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Dok-1 and Dok-2 proteins regulate Natural Killer cell development and function

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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 29 December 2013

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by 2 referees and their comments are provided below.

As you can see, both referees find the analysis interesting. However, they also raise a number of issues that have to be resolved for consideration here. In particular, issues are raised regarding the infection data and the referees find that further analysis is needed to understand why there is increased MCMV replication in Dok1-/- Dok2-/- mice. Should you be able to address the raised concerns in full, then we would like to consider a revised version. 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://www.nature.com/emboj/about/process.html

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

REFEREE REPORTS

Referee #1:

Dok proteins are regulators of immune signaling but their roles for NK cell development and function are largely unexplored. The authors show that Dok-1 and Dok-2 are expressed in freshly isolated human NK cells as well as in various human NK cell lines whereas other Dok genes are not expressed. Crosslinking of activating NK cell receptors led to phosphorylation of Dok-1 which was substantially reduced in the presence of inhibitory signaling (CD94/NKG2A). Overexpression of full-length Dok-2 in a human NK cell line led to reduced phosphorylation of Akt and ERK, reduced IFN-g production and reduced cell-mediated cytotoxicity, effects that were not observed in cells transfected with Dok-2 deleted of its pleckstrin homology domain. Mice double deficient for Dok1 and Dok2 showed reduced numbers of NK cells in spleen, LNs and liver whereas normal numbers were found in the bone marrow. In Dok1-/- Dok2-/- mice, NK cells with an immature phenotype were found in secondary lymphoid organs. Dok1-/- Dok2-/- NK cells produced more IFN-g upon crosslinking of activating receptors whereas they produced less IFN-g in response to combination of IL-12 and IL-18. Dok1-/- Dok2-/- mice failed to control MCMV infection.

This is an interesting paper providing first evidence that Dok1 and Dok2 negatively regulate NK cell function and are required for NK cell maturation. The experiments seem carefully performed and support the major conclusions. The following issues should be addressed in a revised manuscript.

1. The infection data are a crucial facet of the manuscript. It is surprising that Dok1-/- Dok2-/- mice show enhanced IFN-g production in response to triggering of activating immunoreceptors but yet show reduced control of a virus that is mediated by NKG2D. Or can the reduced virus control simply be explained by the lower NK cell numbers found in Dok1-/- Dok2-/- mice? The authors need to provide substantial, additional data assessing IFN-g production, NK cell expansion, cell-mediated cytotoxicity, NK cell maturation and phenotype in the context of MCMV infection. The determination of virus titers by qPCR is unusual. Supporting data from conventional plaque-forming assays should be provided as well.

2. Dok1 and Dok2 knockout mice were generated on a 129/Sv genetic background. Were the mice used in the experiments 129/Sv background or were they partially backcrossed to C57BL/6? Which mouse line was used as 'wildtype' controls? What does DKO+/- mean (Dok1+/- Dok2+/-)?

In that context, I am not entirely clear how the bone marrow chimera experiments were analyzed. Does 'WT' mean analysis of 129/Sv-derived NK cells? It may also be informative to show the results from the (Dok-proficient) C57BL/6 - 129/Sv control chimeras.

3. The authors conclude from the data in Figure 1B that Dok-1 is a direct substrate of SHP-1/2. However, no data are provided to support direct interaction between Dok-1 and SHPs. This could be directly tested or the wording need to be softened.

4. The biochemical data in Figure 2A are very interesting and document that Dok proteins can suppress activation of downstream mediators of NK cell activation (e.g., Akt, Erk). Such biochemical analysis should be provided for Dok1-/- Dok2-/- NK cells as the phospho-flow data in Figure 6D is not very convincing.

5. The authors explain the lower percentage of mature NK cells in Dok-deficient mice with increased apoptosis (Figure 5C). While this may be a viable explanation for the phenotype, it is certainly only one of various models. Would crossing Dok1-/- Dok2-/- mice to Bcl2 tg mice rescue the phenotype observed in Dok1-/- Dok2-/- mice?

Referee #2:

The authors describe how Dok proteins negatively regulate NK cell function downstream of activating receptors. The role of Dok proteins in NK cell development and maturation has not been

previously studied, thus the data are novel and interesting. However, some of the findings appear contradictory and confusing - in particular the final piece of data (Fig 7), where viral infection using a recombinant MCMV containing a specific ligand for an activating NK cell receptor should result in greater protection in the DKO mice (according to the hypothesis that the Dok-deficient NK cells should have enhanced responses), yet they are less protective than WT. The authors explain this by the very small differences in IFN-g production during pro-inflammatory cytokine stimulation observed in Fig 6B, but it is not very convincing or likely that this is the mechanism for a 2 log increase in viral titers. Several other concerns are listed below and must be addressed:

Throughout the paper, data is only shown for the double KO. Is there any phenotype in the single Dok-1 or Dok-2 knockout mice? Even if no phenotype was observed, supplemental data or a comment should be included.

In figure 1B it is suggested that NKG2A may be recruiting SHP1/2 phosphatases to block phosphorylation of DOK-1, but data never actually shown.

In Figure 2B and C, what if both Dok proteins are expressed? Is there greater suppression of IFN-g and killing?

In Figure 5, it may not be fair to compare DKO NK cells on a 129 background to WT NK cells on a B6 background. Rather than backcrossing the DKO, perhaps WT littermate controls from the DKO breeding can be used as a control for the chimeras, as there may be intrinsic developmental and functional defects between 129 and B6 NK cells that we are not aware of.

In Fig 6, are the authors confident in the difference in B, C, and D statistically significant? Some of the error bars are completely overlapping.

Furthermore, are there any differences in cytotoxicity between WT and DKO NK cells?

In Fig 7, shouldn't IFN-g and killing via NKG2D be enhanced in the DKO NK cells? Shouldn't this result in greater protection?

The authors could easily measure IFN-g production by the WT and DKO NK cells ex vivo following infection using intracellular staining. This would strengthen their model that IFN-g and not activating receptor ligation is responsible for protection (although this is not what the literature would suggest).

Additionally, the defect observed during infection could be due to a defect in myeloid cells and completely independent of NK cells. Have the authors considered this possibility? Does depletion of NK cells change the outcome? The best experiment would be to adoptively transfer normalized numbers of WT and DKO NK cells into separate NK-deficient mice (RAG-/- x gC-/-) followed by virus challenge.

Minor:

The title is a bit too general. A more appropriate title may be: Dok-1 and Dok-2 regulate NK cell function downstream of activating receptors.

The condensed title should read: Dok-1 and Dok-2 regulate NK cells.

The introduction opens with the statement that NK cells are ILCs. This is currently debatable, as NK cells are traditionally considered the third lineage of lymphocytes, and very distinct from ILCs in development, function, tissue localization, etc. Thus, this sentence should be reworded or deleted, since ILCs are not brought up again in the paper.

Figure 4B and 5B contain too much data, and should be shown in a more concise manner. The data gets lost in the sheer volume and is even difficult for NK cell experts to look at.

I believe the legend in Fig 6B is wrong. Are the open bars showing WT and black filled bars showing DKO? Please correct, as this data was extremely difficult to follow...

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1st Revision - authors' response 09 April 2014

Point-by-point reply to referee's comments

Thanks to the referees for the comments to improve several aspects of the manuscript, especially on dissecting NK cell behavior in Dok1-/- Dok2-/- mice during MCMV infection.

Referee #1:

This is an interesting paper providing first evidence that Dok1 and Dok2 negatively regulate NK cell function and are required for NK cell maturation. The experiments seem carefully performed and support the major conclusions. The following issues should be addressed in a revised manuscript.

1. The infection data are a crucial facet of the manuscript. It is surprising that Dok1-/- Dok2-/- mice show enhanced IFN-g production in response to triggering of activating immunoreceptors but yet show reduced control of a virus that is mediated by NKG2D. Or can the reduced virus control simply be explained by the lower NK cell numbers found in Dok1-/- Dok2-/- mice? The authors need to provide substantial, additional data assessing IFN-γ production, NK cell expansion, cell-mediated cytotoxicity, NK cell maturation and phenotype in the context of MCMV infection. The determination of virus titers by qPCR is unusual. Supporting data from conventional plaque-forming assays should be provided as well.

We agree with the reviewer and have performed the requested experiments.
The virus titers was assessed by plaque assay (Figure 7A – right panel) and led to results consistent with those obtained by qRT-PCR as shown in the manuscript.
Beside their lower numbers of NK cells, especially of the CD11b+ mature subset (Figure 7B), no major alteration in the antiviral NK cell response to MCMV could be observed in DKO mice as compared to control animals, in terms of IFN-γ (Figure 7C) and Granzyme B expression (Figure 7D), proliferation, apoptosis and NKG2D expression, at all time points examined (d0, d1.5, d2, d3 and d6 post-infection). Similar NKp46, Ly49D and CD122 expression levels were detected in both mouse strains (see « for inspection by the reviewers » Figure 1). Moreover, the enhanced susceptibility of DKO mice to MCMV infection is only transient, as all animals have controlled the virus by day 6, at a time when a major amplification of NK cell numbers as occurred in both WT and DKO mice. Hence, as suggested by the reviewer and now discussed in the paper, the delayed virus control in Dok1-/- Dok2-/- mice might simply be explained by their lower numbers of NK cells, especially of the CD11b+ mature subset, and could be corrected over time during the infection due to the expansion of these cells.

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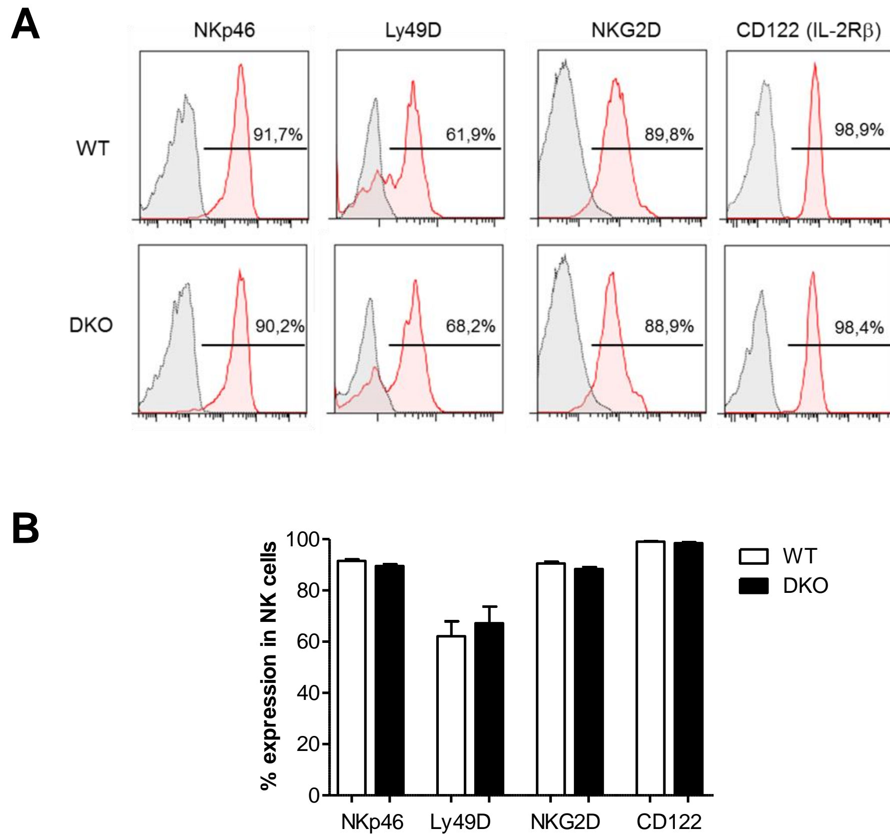

Similar expression levels of a selected panel of receptors were detected at the surface of both WT and Dok-1/Dok-2–deficient (DKO) NK cells.

Splenocytes from WT (white bars) or DKO (black bars) mice were extracellular stained for CD3, CD49b (DX5), and NKp46, Ly49D, NKG2D and CD122. The expression of these markers are analyzed by flow cytometry. **(A)** Histograms are corresponding to the staining of activating receptors, NKp46, Ly49D and NKG2D and cytokine receptors, the common β chain of IL-2R, CD122 in gated NK cells. Percentage of positive cells for these different markers are shown. **(B)** The data are the results of a pool of 3 – 4 independent experiments and are presented as mean ± SD (n= 20).

2. Dok1 and Dok2 knockout mice were generated on a 129/Sv genetic background. Were the mice used in the experiments 129/Sv background or were they partially backcrossed to C57BL/6? Which mouse line was used as 'wildtype' controls? What does DKO+/- mean (Dok1+/- Dok2+/-)?
Excluding the bone marrow chimera experiments, all the other experiments were performed on 129/Sv background. The "wildtype" controls are corresponding to 129/Sv mice. DKO+/- indeed means Dok1+/- Dok2+/-. We clarified these points in the "Materials and Methods" section.

In that context, I am not entirely clear how the bone marrow chimera experiments were analyzed. Does 'WT' mean analysis of 129/Sv-derived NK cells? It may also be informative to show the results from the (Dok-proficient) C57BL/6 - 129/Sv control chimeras.
We agree with the reviewer that the description of the bone marrow chimera experiments could be improved. In these settings, BM chimeric mice were generated using a mix of C57BL/6 CD45.1+ BM cells and of 129/Sv or DKO BM cells. The figure legend and labels were misleading. The percentage of NK cells as shown in the figure was measured within the CD45.2+ compartment (i.e. 129/Sv or DKO). We changed both figure legends and figure labels to make it clearer.

3. The authors conclude from the data in Figure 1B that Dok-1 is a direct substrate of SHP-1/2. However, no data are provided to support direct interaction between Dok-1 and SHPs. This could be directly tested or the wording need to be softened.
It has been reported that Dok-1 is a direct substrate of SHP-1 in macrophages [SHP-1 regulation of p62(DOK) tyrosine phosphorylation in macrophages. Berg KL, Siminovitch KA, Stanley ER. J Biol Chem. 1999 Dec 10;274(50):35855-65.], however this regulation is not demonstrated in our work. We agree with the comment and replace the sentence in the text by « *the level of NKp30-induced Dok-1 tyrosine phosphorylation decreased upon co-engagement of NKp30 and CD94/NKG2A (Figure 1B). One hypothesis would be that Dok-1/2 are substrates of the SHP-1/2 protein tyrosine phosphatases reported to be associated to the CD94/NKG2A inhibitory receptor signaling.* »

4. The biochemical data in Figure 2A are very interesting and document that Dok proteins can suppress activation of downstream mediators of NK cell activation (e.g., Akt, Erk). Such biochemical analysis should be provided for Dok1-/- Dok2-/- NK cells as the phospho-flow data in Figure 6D is not very convincing.
Thank you for your comments on our biochemical data performed on human NK cell lines, KHYG-1 and NKL (respectively Figure 2 and S2). In the mouse model, we are facing a problem of an alteration in the NK cell development (lower total number of NK cells and less mature NK cells in Dok1-/- Dok2-/- mice). Thus, it is necessary to use an assay working at a single cell level where cell signaling events are detected in both mouse strains (129/Sv and 129/Sv. Dok1-/- Dok2-/- mice) for the mature NK cells such as CD11b high NK cells.
We develop a phosphoflow analysis [Analysis of signaling events by dynamic phosphoflow cytometry. Firaguay G, Nunès JA. Sci Signal. 2009 Sep 1;2(86):pl3] that is validated in primary human immune cells [Differential role for CD277 as a co-regulator of the immune signal in T and NK cells. Messal N, Mamessier E, Sylvain A, Celis-Gutierrez J, Thibult ML, Chetaille B, Firaguay G, Pastor S, Guillaume Y, Wang Q, Hirsch I, Nunès JA, Olive D. Eur J Immunol. 2011 Dec;41(12):3443-54. HCV glycoprotein E2 is a novel BDCA-2 ligand and acts as an inhibitor of IFN production by plasmacytoid dendritic cells. Florentin J, Aouar B, Dental C, Thumann C, Firaguay G, Gondois-Rey F, Soumelis V, Baumert TF, Nunès JA, Olive D, Hirsch I, Stranska R. Blood. 2012 Nov 29;120(23):4544-51.].
In Figure 6D, we reported an increase of Akt and Erk phosphorylation upon NKp46 triggering in mature murine NK cells in both mouse strains. We agree that the window is quite narrow (only at low doses of NKp46 mAb, 2μg/ml) for the difference between Dok1-/- Dok2-/- NK cells and WT NK cells. However this difference is always detectable in all the samples. We also added new results from phosphoflow data with another activating NK cell receptor, Ly49D (Figures S3 & S4) that are supporting the data showed in Figure 6D for NKp46.

5. The authors explain the lower percentage of mature NK cells in Dok-deficient mice with increased apoptosis (Figure 5C). While this may be a viable explanation for the phenotype, it is certainly only one of various models. Would crossing Dok1-/- Dok2-/- mice to Bcl2 tg mice rescue the phenotype observed in Dok1-/- Dok2-/- mice?
We agree that the experiment proposed by the reviewer would make the point, but this additional experiment requiring the generation of a novel mouse strain with two homozygous KO mutations and a transgenic background was not realistic in the time frame allowed for the revision of the

manuscript. We thus could not perform the requested experiment.

Referee #2:

The authors describe how Dok proteins negatively regulate NK cell function downstream of activating receptors. The role of Dok proteins in NK cell development and maturation has not been previously studied, thus the data are novel and interesting. However, some of the findings appear contradictory and confusing - in particular the final piece of data (Fig 7), where viral infection using a recombinant MCMV containing a specific ligand for an activating NK cell receptor should result in greater protection in the DKO mice (according to the hypothesis that the Dok-deficient NK cells should have enhanced responses), yet they are less protective than WT. The authors explain this by the very small differences in IFN-γ production during pro-inflammatory cytokine stimulation observed in Fig 6B, but it is not very convincing or likely that this is the mechanism for a 2 log increase in viral titers.

In Fig 7, shouldn't IFN-g and killing via NKG2D be enhanced in the DKO NK cells? Shouldn't this result in greater protection?
The authors could easily measure IFN-γ production by the WT and DKO NK cells ex vivo following infection using intracellular staining. This would strengthen their model that IFN-γ and not activating receptor ligation is responsible for protection (although this is not what the literature would suggest).
We have performed additional studies to attempt to answer this concern. Beside their lower numbers of NK cells, especially of the CD11b+ mature subset, no major alteration in the antiviral NK cell response to MCMV could be observed in DKO mice as compared to control animals, in terms of IFN-γ and Granzyme B expression, proliferation, apoptosis and NKG2D expression, at all time points examined (d0, d1.5, d2, d3 and d6 post-infection). Moreover, the enhanced susceptibility of DKO mice to MCMV infection is only transient, as all animals have controlled the virus by day 6, at a time when a major amplification of NK cell numbers has occurred in both WT and DKO mice. Hence, as suggested by the other reviewer and now discussed in the manuscript, the delayed virus control in Dok1-/- Dok2-/- mice might simply be explained by their lower numbers of NK cells, especially of the CD11b+ mature subset, and could be corrected over time during the infection due to the expansion of these cells.

Several other concerns are listed below and must be addressed:

Throughout the paper, data is only shown for the double KO. Is there any phenotype in the single Dok-1 or Dok-2 knockout mice? Even if no phenotype was observed, supplemental data or a comment should be included.
Single Dok-1 or Dok-2 deficient mice did not show obvious phenotypes possibly because of overlapping functions of Dok-1 and its closest family member, Dok-2 [The roles of Dok family adapters in immunoreceptor signaling. Mashima R, Hishida Y, Tezuka T, Yamanashi Y. Immunol Rev. 2009 Nov;232(1):273-85.]. We included this comment in the text in the results session.

In figure 1B it is suggested that NKG2A may be recruiting SHP1/2 phosphatases to block phosphorylation of DOK-1, but data never actually shown.
This kind of cell signaling regulation is not demonstrated in our work. We agree with the comment and replace the sentence in the text by « *the level of NKp30-induced Dok-1 tyrosine phosphorylation decreased upon co-engagement of NKp30 and CD94/NKG2A (Figure 1B). One hypothesis would be that Dok-1/2 are substrates of the SHP-1/2 protein tyrosine phosphatases reported to be associated to the CD94/NKG2A inhibitory receptor signaling.* »

In Figure 2B and C, what if both Dok proteins are expressed? Is there greater suppression of IFN-γ and killing?
They are overlapping functions of Dok-1 with its closest family member, Dok-2 [The roles of Dok family adapters in immunoreceptor signaling. Mashima R, Hishida Y, Tezuka T, Yamanashi Y. Immunol Rev. 2009 Nov;232(1):273-85.]. In a precedent study in T cells [Functional interaction of RasGAP-binding proteins Dok-1 and Dok-2 with the Tec protein tyrosine kinase. Gérard A, Favre C, Garçon F, Némorin JG, Duplay P, Pastor S, Collette Y, Olive D, Nunès JA. Oncogene. 2004 Feb 26;23(8):1594-8.], we tried experiments with a co-overexpression of Dok-1 and Dok-2 in Jurkat T

cell line, the inhibitory effects on IL-2 promoter are not stronger when both Dok-1 and Dok-2 are expressed compared to a single Dok overexpression.

In Figure 5, it may not be fair to compare DKO NK cells on a 129 background to WT NK cells on a B6 background. Rather than backcrossing the DKO, perhaps WT littermate controls from the DKO breeding can be used as a control for the chimeras, as there may be intrinsic developmental and functional defects between 129 and B6 NK cells that we are not aware of.

We agree with the reviewer that this part was not very clear. In this experiment, BM chimeric mice were generated using a mix of C57BL/6 CD45.1+ BM cells and of 129/Sv or DKO BM cells. The figure legend and labels were misleading. The percentage of NK cells as shown in the figure was measured within the CD45.2+ compartment (i.e. 129/Sv or DKO). We changed both figure legends and figure labels to make it clearer.

In Fig 6, are the authors confident in the difference in B, C, and D statistically significant? Some of the error bars are completely overlapping.

The mean values of the data shown in the Figure 6, are associated to p values less than 5%; the differences are statistically significant. Visually, we agree that when there are overlaps of error bars, it's not the easiest way to show these differences; it occurred sometime when the data are expressed as mean ± SD instead of mean ± SEM (see for instance the figure 1 of this paper published in Nature Methods http://www.nature.com/nmeth/journal/v10/n10/full/nmeth.2659.html). The data in the new figure 6 B-D represent the mean values ± SEM instead of mean ± SD.

Furthermore, are there any differences in cytotoxicity between WT and DKO NK cells?

We didn't assess NK cell cytotoxicity in these mouse strains. However, there are more NK cells expressing both CD107a and IFN-γ in spleen from DKO mice as compared to WT mice upon Ly49D stimulation (see « for inspection by the reviewers» Figure 2).

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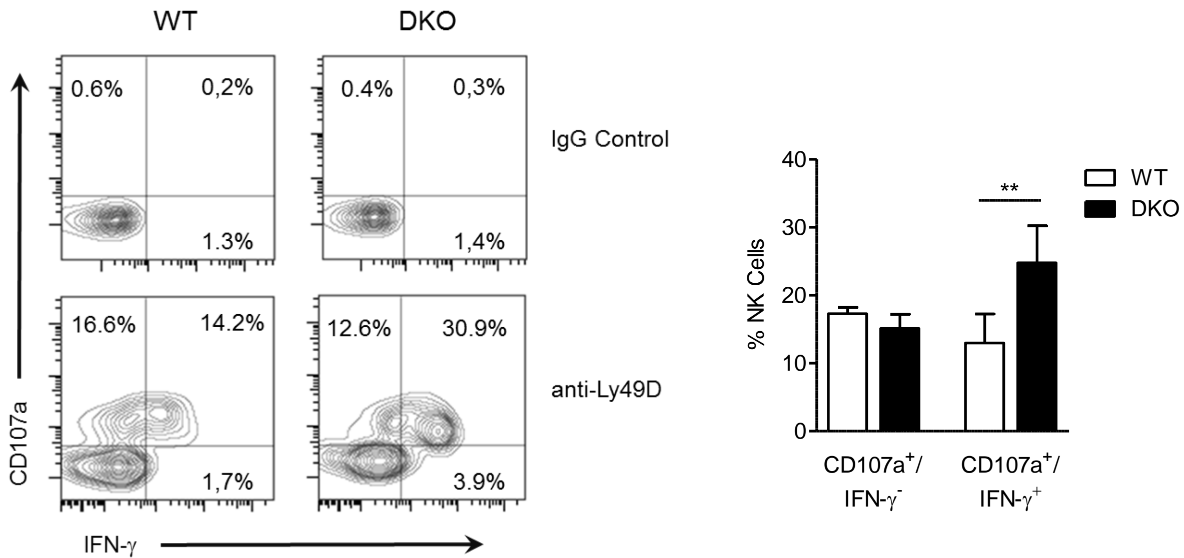

The frequency of double positive CD107a⁺/IFN-γ⁺ cells is increased in Dok-1/Dok-2–deficient NK cells upon Ly49D triggering. Splenocytes from DKO or WT mice were incubated on antibody – coated plates using anti-Ly49D (5μg/ml) antibodies during 4 – 5 hours. At the end of incubation, the splenocytes were stained for CD3, NKp46, CD11b and CD107a then analyzed by flow cytometry. IFN-γ expression was determined by intracellular staining. Representative flow cytometry dot plots in WT or DKO NK cells incubated with anti-Ly49D antibodies are shown for IFN-γ and CD107a parameters (**left panel**). Histograms represent the WT (white bars) or DKO (black bars) CD107a+/IFN-γ^- or CD107a$^+$/IFN-γ^+ NK cell frequency ± SD (**right panel**). Representative data of 3 independent experiments (n= 6-7/group/experiment). ** $P < 0.01$.

Additionally, the defect observed during infection could be due to a defect in myeloid cells and completely independent of NK cells. Have the authors considered this possibility? Does depletion of NK cells change the outcome? The best experiment would be to adoptively transfer normalized numbers of WT and DKO NK cells into separate NK-deficient mice (RAG-/- x gC-/-) followed by virus challenge.

We have examined the production of IFN-β and of its downstream target gene *Mx1* in DKO mice as compared to control animals (Supplemental Figure 6). Both genes were induced to the same levels in both mouse strains. Hence, we can exclude any deficit of myeloid or infected cells in the induction of the antiviral type I interferons responses in DKO animals. Due to time and resource constraints in the frame of the revision of our manuscript, it was not possible to perform the *in vivo* depletion of NK cells nor the complex and costly adoptive transfer experiment suggested by the reviewer.

Minor:

The title is a bit too general. A more appropriate title may be: Dok-1 and Dok-2 regulate NK cell function downstream of activating receptors.
The condensed title should read: Dok-1 and Dok-2 regulate NK cells.

Thank you for your suggestion. We changed the title and the running title.

The introduction opens with the statement that NK cells are ILCs. This is currently debatable, as NK cells are traditionally considered the third lineage of lymphocytes, and very distinct from ILCs in development, function, tissue localization, etc. Thus, this sentence should be reworded or deleted, since ILCs are not brought up again in the paper.

The text has been changed accordingly.

Figure 4B and 5B contain too much data, and should be shown in a more concise manner. The data gets lost in the sheer volume and is even difficult for NK cell experts to look at.

We simplified the figures; we deleted the data from the liver and lymph nodes (Figure 4B, 5A and 5B).

I believe the legend in Fig 6B is wrong. Are the open bars showing WT and black filled bars showing DKO? Please correct, as this data was extremely difficult to follow...

We corrected the legend in Figure 6B.

Thank you for these constructive comments that have allowed us to improve the manuscript. We hope that these changes will fit to the *EMBO Journal*, allowing the manuscript publication.

2nd Editorial Decision 12 May 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by two referees and their comments are provided below. As you can see below both referees appreciate the introduced changes and support publication here. There are a just a few minor issues that should be resolved before formal acceptance here.

REFEREE REPORTS

Referee #1:

This is a revised version of this manuscript. The authors have addressed my main concerns and the manuscript is much improved. I consider the title a bit of a problem as the MCMV data shows largely normal NK cell function but impaired virus control likely due to defects in NK cell differentiation and/or maturation. The title should be changed to reflect the major findings.

Referee #2:

The authors have addressed the majority of my concerns, however there are 2 remaining concerns about the MCMV experiment in the final figure.

1. In panel 7A, the 2 log difference in viral RNA levels is not confirmed by conventional viral plaque assay within the same fig panel. How can one measurement show a 2 log difference while the other assay shows "ns"? Which is correct?

Given that standard plaque assays have been used in the MCMV field for decades, this is likely reflective of the true viremia within the mice. Thus, the main message of figure 7 is likely different and needs to be rewritten.

2. The lack of a difference in viral titers and Ly49H-specific NK cell response at day 6 PI should be shown in the main figures. It is critical to point out the fact that although the NK cell numbers are decreased in the DKO mice, the number of Ly49H NK cells catches up after MCMV infection, and this may correlate with similar viral clearance.

2nd Revision - authors' response 26 May 2014

Referee #1:

This is a revised version of this manuscript. The authors have addressed my main concerns and the manuscript is much improved. I consider the title a bit of a problem as the MCMV data shows largely normal NK cell function but impaired virus control likely due to defects in NK cell differentiation and/or maturation. The title should be changed to reflect the major findings.

Following this advice, we have now changed the title "Dok-1 and Dok-2 proteins regulate Natural Killer cell development and function".

Referee #2:

The authors have addressed the majority of my concerns, however there are 2 remaining concerns about the MCMV experiment in the final figure.

1. *In panel 7A, the 2 log difference in viral RNA levels is not confirmed by conventional viral plaque assay within the same fig panel. How can one measurement show a 2 log difference while the other assay shows "ns"? Which is correct?*
Given that standard plaque assays have been used in the MCMV field for decades, this is likely reflective of the true viremia within the mice. Thus, the main message of figure 7 is likely different and needs to be rewritten.

We have changed the phrasing of the message of the Figure 7A to precise that the plaque assay also shows a trend towards lower viral loads in the spleen of DKO mice at day 3 post-infection although it did not reach significance.

2. *The lack of a difference in viral titers and Ly49H-specific NK cell response at day 6 PI should be shown in the main figures. It is critical to point out the fact that although the NK cell numbers are decreased in the DKO mice, the number of Ly49H NK cells catches up after MCMV infection, and this may correlate with similar viral clearance.*

We have now added an additional panel in Figure 7 (new panel B), to show that control of viral replication is only delayed in DKO mice, since at day 6 post-infection viral loads are decreased in DKO mice to very low to undetectable levels similar to those observed in WT animals. We also show in this panel the percentages of NK cells in total splenocytes in the two mouse strains at d0, d3 and d6, illustrating that the number of NK cells catches up in DKO mice after MCMV infection. In the main text, as requested by the reviewer, we have further emphasized the point that although the

NK cell numbers are strongly decreased in the DKO mice under steady state conditions, NK cells accumulate in the spleen upon MCMV infection in both WT and DKO animals to reach high levels similar between the two mouse strains between days 3 and 6 which may correlate with the delayed but ultimately similar ability of DKO mice to control viral replication.

We hope that these changes will fit to the *EMBO Journal*, allowing the manuscript publication.