: spontaneous death of immature cells? lack of survival of mature cells? impaired proliferation?

RESPONSE: We have addressed this question in a number ways and find that neither cell survival nor proliferation is impaired in the absence of Aiolos (Figure 2E, F, G and data not shown), and in certain circumstances Aiolos-deficient NK cells actually have a proliferative advantage over wild type cells. Thus we have concluded that Aiolos-deficiency results in a “development block”, due to the cells being incapable of normally executing their differentiation program (pages 8-9).

2. The authors describe the appearance of an ‘aberrant’ CD27+ KLRG1+ population in Aiolos deficient mice, with low expression of CD11b and Ly49C but high expression of cKit and CD51. Considering that KLRG1 expression is upregulated on proliferating NK cells, the use of this marker in phenotypic analyses of NK cell development might blur the actual description of the different NK cells differentiation stages. Indeed, another possibility is that this KLRG1+ phenotype just results from an NK-penia driven homeostatic proliferation of normal immature NK cells (CD122+ NK1.1+ Dx5+ CD27+ ckit+ CD11blo NKG2+ Ly49D/H+ Ly49C-).

RESPONSE: We agree with the reviewer that KLRG1 can be induced by some cytokines and by lymphopenia induced homeostatic proliferation. We have now added a series of panels showing CD27 versus CD11b in +/- and Ikzf3- NK cells from bone marrow and spleen (Figure 2C). This analysis nicely shows that NK cells do not progress normally to the CD27 stage without Aiolos and instead become CD27 KLRG1+ (Figure 2C). While we and others have reported that KLRG1 expression is induced when NK cells are transferred into a lymphopenic environment, we do not think that occurs in the absence of Aiolos for the following reasons; 1) The equivalent NK cell numbers between the genotypes (Figure 2A-B), 2) The equivalent proliferation rates when +/- and Ikzf3- NK cells are transferred into Rag1- hosts (Figure 2F), 3) Bone marrow chimeras show that Aiolos-deficient NK cells compete normally with wild type NK cells, while maintaining KLRG1 expression in CD27+ cells. Thus we conclude that the accumulation of CD27+ KLRG1+ NK cells without Aiolos is indicative of a defect in peripheral NK cell differentiation.

3. As the authors point out a few times, although not really precisely described to date, NK cell subpopulations are known to have distinct functional patterns in terms of cytokine secretion, cytotoxic potential and trafficking. Considering the very significant different repartition of the various cell subpopulations between Aiolos knockout mice and wild type mice, and the fact that all functional assays are done on total NK cells, it is difficult to interpret the functional differences. So, in order to carefully study the potential role of Aiolos in NK cell functions, experiments using sorted cell populations would have to be performed. As an example, in Fig 4 in vitro experiments reveal a modest, if any, increase in IL-15 driven proliferation, and argue that this can stem from the known enhanced proliferative capacity of CD27+ cells. Moreover, in Fig3D, the author show that CD122 seems to be enhanced on Aiolos KO spleen cells compared to WT cells thus suggesting that IL-15 sensitivity can be different between the 2 cell populations. It would be interesting to know if CD122 expression is really enhanced as a consequence of Aiolos deficiency, or if a normal differential expression between immature and mature subpopulations might explains this results considering the absence of mature cells in Aiolos KO mice.

RESPONSE: While the reviewer raises a valid point we feel that the deregulated markers observed without Aiolos (CD27, KLRG1, kit, CD51 and to a lesser degree CD11b all show altered expression) reduces the usefulness of this sort of comparison. Moreover we have compared the list
of differentially expressed genes between CD11b<sub>low</sub>CD27<sup>+</sup> and CD11b<sub>high</sub>CD27<sup>-</sup> splenic NK cells (Chiossone et al Blood 111, 5488-5496 (2009)) with our list of Aiolos regulated genes (Supplementary Table 1) and found only 11% overlap. We interpret this low correlation to indicate that Aiolos is not simply controlling the transition from the CD27<sup>+</sup> to CD27<sup>-</sup> stage. We have also found that the in vitro cytotoxicity and secretory functions are, on the whole, Aiolos independent, and thus we feel that an analysis of the individual stages in vitro is not likely to clearly elucidate how Aiolos controls NK cell maturation. As the reviewer notes we did show a very small increase in CD122 expression in Aiolos-deficient NK cells (this panel was intended as a control to show that the cells express CD122 relatively normally compared to other markers such as c-kit, and has been removed for space reasons). Our RNAseq data suggest that this small change in CD122 is post-transcriptional and may, as the reviewer suggests, result from the lack of the CD27<sup>-</sup> cells (see reviewer Figure 1 below).

Reviewer Figure 1. Expression of CD122 in splenic NK cell subsets. Left plot, flow cytometric analysis of gated NK1.1<sup>+</sup>CD49b<sup>+</sup>TCRb<sup>-</sup> NK cells from a C57Bl/6 spleen. The previously defined Immature (Imm), Mature 1 (M1) and Mature 2 (M2) subsets are indicated (Huntington et al J Immunol (2007), 178: 4764–4770). Note Imm and M1 fractions are CD27<sup>+</sup> and M2 cells are CD27<sup>-</sup> (not shown). Right histogram, flow cytometric analysis for CD122 on the NK cell populations defined in the left panel. Expression of CD122 is equivalent between Imm and M1 cells, but down regulated on the M2 population.

4. Figure 4 legend's indicates that the cytotoxic activity is tested against RMA-S-Rae1 cells (MHC-I deficient AND NKG2D triggering) and not RMA-S cells. As opposed to the author's statement in the manuscript, NK cell cytotoxic activity in this assay therefore does not only results from a lack of MHC-I expression.

RESPONSE: We thank the reviewer for pointing out this oversight. We have now modified the text accordingly.

5. Throughout the manuscript, the authors should present the stages of NK cell maturation by using the most usual way (CD27 and CD11b histogram plot) rather than CD27 and KLRG1.

RESPONSE: We agree with the reviewer and have added a series of panels showing CD27 versus CD11b in +/- and Ikar<sup>-</sup> NK cells from bone marrow and spleen (New Figure 2C), as well as determined Aiolos protein expression using these markers (Figure 1D). Splenic NK cells go through 3 stages 1) CD11b<sup>low</sup>CD27<sup>-</sup>KLRG1<sup>+</sup> 2) CD11b<sup>high</sup>CD27<sup>-</sup>KLRG1<sup>+</sup> and CD11b<sup>high</sup>CD27<sup>-</sup>KLRG1<sup>+</sup>. The comparison of CD27 and KLRG1 nicely delineates the last 2 stages, which are the site of the phenotype in Aiolos-deficient mice.

Referee #2:

…General comments
This is an interesting manuscript investigating the role of the transcription factor Aiolos for differentiation and function of NK cells, which has been largely ignored in the past. While the experiments seem carefully designed, the data does not provide much mechanistic insights into how Aiolos controls differentiation and function of NK cells. A revised manuscript addressing the specific points below may be suitable for publication in EMBO J.

Specific Comments
1. In general, the observed phenotype is not mechanistically explained. What is regulated by Aiolos and what regulates Aiolos? No direct target genes are presented. Transcriptome analysis of NK cells from wildtype and Ikzf3 knockout mice could help to better understand the phenotype and raise enthusiasm.

RESPONSE: We agree with the reviewer and have now performed RNAseq on splenic NK cells from wild type and Ikzf3−/− mice (2 biological replicates) to provide the requested transcriptome analysis. Given the deregulated markers (as discussed in the response to reviewer 1), we decided to simply sort mature NK cells for this analysis (NK1.1−NKp46+CD11bhighTCRb+). This data presented in Figure 3 and Supplementary Table 1, shows 331 differentially expressed genes (220 upregulated without Aiolos and 111 down regulated). We provide validation of the potential target genes in Figure 3. This analysis revealed that Aiolos does not exert its function through the regulation of any known transcriptional regulators of the NK cell lineage (Figure 3F) and that the other members of the Ikaros family were not upregulated in compensation for Aiolos loss. (Figure 3F). Moreover, despite the similarities in the knockout phenotypes Aiolos does not appear to be regulated by either T-bet, Blimp1 or both.

2. There is a tendency to higher absolute NK cell numbers in the spleen of Ikzf3-deficient mice (Figure 3E) which is in contrast to lower proliferation in vitro in response to IL-15 (Figure 5A,B). How can this be explained?

RESPONSE: The original Figure 5A, B (now removed from the manuscript) actually showed modestly increased proliferation in the absence of Aiolos and thus agreed with the higher absolute NK cell numbers in these mice. We have replaced that data with the more informative CFSE/CTV experiments (Figure 2E-F) that also show increased IL-15 responsiveness.

3. Ikzf3-deficient NK cells did not show reduced cytotoxicity (Figure 5C,D) but tumor rejection in vivo was significantly impaired (Figure 7B-E). What is the mechanism of tumor rejection if not cytotoxicity?

RESPONSE: The reviewer has misinterpreted the in vivo data, which shows increased tumor control in Aiolos-deficient mice. Cytotoxicity in NK cells depends not only on the presence of the Perforin/Granzyme containing granules and death ligands, which are present in the Aiolos-deficient cells, but also on the activity of various activating or inhibitory pathways, as well as the frequency and location of the NK cells. We show in new Figure 2G that the Ikzf3−/− NK cells hyper-respond to IL-15 and to exogenous IL-2/anti-IL2 mAb complexes (page 8-9) and in Figure 6F that the Aiolos-deficient NK cells that are recruited to the lungs upon tumor challenge maintain their CD27+ phenotype (page 14). As we have previously shown that the CD27+ stage contains most of the cytotoxic potential in vitro (Hayakawa and Smyth J Immunol 176,1517-1524 (2006) and Huntington et al J Immunol 178,4764-4770 (2007)), we propose that the common g chain receptor hypersensitivity and block at the CD27+ stage underpins the improved tumor control.

4. The increased susceptibility to MCMV has to be further investigated. NK cell function (IFN-g production, NK population expansion, cytotoxicity, migration/recruitment) needs to be analyzed. The reduced virus control in salivary glands is puzzling because virus control in salivary glands is believed to be CD4 T cell-dependent. Could Ikzf3 deficiency in CD4 T cells contribute to the observed phenotype?

RESPONSE: The reviewer raises 2 issues: (1) is there some impact of Aiolos-deficiency on adaptive immune responses and (2) is there any measurable difference in NK cell function.

1) Our data don’t support any defect in the adaptive immune response being involved in the early response to MCMV. In C57/B16 mice T cells do not participate in the early control of acute infection (Sumaria et al. Immunol Cell Biol 87, 559–566 (2009)). Most importantly, we saw no difference in the expansion of CD8+ and CD4+ T cells and no difference in MCMV-specific (M45 tetramer binding CD8+ T cells) in the absence of Aiolos. The increase in T cell number in the spleen at day 0 is due to the mild splenomegaly that has been previously reported for these mice and reflected in the mildly increased splenic NK cell numbers (Figure 2B). This data is now provided as a Supplementary Figure 1 and discussed on page 13 of the text. The increase in viral titer in the salivary gland could be a result of more virus being present early during infection – we have
observed this phenotype before in IFNg-deficient mice at day 10 post-infection and attributed to a combination of differences in viral dissemination and local effector mechanisms (latter probably CD4+ T cell mediated, Sumaria et al. Immunol Cell Biol 87, 559–566 (2009)).

2) The results also suggest that NK cytotoxic function is largely intact, since there is no difference in viral replication in the spleen (Figure 7A), where virus control in C57/Bl6 mice is completely dependent on perforin-dependent NK cell mediated activity (Sumaria et al Immunol Cell Biol 87, 559–566 (2009)). We also analyzed NK cell responses in the wild type: Ikzf3+/− bone marrow chimeric mice where each compartment is exposed to the identical viral load. We observed no difference in expansion of Ly49H+ NK cells in the liver and spleen (Supplementary Figure 1 and data not shown). We have discussed these points on page 13 of the text.

Referee #3:

The authors describe how the transcription factor Aiolos regulates maturation and function of peripheral NK cells. The role of Aiolos in NK cell development and maturation has not been previously studied, thus the data are novel. However, many of the findings appear contradictory and confusing. The lack of consistency throughout the manuscript is concerning. There are at least 3 pieces of data that are not consistent. In particular data in Figure 4 suggests differences in cytokine production and cytotoxicity between WT and KO NK cells, whereas Figure 5 then shows normal killing and cytokine production. Furthermore, it is confusing why Aiolos-deficient mice are more susceptible to virus but more resistant to tumors. The explanation in the discussion is not convincing because it is known that cytotoxicity via activating Ly49H receptor and perforin/granzyme release that mediates resistance against MCMV. Take away the Ly49H receptor or effector molecules, and mice are extremely susceptible to MCMV infection. Lastly, Aiolos+/− NK cells mediate the same degree of cytotoxicity against missing-self tumors in vitro in Figure 5, but kill missing-self tumors better in vivo in figure 7. Why is this so? The manuscript contains many findings that lack consistency.

RESPONSE: As outlined in our response to reviewer 2, we do not propose that Aiolos is regulating cytotoxicity per se (for example the RNAseq showed normal Perforin, Fas, DR5, modestly reduced GzmB and increased GzmC, M and K, Supplementary Table 1 and data not shown), but instead use this as a read out of the ability of the Ikzf3−/− NK cells to respond to various stimuli in vitro and in vivo. The nature of these stimuli as well as the expression of the various activating or inhibitory receptors (and the signaling pathways emanating from these receptors) and the frequency and location of the NK cells determines the effector response. We show in the new Figure 2G (page 9) that the Ikzf3−/− NK cells hyper-respond to exogenous IL-2/anti-IL2mAb complexes and that the Aiolos-deficient NK cells that are recruited to the lungs upon tumor challenge maintain their CD27+ phenotype (Figure 6F, page 14). Thus we propose that the common g chain receptor hyper-responsiveness and block at the CD27+ stage in the absence of Aiolos underpins the improved tumor control.

Specifically

1. “data in figure 4 suggests differences in cytokine production and cytotoxicity between WT and KO NK cells, whereas figure 5 then shows normal killing and cytokine production”. The data in Figure 4 compares the expression of two effector molecules IFNg and GzmB directly ex vivo, with those cultured for 7 days in cytokines (Figure 5). While reduced GzmB is a surrogate marker for cytotoxic potential, it is known that loss of GzmB alone does not significantly impact of these in vitro killing assays or tumor control (Smyth et al J Immunol 171,515-518 (2003). Thus these are distinct assays that measure different NK cell capabilities.

2. “confusing why Aiolos-deficient mice are more susceptible to virus but more resistant to tumors” While both these pathways use Perforin/Gzm mediated killing as the effector mechanism (MCMV control also requires IFNg (Sumaria et al. Immunol Cell Biol 87, 559–566 (2009)), it is entirely possible that in the absence of Aiolos NK cells don’t respond well to MCMV (through Ly49H is expressed normally in Ikzf3−/− NK cells) but do respond to triggering by the MHCI-I mechanism. The mechanism for this may involve the increased NK cell responsiveness to cytokines (Figure 2E-G) or subset specific functions that we discuss on page 18.
3. “Aiolos-/- NK cells mediate the same degree of cytotoxicity against missing-self tumors in vitro in figure 5, but kill missing-self tumors better in vivo in figure 7. Why is this so?”. As discussed in point #2, we don’t claim any function for Aiolos in directly controlling the killing mechanism and instead propose that the blocked differentiation at a stage known to have more killing activity in vitro and increased cell responsiveness results in better tumor control in vivo.

Perhaps the discrepancy between virus and tumor can be explained by differences in adaptive immunity between WT and Aiolos-/- mice. Are T and B cell responses defective in Aiolos-/- mice during virus and tumor challenge? In the viral challenge model, the differences in viral titer between the two groups of mice at day 10 and 18 can be explained by differences in CD8+ T cell responses. Have these been measured? The late influences (beyond the first couple of days) of Aiolos-deficiency can also be ruled out by crossing mice to a RAG-deficient background.

RESPONSE. As discussed in detail in our response to reviewer 2, we have measured CD4+ and CD8+ T cell responses (including antigen specific CD8+ T cells using the M45 tetramer) and found these to be normal in Ikrj3-/- NK cells (Supplementary Figure 1). This agrees with a publication by some of us (Sumaria et al. Immunol Cell Biol 87, 559–566 (2009)) that showed no early role for T cells. The tumor models we are using have been shown by us in many studies to be controlled in an NK cell dependent manner and that tumor growth is identical in Rag1-/- hosts (for example Smyth et al J Immunol 165,2665-2670 (2000) and Brady et al J Immunol 172,2048-2058 (2004).

The clonal-like expansion of a specialized subset of NK cells expressing the Ly49H receptor is responsible for protection against virus. Is this Ly49H NK cell expansion defective in Aiolos-/- mice? This could be another explanation for the higher viral titers.

RESPONSE: We agree with the reviewer that reduced Ly49H would result in impaired control of MCMV. We have analyzed NK cell responses in the wild type: Ikrj3-/- bone marrow chimeric mice where each compartment is exposed to the identical viral load. We observed no difference in expansion of Ly49H+ NK cells in the liver and spleen (Supplementary Figure 1 and data not shown). We also found Klra8 (encoding ly49H) to be equally expressed between the genotypes in steady state NK cells (RNAseq data not shown). We have discussed these points on page 13.

It is surprising that decreased overall expression of IFNγ and granzyme B in Aiolos-/- mice leads to increased tumor control of RMA/s and B16, especially due to the fact that the in vitro killing of RMA/s RAE1b is similar between WT and KO. Could this be due to enhanced infiltration of immature NK cells in these tumors? Or loss of inhibition by downregulation of KLRG1? Characterization of the tumor-infiltrating NK cells would greatly strengthen this paper.

RESPONSE: The reviewer raises a good point. We have analyzed the recruitment and phenotype of NK cells in the lungs of tumor injected mice (Figure 6F). We found this approach to be much cleaner than isolating the infiltrating NK cells from subcutaneous tumor masses that depended greatly on variables such as tumor size and the inflammatory environment. Using the B16 lung metastasis model we found a very pronounced increase in NK cell numbers after tumor establishment (day 14). This data shows that the tumor associated Aiolos-deficient NK cells that are recruited to the lungs upon tumor challenge maintain their CD27 KLRG1+ phenotype, while wild type NK cells were CD27+ KLRG1-. Thus loss of KLRG1 inhibition cannot be the cause of the enhanced tumor control and we conclude that the CD27+ NK cells observed in the Aiolos-deficient mice are on a per cell basis better at tumor control than CD27- wild type cells.

Minor:
The discussion section uses CD27 and CD11b to describe the maturation stages of NK cells. However, throughout the manuscript, the authors make no use of these markers together to examine NK cell maturation. As this group first described use of these markers in combination, it would be beneficial to show some of this staining. It is unclear why this group has chosen to substitute KLRG1 for CD11b in all of their phenotyping data. Why was this done? Are these 2 markers interchangeable? CD27 should be shown versus CD11b in the panels of these figures to reach consensus with the majority of published literature on NK cell maturation.
RESPONSE: We agree with the reviewer and have added a series of panels showing CD27 versus CD11b in +/+ and IκBα−/− NK cells from bone marrow and spleen (New Figure 2C), as well as determined Aiolos protein expression using these markers (Figure 1D). Splenic NK cells go through three stages: 1) CD11b+CD27−KLRG1−, 2) CD11b+CD27+KLRG1−, and 3) CD11b+CD27−KLRG1+. The comparison of CD27 and KLRG1 nicely delineates the last 2 stages which is the site of the phenotype in Aiolos-deficient mice.

In the final paragraph of their discussion, the authors talk about how less mature NK cells (CD27hi) have a higher proliferative potential during IL-15 in vitro stimulation or during lymphopenia, and how this might explain the greater protection against tumor challenge. This does not make sense. Why would the tumor microenvironment mimic IL-15 in vitro or lymphopenia? Presumably more mature NK cells possessing higher cytotoxicity would favor tumor clearance. Is there evidence that CD27+KLRG1− NK cells are more protective than CD27−KLRG1+ NK cells against tumors in vitro or in vivo?

RESPONSE: There are very clear data to show that CD27+ NK cells are more protective than CD27− NK cells against tumors in vitro. We have previously shown that CD27+ NK cells are more potent killers of tumor cells in vitro (Hayakawa and Smyth J Immunol 176,1517-1524 (2006)) and have higher proliferative capacity (Hayakawa and Smyth J Immunol 176,1517-1524 (2006) and Huntington et al J Immunol 178,4764-4770 (2007)). Moreover Blimp1-deficient NK cells that have a very similar developmental block then the Aiolos-deficient NK cells also show increased tumor control in vivo (Kallies et al Blood 117,1869-1879 (2011)). Thus, while it might appear paradoxical, the evidence suggests that the bulk of the NK cell cytotoxic activity in vivo resides in the CD27+ fraction. It also should be noted that while the CD27+ cells derive from CD27+ precursors both fractions are CD11bhi and represent mature NK cell stages.

Figures 1 and 2 should be combined. In figure 1, although the authors state that the cell sorting strategy will be explained in a supposed future publication, it would be beneficial to include the representative gating strategy in order to fully understand and interpret the RNA seq data.

RESPONSE: We have combined Figures 1 and 2, into a single Figure 1. The gating strategies of the NK cell populations and the generation of the RNAseq data used in Figure 1 in are now published in Seillet J. Immunol 92:2667-2676 (2014) (for NKP, iNK, mNK) and Carotta Blood 117:5449-5452 (2011) (for pre-pro-NK). These have been fully referenced in the Methods.

The numbering of Figure 3 doesn’t make sequential sense. Figure 3E should be relabeled 3B.

RESPONSE: We have ordered Figure 2 (formerly Figure 3) as the reviewer suggests.

2nd Editorial Decision 27 August 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has now been re-reviewed by the three referees.

As you can see below, the referees appreciate the introduced changes and support publication in The EMBO Journal. I am therefore very pleased to accept the manuscript for publication here. There are just a few text edits to attend to before formal acceptance here. I have provided the link below so that you can upload the revised version.

REFEREE REPORTS

Referee #1:

The revised version is great.
I recommend publication in the Embo J.

Referee #2:
This is a revised version of this manuscript which is considerably improved. Specifically, the authors have added RNA seq analysis of Aiolos-deficient NK cells which revealed important information. The authors should better discuss their data in the context of the recently discovered complexity of NKp46+ NK1.1+ cells (NK cells vs. ILC1/TRAIL+ DX5- "NK" vs. plastic ILC3) (Daussy, J Exp Med 2014; Klose, Cell 2014; Sojka, Elife 2014; etc.) as they have not ruled out that the composition of the population of NKp46+ NK1.1+ cells is not substantially changed in Aiolos-deficient mice.

Referee #3:

The authors have satisfactorily clarified or addressed the majority of my concerns. There are several key references missing in the introduction that are highlighted below.

In the second paragraph of the introduction, Firth et al (JEM 2013) and Cortez et al (JI 2014) should be cited in the discussion of NF-IL3 (E4BP4). Boos et al (JEM 2007) should be cited when Id2 is mentioned. Lastly, the transcription factor Zbtb32 has been recently implicated in NK cell differentiation (Beaulieu et al, Nat Immunol 2014) and should be discussed in the last sentence of the second paragraph, as Zbtb32 is thought to target Blimp-1 during NK cell activation.

With these minor revisions, I believe this manuscript is worthy of publication in The EMBO Journal.

2nd Revision - authors' response 01 September 2014

We are delighted that our manuscript titled “Peripheral Natural Killer Cell Maturation Depends On The Transcription Factor Aiolos” (EMBOJ-2014-87900R) was accepted for publication in The EMBO Journal. Below is our response to the reviewers remaining minor comments

Reviewer 2.

The authors should better discuss their data in the context of the recently discovered complexity of NKp46+ NK1.1+ cells (NK cells vs. ILC1/TRAIL+ DX5- "NK" vs. plastic ILC3) (Daussy, J Exp Med 2014; Klose, Cell 2014; Sojka, Elife 2014; etc.) as they have not ruled out that the composition of the population of NKp46+ NK1.1+ cells is not substantially changed in Aiolos-deficient mice.

RESPONSE: We had already referenced the Daussy et al paper and have now also referenced Sojka et al. We are confident that the FACS marker combinations we used do rule out the possible change in the composition Aiolos-deficient NK cell populations. We now mention on page 9 (bottom) that “This strategy also excludes any potential contamination by ILC1s, which are CD11b-” (Daussy et al, 2014; Sojka et al, 2014).”

Reviewer 3.

Addition of new references.

RESPONSE: We have added the suggested references in the introduction.