NK cell receptor NKG2D sets activation threshold for the NCR1 receptor early in NK cell development

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The activation of natural killer (NK) cells depends on a change in the balance of signals from inhibitory and activating receptors. Here, we found that the activating receptor NKG2D specifically set the activation threshold for the activating receptor NCR1 through a process that required the adaptor DAP12. As a result, NKG2D-deficient (Klrk1−/−) mice controlled tumors and cytomegalovirus infection better than wild-type controls through the NCR1-induced production of the cytokine IFN-γ. Expression of NKG2D before the immature NK cell stage increased expression of the adaptor CD3ζ. Reduced expression of CD3ζ in Klrk1−/− mice was associated with enhanced signal transduction through NCR1, and CD3ζ deficiency resulted in hyper-responsiveness to stimulation via NCR1. Thus, an activating receptor developmentally set the activity of another activating receptor on NK cells and determined NK cell reactivity to cellular threats.

NK cells detect and eliminate ‘stressed’ cells, for example, following infection or malignant transformation. Because of their ability to respond without prior sensitization, activation of NK cells needs to be tightly regulated to ensure proper immunosurveillance while avoiding hyperactivity that may lead to inflammatory or autoimmune disorders. Proper responsiveness is mediated through an ‘education’ process during NK cell development. After engagement of inhibitory receptors, NK cells gain full reactivity and develop tolerance toward self. NK cell responsiveness is further fine-tuned by continuous cues that mature NK cells encounter in the periphery. NK cell activation depends on a shift in the signaling balance between inhibitory and activating receptors. Under homeostatic conditions, inhibitory signals prevail when NK cells interact with peripheral tissues. In response to a cellular threat, host cells downregulate MHCI molecules and/or overexpress stress-induced or nonself-ligands. Upon encounter of stressed target cells, a lack of signals through the inhibitory receptors and/or increased stimulation of activating receptors shifts the balance toward NK cell activation. The factors that control the bandwidth of the equilibrium between inhibitory and activating cues are not completely characterized.

NKG2D and NCR1 are activating receptors expressed on all NK cells. They primarily mediate tissue stress surveillance by recognizing stress-induced self-ligands on target cells. NCR1 (NKp46) is the only member of the NCR family expressed in mice. The role of NCR1 and NKG2D in the control of tumors and infection is well documented. Beyond its effector functions, NKG2D is expressed from the earliest precursors onward during NK cell development. NKG2D-deficient mice have bone marrow (BM) NK cell progenitors that have enhanced proliferation, faster maturation and augmented sensitivity to apoptosis, indicating a role for NKG2D signaling in NK cell development. Moreover, as expected, NKG2D deficiency results in reduced NK cell responsiveness to target cells expressing NKG2D ligands. However, in response to specific activating stimuli, NKG2D-deficient NK cells display a hyper-reactive phenotype in terms of production of the cytokine IFN-γ and better control of mouse cytomegalovirus (mCMV) infection compared to NKG2D-sufficient NK cells. How NKG2D deficiency drives NK cell hyper-reactivity is unclear.

The activating receptors NCR1 and NKG2D require adaptors to convert signals into the cell. NKG2D associates with the adaptors DAP10 or DAP12 (ref. 1), and NCR1 docks the adaptors CD3ζ and FceRy11. DAP10 has a YxxM motif through which the PI3K and Grb2-Vav1-SOS1 are engaged. CD3ζ, FceRy and DAP12 possess ITAM motifs, whose phosphorylation activates signaling proteins Syk and ZAP70, resulting in cytotoxicity and/or cytokine production. It is commonly believed that adaptors with ITAM motifs transduce activating signals. However, increasing evidence shows that they can also negatively impact signaling cascades. How adaptors of activating NK cell receptors contribute to negative regulation of signaling is mostly unknown.

Here we demonstrate that NKG2D deficiency or blocking of NKG2D signaling early during NK cell development caused hyper-reactivity of the NCR1 receptor, resulting in enhanced control of mCMV infection and tumors expressing NCR1 ligands. Deficiency of NKG2D or DAP12 resulted in downregulation of CD3ζ and ZAP-70 yet stronger signaling after NCR1 stimulation. Ablation of NKG2D in immature CD122+NK1.1+NCR1+C11b−c-Kit− NK cells completely abrogated the hyperactive phenotype of NK cells, indicating that this regulation occurs early during NK development.

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Klrk1−/− NK cells have specific hyper-reactivity through NCR1.

To analyze the impact of KNG2D deficiency on target-cell engagement, we performed a conjugation assay with B16 melanoma. We observed no difference in the amount of NK target cell complexes between C57BL/6J and Klrk1−/− NK cells (Fig. 2a). To test whether increased IFN-γ production in Klrk1−/− NK cells resulted from enhanced signaling through activating receptors, we stimulated splenic NK cells from Klrk1−/− mice or Klrk1+/− littermates through different activating receptors. Engagement of receptors NK1.1, DNAM-1, Ly49D or Ly49H by mAb or incubation of NK cells with IL-12 and IL-18 cytokines resulted in similar production of IFN-γ in Klrk1−/− and Klrk1+/− NK cells (Fig. 2b,c and Supplementary Fig. 1f). In contrast, stimulation with mAb against the NCR1 receptor resulted in a higher percentage of IFN-γ+ Klrk1−/− NK cells compared to IFNy+ Klrk1+− NK cells (Fig. 2b,c). NCR1 expression and stimulation-induced degranulation by mAb (Fig. 2d) were similar in Klrk1−/− and Klrk1+/− NK cells. Thus, KNG2D-deficient NK cells show hyper-responsiveness to stimulation through the NCR1 receptor.

NCR1 is known to have a role in the control of B16 melanoma. Labeling with NCR1-ig fusion proteins showed high expression of NCR1 ligands on B16 cells (Supplementary Fig. 1k). To investigate whether NCR1 was involved in the enhanced tumor control by Klrk1−/− mice, we used Ncr1−/−mice, which are deficient in NCR1. Ncr1−/−mice showed reduced survival in comparison to C57BL/6J mice after i.v. injection of B16 melanoma cells (Fig. 2e). Klrk1−/− mice showed better survival in comparison to C57BL/6J controls, whereas Klrk1−/−Ncr1−/−mice had survival comparable to that of Ncr1−/−mice (Fig. 2f). Depletion of NK cells by mAb abrogated any difference in survival between all mice (Fig. 2e). These results show that the enhanced tumor control in Klrk1−/− mice is dependent on NCR1 engagement by NK cells.

Compared to C57BL/6J mice, Klrk1−/− mice have better control of MCMV infection (Supplementary Fig. 2), which is NK cell dependent. To test whether this effect was mediated through NCR1, we infected C57BL/6J, Ncr1−/−, Klrk1−/− and Ncr1−/−Ncr1−/−mice with Δm157 MCMV, a mutant strain of MCMV lacking ligand for NK cell receptor Ly49H. We used this MCMV strain to avoid the Ly49H-mediated control of viral replication, which may occlude the effects of NCR1 (ref. 29). Klrk1−/−mice showed better control of Δm157 MCMV in the spleen compared to all other mice, and this was lost after depletion of NK cells by mAb (Fig. 2f). These results show that the enhanced control of MCMV infection by NKG2D-deficient mice is dependent on NCR1 engagement by NK cells.

NKG2D sets NCR1 activation threshold during NK cell development. During NK cell development, NKG2D is expressed from the Lin−Cd117−Sca1+Flt3−CD127+ cells onwards, which represents the earliest NK cell committed precursor (pre-pro NK). Because NKG2D deficiency impacts development of NK cells in the bone marrow (BM) as well as NK cells effector responses in the periphery, we asked whether the hyper-reactivity of Klrk1−/− NK cells to NCR1 stimulation was acquired during development or later on in mature NK cells in the periphery. We crossed Klrk1−/− mice with Ncr1−/− mice (Supplementary Fig. 3a) to generate Ncr1−/−Klrk1−/−mice, in which Cre-mediated deletion of Klrk1 occurs in Cd122−NK1.1+Ncr1−/−Cd11b−c-Kit− NK cells. Spleen NK cells from Ncr1−/−Klrk1−/−mice showed comparable production of IFN-γ to Klrk1−/−mice after stimulation of NCR1 by mAb in vitro (Supplementary Fig. 3a). We did not observe differences in survival between Ncr1−/−Klrk1−/−mice and Klrk1−/−mice after i.v. injection of B16 cells (Fig. 3b). In contrast, a higher percentage of spleen NK cells from Klrk1−/− mice, which had a germline deletion of Klrk1 and were generated from the cross between deleter (tg-cmvNcr1×) and Klrk1−/− mice, produced IFN-γ to NCR1 stimulation by mAb.
compared to C57BL/6J control (Supplementary Fig. 3b). These results indicate that NKG2D sets the activation threshold for NCR1 developmentally before CD122+NK1.1+NCR1+CD11b−c-Kit− NK cells.

Next we tested whether NKG2D played a role during early NK cell development in a model independent of genetic modification of Klrk1. In Rag1<sup>Cre</sup>−/−<sup>EYFP<sup>loxp<sup>−</sup>Flt3<sup>L</sup>/mice, all cells derived from Rag1<sup>+</sup> hematopoietic precursors, including T cells, B cells and a large fraction of NK cells, expressed EYFP and diphtheria toxin receptor (DTR) upon Cre-mediated deletion of the transcriptional ’stop’ sequence and can be eliminated by diphtheria toxin (DT) injection (Supplementary Fig. 3c). Rag1<sup>Cre</sup>−/−<sup>EYFP<sup>loxp<sup>−</sup>Flt3<sup>L</sup>/mice were i.p. injected with DT on the first two consecutive days to deplete all NK cells originating from the Rag1<sup>+</sup> precursors. To inhibit NKG2D signaling on all newly generated NK progenitors, the mice were treated from the second day onward with NKG2D-blocking mAb or isotype control (Supplementary Fig. 3c), and the receptor responsiveness of EYFP<sup>+</sup> NK cells was analyzed 2 weeks later. Spleen NK cells from Rag1<sup>Cre</sup>−/−<sup>EYFP<sup>loxp<sup>−</sup>DTR/ mice that had received i.v. NKG2D-blocking mAb showed increased IFN-γ production after stimulation of NCR1 but not following stimulation of NK1.1 or Ly49H by mAbs (Fig. 3c), indicating that NKG2D sets the activation threshold for NCR1 early in NK cell development. These observations prompted us to analyze the impact of NKG2D deficiency on hematopoiesis. NKG2D deficiency does not affect hematopoietic stem cells or more differentiated precursors such as the common lymphoid and myeloid precursors<sup>10,11</sup> (Fig. 3d). However, there was a reduction in the number of BM Lin−CD117<sup>dim</sup>Sca1<sup>+</sup>Flt3<sup>L</sup>−CD127<sup>+</sup> NK progenitors in Klrk1<sup>−/−</sup> mice compared to Klrk1<sup>+/+</sup> littermates (Fig. 3d). Further analysis of NK cell development revealed an increase in percentage of CD122<sup>+</sup>NK1.1<sup>−/−</sup>NCR1<sup>−/−</sup>CD11b<sup>−/−</sup>-c-Kit<sup>−</sup> and decrease of CD122<sup>+</sup>NK1.1<sup>−/−</sup>NCR1<sup>−/−</sup>CD11b<sup>−/−</sup>-c-Kit<sup>−</sup> NK progenitors in Klrk1<sup>−/−</sup> mice compared to Klrk1<sup>+/+</sup> littermates (Fig. 3e and Supplementary Fig. 4a). To confirm the role of NKG2D in changing NK progenitors in a second model, we analyzed their BM of Rag1<sup>Cre</sup>−/−<sup>EYFP<sup>loxp<sup>−</sup>DTR/mice 15 d after DT injection and treatment with NKG2D-blocking mAb. Similar to Klrk1<sup>−/−</sup> mice, we observed an increase in percentage of CD122<sup>+</sup>NK1.1<sup>−/−</sup>NCR1<sup>−/−</sup>CD11b<sup>−/−</sup>-c-Kit<sup>−</sup> and decrease of CD122<sup>+</sup>NK1.1<sup>−/−</sup>NCR1<sup>−/−</sup>CD11b<sup>−/−</sup>-c-Kit<sup>−</sup> NK progenitors compared to isotype control-treated Rag1<sup>Cre</sup>−/−<sup>EYFP<sup>loxp<sup>−</sup>DTR mice (Fig. 3f). Together, these results indicate a role of NKG2D in NK cell development at the time of NCR1 appearance on NK progenitors.

NKG2D-mediated NK cell education differs from known mechanisms. NK cells that never expressed Rag1 during...
development showed cell-intrinsic hyper-responsiveness in comparison to NK cells generated from Rag1+ progenitors\(^2\). We therefore asked whether NKG2D promoted NK cell development from a Rag1+ progenitor. We generated \(Klrk1^-/-\) \(Rag1^{Cre}\) \(EYFP^{Stop-Flox}\) and \(Klrk1^-/-\) \(Rag1^{Cre}\) \(EYFP^{Stop-Flox}\) mice, in which expression of Rag1 was marked through the expression of EYFP. As shown previously\(^2\), we observed that a higher percentage of EYFP- NK cells from \(Klrk1^-/-\) \(Rag1^{Cre}\) \(EYFP^{Stop-Flox}\) mice were \(Klrk1^+\) and \(CD11b^+\) compared to EYFP+ NK cells (Supplementary Fig. 4b).

We made the same observations in \(Klrk1^-/-\) \(Rag1^{Cre}\) \(EYFP^{Stop-Flox}\) mice (Supplementary Fig. 4c). However, there were no differences in percentages of EYFP+ or EYFP- NK cells between \(Klrk1^+/-\) \(Rag1^{Cre}\) \(EYFP^{Stop-Flox}\) and \(Klrk1^-/-\) \(Rag1^{Cre}\) \(EYFP^{Stop-Flox}\) mice (Fig. 3g). Furthermore, regardless of EYFP expression, NK cells from \(Klrk1^+/-\) \(Rag1^{Cre}\) \(EYFP^{Stop-Flox}\) mice produced more IFN-\(\gamma\) than NK cells from \(Klrk1^-/-\) \(Rag1^{Cre}\) \(EYFP^{Stop-Flox}\) mice upon NCR1 stimulation by mAb, whereas responsiveness to NK1.1 was similar (Fig. 3h), indicating that NKG2D influences NCR1 signaling independently of the Rag-driven developmental pathway.

To investigate the role of Ly49-mediated education in NCR1 signaling, we compared the production of IFN-\(\gamma\) in Ly49I+ to that in Ly49I- \(Klrk1^+/-\) and \(Klrk1^-/-\) NK cells following NCR1 stimulation by mAb. \(Klrk1^-/-\) Ly49I+ NK cells produced more IFN-\(\gamma\) compared to \(Klrk1^+/-\) Ly49I- NK cells (Fig. 3i). However, \(Klrk1^-/-\) NK cells produced more IFN-\(\gamma\) compared to \(Klrk1^+/-\) NK cells regardless of their Ly49I expression (Fig. 3i). Also, there was no difference in the frequency of Ly49I+ NK cells between \(Klrk1^-/-\) and \(Klrk1^+/-\) cells (Supplementary Fig. 4d). These observations indicate that the
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threshold for the NKG2D-dependent activation of NCR1 is independent of Ly49-mediated education.

SHP-1 plays a role in NK cell education, and SHP-1-deficient NK cells are hypo-responsive to MHC-I-deficient transplants and tumors. To test whether SHP-1 plays a role in the NKG2D-mediated education, we treated Klrk1−/− and Klrk1+/+ spleen NK cells in vitro with the SHP-1 inhibitor NSC-87877 (ref. 35) and then stimulated them through the NCR1 receptor by mAb. SHP-1 inhibition resulted in an increase of IFN-γ production in both Klrk1−/− and Klrk1+/+ NK cells. However, production of IFN-γ was higher in Klrk1−/− NK cells compared to Klrk1+/+ (Fig. 3i), indicating that NKG2D sets the activation threshold for NCR1 independently of SHP-1/2.

The NKG2D-DAP12 signaling axis regulates NCR1 activity. To investigate the mechanism through which NKG2D regulates the activity of NCR1, we focused on the adaptors DAP10 and DAP12 (ref. 13). We used mice lacking either DAP10 (Hcst−/−) or DAP12 (Tyrobp−/−). There were no differences in the production of IFN-γ between C57BL/6, Klrk1−/−, Hcst−/− and Tyrobp−/− spleen NK cells after stimulation through NK1.1 by mAb (Fig. 4a). Ly49H and Ly49D use DAP12 for signal transduction. IFN-γ production from Tyrobp−/−, but not Klrk1−/− or Hcst−/− NK cells was reduced compared to C57BL/6 NK cells after stimulation through these receptors (Fig. 4a). Notably, after stimulation through NCR1, Tyrobp−/−, but not Hcst−/−, NK cells showed an increase in IFN-γ production compared to C57BL/6 controls, similarly to Klrk1−/− NK cells (Fig. 4a). Similar observations were made after NCR1 stimulation of spleen NK cells from Tyrobp−/− mice and Tyrobp−/− littermates (Supplementary Fig. 5a). When B16 cells were injected i.v. in Klrk1−/−, Hcst−/−, Tyrobp−/− and C57BL/6 mice, Tyrobp−/− mice showed prolonged survival in comparison to Hcst−/−, C57BL/6 and even Klrk1−/− mice (Fig. 4b), indicating that signaling through DAP12 only was important for NK cell hyper-reactivity to NCR1 stimulation.

We next questioned whether the hyper-responsiveness of DAP12-deficient NK cells was specific for NKG2D or whether...
it was observed following deletion of any receptor that signals through this adaptor. When Klrk1<sup>−/−</sup>, Ly49H<sup>−/−</sup> or C57BL/6J spleen NK cells were stimulated through NK1.1 or NCR1 by mAbs or with the cytokine IL-12, Ly49H<sup>−/−</sup> NK cells did not show increased IFN-γ production after any of these stimulations relative to C57BL/6J NK cells (Fig. 4c). In mice, NKG2D has a long (L) and a short (S) isoform, of which only the latter associates with DAP12 (ref. 15). We therefore investigated whether NKG2D-S and DAP12 were expressed during early NK cell development in wild-type mice. qPCR in sorted CD122<sup>+</sup>NK1.1<sup>−</sup>NCR1<sup>−</sup>CD11b<sup>−</sup>c-Kit<sup>−</sup> and CD122<sup>+</sup>NK1.1<sup>−</sup>NCR1<sup>−</sup>CD11b<sup>−</sup>c-Kit<sup>−</sup> BM NK progenitors detected transcripts for the Tyrobp and short isoform of Klrk1, whose expression increased from CD122<sup>+</sup>NK1.1<sup>−</sup>NCR1<sup>−</sup>CD11b<sup>−</sup>c-Kit<sup>−</sup> to CD122<sup>+</sup>NK1.1<sup>−</sup>NCR1<sup>−</sup>CD11b<sup>−</sup>c-Kit<sup>−</sup> NK progenitors (Supplementary Fig. 5b,c). Thus, the NKG2D-mediated control of NCR1 signaling occurs through the NKG2D–DAP12 axis early in NK cell development.

**CD3ζ and ZAP-70 are involved in inhibition of NCR1 signaling.** Because NCR1 uses CD3ζ and FceRy for signal transduction<sup>16</sup>, we tested whether the NKG2D–DAP12 axis affects signaling through these adaptors. Flow cytometry analysis indicated that expression of CD3ζ was reduced in both Klrk1<sup>−/−</sup> and Tyrobp<sup>−/−</sup> NK cells in comparison to C57BL/6J NK cells, whereas expression of FceRy was comparable in all groups (Fig. 5a,b and Supplementary Fig. 5d,e). In addition, expression of ZAP-70, a signaling component downstream of CD3ζ, was reduced in Klrk1<sup>−/−</sup> and Tyrobp<sup>−/−</sup> spleen NK cells relative to C57BL/6J NK cells, whereas expression of the Syk kinase, also downstream of CD3ζ, was comparable in all groups (Fig. 5a,b and Supplementary Fig. 5e). Immunoblot analysis also showed reduced expression of CD3ζ and ZAP-70 in Klrk1<sup>−/−</sup> spleen NK cells compared to C57BL/6J NK cells (Fig. 5c). In contrast, Ncr1<sup>−/−</sup>Klrk1<sup>fl/fl</sup> spleen NK cells had no alterations in the expression of CD3ζ or ZAP-70 compared to Klrk1<sup>fl/fl</sup> NK cells (Fig. 5d).

Because CD3ζ-deficient mouse spleen NK cells are hyper-responsive to CD16 stimulation<sup>15</sup>, we asked whether NKG2D and DAP12 mediated the responsiveness of NK cells to CD16 engagement. Klrk1<sup>−/−</sup> and Tyrobp<sup>−/−</sup> spleen NK cells showed enhanced IFN-γ production following CD16 stimulation compared to C57BL/6J NK cells (Supplementary Fig. 5f).

As determined by qPCR, Cd247 or Fcεr1g mRNA was similar in Klrk1<sup>−/−</sup> and C57BL/6J NK cells (Fig. 5e), suggesting altered post-transcriptional regulation. To identify candidates that might impact the expression of CD3ζ and/or ZAP-70, we compared the transcriptome of spleen CD3ζ<sup>−/−</sup>NK1.1<sup>−</sup>NCR1<sup>−</sup> NK cells from C57BL/6J, Klrk1<sup>−/−</sup>, and Tyrobp<sup>−/−</sup> mice by RNA sequencing. Ninety-four genes were differentially expressed between C57BL/6J and Klrk1<sup>−/−</sup> NK cells, whereas expression of 543 genes was different between Tyrobp<sup>−/−</sup> and C57BL/6J NK cells (Fig. 6a). We performed qualified cluster analysis of genes differentially expressed in Klrk1<sup>−/−</sup> NK cells versus C57BL/6J cells on the basis of data mining of known protein–protein interactions to establish a potential link with CD3ζ and/or ZAP-70 (Supplementary Fig. 6a). Next, we determined which of these genes showed a shared expression pattern between Klrk1<sup>−/−</sup> and Tyrobp<sup>−/−</sup> mice (Fig. 6a,b). Prf, encoding perforin, and Sla, encoding adaptor SLAP-1, were identified as potential candidates. Using flow cytometry analysis we did not detect a difference in perforin protein expression between Klrk1<sup>−/−</sup> and C57BL/6J spleen NK cells (Fig. 6c). SLAP-1 is known to target CD3ζ for degradation in thymocytes<sup>17</sup>. Sla transcripts were upregulated in Klrk1<sup>−/−</sup> and Tyrobp<sup>−/−</sup> NK cells compared to C57BL/6J NK cells (Fig. 6b). Cell surface expression of SLAP-1 on Klrk1<sup>−/−</sup> NK cells was increased compared to C57BL/6J NK cells (Fig. 6c and Supplementary Fig. 6b). In addition, expression of SLAP-1 in splenic EYFP<sup>+</sup> NK cells

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**Fig. 4**| The NKG2D–DAP12 signaling axis regulates NCR1 activity. (a) NK cells from Tyrobp<sup>−/−</sup> (n = 3 or 4 mice), Hcst<sup>−/−</sup> (n = 3 or 5 mice), Klrk1<sup>−/−</sup> (n = 3 or 5 mice) and C57BL/6J (n = 3 or 5 mice) were stimulated for 4 h through the NK1.1, Ly49H, Ly49D or NCR1 receptor by mAb and IFN-γ production was analyzed after 4 h. (b) Survival curves of indicated groups of mice after i.v. injection of B16 cells (Kaplan–Meier model followed by Log-rank (Mantel-Cox) test; n = 10 mice per group). (c) NK cells from Ly49h<sup>−/−</sup> (n = 3 mice), Klrk1<sup>−/−</sup> (n = 4 mice) and C57BL/6J (n = 4 mice) mice were stimulated through the NK1.1 or NCR1 receptor by mAb or with IL-12 cytokines and IFN-γ production was analyzed after 4 h. Shown are representative plots of three (a) or two (b,c) experiments. For analysis of (a) ANOVA, with Bonferroni’s post-test correction for multiple comparisons was used. Shown are means ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.
from Rag1<sup>−/−</sup> EYFP<sup>Cre+/−</sup> DTR mice 15 d after DT injection and treatment with NKG2D-blocking Ab was increased compared to EYFP<sup>+</sup> NK cells from DT and isotype-treated Rag1<sup>−/−</sup> EYFP<sup>Cre+/−</sup> DTR mice (Fig. 6d). Next, we asked whether SLAP-1 downregulates CD3ζ in NK cells. We observed increased expression of CD3ζ protein in spleen NK cells from Sla<sup>−/−</sup> mice<sup>37</sup> compared to wild-type controls, whereas expression of NCR1, FceRy, Syk and ZAP-70 were not affected (Fig. 6e and Supplementary Fig. 6c). Thus, NKG2D deficiency results in an increase of SLAP-1 protein in NK cells, which reduces the expression of CD3ζ.

In thymocytes, CD3ζ and ZAP-70 mediate activation upon TCR stimulation, but they are also important for the shutdown of signal transduction through recruitment of proteins involved in proximal negative feedback mechanisms.<sup>35,38</sup> We therefore asked whether NCR1 stimulation in Klrk1<sup>−/−</sup> cells results in enhanced proximal signaling. Phosphorylation of Syk, the kinase directly downstream of FceRy, was both increased and prolonged in Klrk1<sup>−/−</sup> NK cells after NCR1 stimulation by mAbs compared to Klrk1<sup>+/+</sup> NK cells (Fig. 6f). Similar observations were made in Tyrobp<sup>−/−</sup> cells (Fig. 6g and Supplementary Fig. 6d). Importantly, phosphorylation of PLC-γ, which is a target of Syk, was prolonged in Klrk1<sup>−/−</sup> NK cells compared to Klrk1<sup>+/+</sup> NK cells, whereas maximal phosphorylation of PLC-γ was only slightly increased (Fig. 6h), indicating a reduced negative feedback loop in proximal NCR1 signaling. Finally, we asked whether CD3ζ deficiency resulted in hyper-responsive NK cells. Cd4<sup>+</sup> spleen NK cells produced more IFN-γ after stimulation through the NCR1 receptor by mAb, but not through NK1.1, Ly49H or Ly49D, compared to C57BL/6 J spleen NK cells (Fig. 6i), indicating a role for CD3ζ in the negative regulation of NCR1 signaling. NCR1 expression was similar in all the cells analyzed (Supplementary Fig. 6f). As such, NKG2D sets an activation threshold for the NCR1 receptor by increasing CD3ζ protein and by lowering expression of SLAP-1 during NK cell development.

**Discussion**

Here we show that NKG2D sets activation threshold for NCR1 early in NK cell development, which determines the sensitivity of NK cells to cellular targets expressing NCR1 ligands. This process operates through a NKG2D–DAP12 signaling axis that drives down-regulation of CD3ζ and ZAP-70, involved in negative regulation of NCR1 signaling. Thus, we identified a developmental NK cell regulation of CD3ζ that is distinct from previously described mechanisms of education in which one activating receptor regulates the activity of another activating receptor.

Our results indicated that the role of NKG2D in the regulation of NCR1 activation was mediated by DAP12. Although we did not
Fig. 6 | Hyper-reactivity of Klrk1<sup>−/−</sup> NK cells in response to NCR1 stimulation is a consequence of reduced expression of CD3ζ. (a,b) RNAseq was performed on sorted NK cells isolated from the spleens of Klrk1<sup>−/−</sup>, Tyrobp<sup>−/−</sup> and C57BL/6<sup>+</sup> mice. Venn diagram (a) and heat map (b) for differentially expressed genes. (c) Analysis of splenic NK cells from C57BL/6<sup>+</sup> (n = 5 mice) and Klrk1<sup>−/−</sup> (n = 4 mice) mice for expression of perforin (left) and SLAP-1 (right). Shown are geometric mean values. (d) Analysis of NK cells from Rag<sup>−/−</sup>EYFP<sup>top-fox</sup>DT<sup>−/−</sup> mice injected with DT and in addition with NKG2D-blocking Ab. (e) NK cells from C57BL/6<sup>+</sup> (n = 4 mice) mice were stimulated through the NCR1 receptor by mAb and phosphorylation of Syk (f) and PLC-γ (h) was analyzed. FACS plots are gated for NK cells (CD3<sup>−</sup> NK1.1<sup>+</sup>). Splenic NK cells from Balb/c (n = 5 mice) and Slp<sup>−/−</sup> (n = 3 mice) mice were analyzed for expression of Syk, FcγR, ZAP-70 and CD3ζ (n = 10 Balb/c and 8 Slp<sup>−/−</sup> mice). Graphs show geometric mean values. (f-h) NK cells from C57BL/6<sup>+</sup> (n = 4), Tyrobp<sup>−/−</sup> (n = 4) or Klrk1<sup>−/−</sup> (n = 5) mice were stimulated through the NCR1 receptor by mAb and phosphorylation of Syk (f) and PLC-γ (h) was analyzed. FACS plots are gated for NK cells (CD3<sup>−</sup> NK1.1<sup>+</sup>). (f) RNK cells from C57BL/6<sup>+</sup> and Cd247<sup>−/−</sup> (n = 4 mice per group) mice were stimulated through NCR1 by mAb and IFN-γ production was analyzed. Shown are representative plots of one (a,b), two (d,e,i) or three (c,f,g) experiments. (f) and (h) are performed using littermates. Two-tailed unpaired t-test was used to analyze data in c–i. Shown are means ± s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001.

Formally show that NKG2D and DAP12 were directly engaged to mediate their regulatory role during NK cell development, the absence of a hyper-responsive phenotype in Ly49H-deficient mice makes it highly unlikely that these two molecules have an identical yet independent effect. The role of DAP12 in setting activating thresholds does not appear to be unique to NK cells. DAP12-deficient mice have macrophages<sup>49</sup> and pDCs<sup>40</sup> that produce higher amounts of cytokines after TLR stimulation in comparison to...
wild-type mice. The mechanism by which DAP12 sets activation thresholds in these cells is unknown, but it may be similar to the NKG2D-mediated regulation in NK cells. Because human NKG2D does not bind to DAP12, our findings can not be directly extrapolated. However, several KIRs signal through DAP12 (refs 41-44). It has been shown that activating KIRs downmodulate human NK cell-responsiveness in individuals carrying self ligands45. It will therefore be interesting to see whether regulation of NKP46 activation thresholds in humans is regulated through NKG2D or through Dap12-binding KIRs.

Our data indicated the involvement of CD3ζ and ZAP-70 in the negative regulation of NCR1 signaling. NK cells from CD3ζ-deficient mice had higher production of IFN-γ after stimulation through the NCR1 receptor. These observations are in line with reports of an important role for CD3ζ and ZAP-70 in the negative regulation of signaling in T cells4,39 and NK cells46. Following T cell receptor engagement, CD3ζ-ZAP-70 recruit ubiquitinase Nrdp3 and phosphatases Sts-1 and Sts-2, which dephosphorylate ZAP-70 and cause cessation of the activating signal. Deficiency for Nrdp3, Sts-1 and Sts-2 causes prolonged signal transduction46. We observed that KIrkl+ mice had delayed signal inhibition following NCR1 stimulation, especially at the level of PLC-γ phosphorylation. Thus, we propose that a reduction in expression of CD3ζ-ZAP-70 causes impaired recruitment of factors that terminate the activating signal directly downstream of the NCR1 receptor. Although a direct interaction between CD16 and CD3ζ has only been shown in human cells, deficiency of CD3ζ or DAP12 causes hyper-reactivity in murine NK cells in response to CD16 stimulation19-21. We saw increased production of IFN-γ in KIrkl+ NK cells after stimulation through the CD16 receptor, suggesting that NKG2D may also regulate the responsiveness of CD16.

Chronic exposure to NKG2D ligands can result in cross-tolerance of multiple distinct NK cell activation pathways22-24. In addition, weak signaling through NKG2D leads to reduced activity of NK cells, which can be circumvented by administration of soluble high-affinity ligands22. However, peripheral ‘desensitisation’ through NKG2D generates a general hyporesponsiveness of NK cells to activating stimuli. Therefore, the developmental and peripheral regulation of activation thresholds by NKG2D appear to use distinct molecular mechanisms.

The NKG2D-mediated ‘education’ of NCR1 is distinct from previously described mechanisms. Education through molecules such as Ly49 receptors25 or via modification of the SHP phosphatases26 results in a general hyporesponsiveness to activating stimuli. The impact of NKG2D-mediated education, in contrast, appears restricted to NCR1. Importantly, we identify a temporal window in which NKG2D permanently controls NCR1 responsiveness. Deletion of KIrkl from the CD122*KN1.1*NCR1*CD11b-c-KT- NK progenitors onward did not cause NK cell hyperresponsiveness to NCR1 as was the case in mice with germline deletion of KIrkl. Ly49 molecules are expressed from the CD122*KN1.1*NCR1*CD11b-c-KT- NK progenitors onward. Indeed, lack of Ly49H1, which also signals through DAP12, did not result in NK cell hyperresponsiveness to NCR1. How permanent regulation of CD3ζ expression by NKG2D signaling at a highly restricted stage of NK cell development is accomplished mechanistically remains unclear. Our data suggest that post-transcriptional regulation of CD3ζ, RNA-seq analysis revealed Sla as a candidate involved in this process. Sla encodes SLAP-1, an adaptor that targets CD3ζ for ubiquitin ligase c-Cbl-dependent degradation following receptor activation.9. NKG2D deficiency, or blocking of NKG2D during NK cell development, caused a permanent increase in Sla gene expression, implying increased degradation of CD3ζ and subsequently altered signal transduction through NCR1. Indeed, SLAP-1-deficient NK cells expressed higher amounts of CD3ζ, CD244, another NK activating receptor expressed on multipotent hematopoietic progenitors h, was shown to regulate immune cell function through epigenetic silencing of chromatin regions9. Although methylation analysis of the Sla locus in KIrkl+ NK cells did not reveal differences compared to wild-type NK cells (unpublished data by T.D.H. and Y.C.B.), this does not exclude another ways of epigenetic regulation of Sla expression.

Online content Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41590-018-0209-9.

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Author contributions

V.J. carried out most of the experiments and analyzed data. M.S., I.K., M.L., S.M., B.L. and EM.W performed and analyzed experiments. B.P. directed the research. B.P., V.J. and EM.W designed experiments and wrote the paper. Y.S. and M.P. designed and performed qPCR on NK precursors. T.D.H. and Y.T.B. designed and performed RNA-seq experiments.

Competing interests

The authors declare no competing interests.

Additional information

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Mice. Mice were strictly age and sex matched within experiments and were kept in SPF conditions and handled in accordance with institutional, national and/or EU guidelines. Permission for our experiments was given by Ethical Committee of the Faculty of Medicine, University of Rijeka and Croatian Ministry of Agriculture, Veterinary and Food Safety Directorate (UP1/3-22/01-16/01, 525-10/0255-16-7). Mice used in experiments were between 6 and 12 weeks of age. Klrk1−/− and Klrk1+/− mice were generated as described previously12. Wild-type C57BL/6J (strain 000664), Balb/c (strain 00651) and Ifng−/− (strain 002287) mice were from the Jackson Laboratory. Ncr1−/− mice were provided by K. Rajewsky. Cd247−/− mice were kindly provided by A. Waisman (Mainz, Germany). Ncr1−/− mice were provided by M. Busslinger (Vienna, Austria). Rosa26-stop FOXD (EYFP) mice were generated as described previously9,18. Wild-type C57BL/6 J (strain 002287) mice were from the Jackson Laboratory. 

In vitro analysis of NK cells. For NK killer assay, B16 cells were labeled with PK136. In these experiments we used ten mice per group and experiments were repeated two times.

Antibodies used were 250 μM PK136. Digital caliper was used to measure tumor size. X-ray pictures of tumors in mice were kindly provided by K. Rajewsky. M. Busslinger (Vienna, Austria). Rosa26-stop FOXD (EYFP) mice were generated as described previously9,18. Wild-type C57BL/6 J (strain 002287) mice were from the Jackson Laboratory. 

Cells. B16 cell line was purchased from the American Type and Culture Collection (ATCC). Cells were cultured in complete DMEM, supplemented with 10 mM HEPES (pH 7.2), 2 mM l-glutamine, 105 U/L Penicillin, 0.1 g/L Streptomycin, and 10% FCS. Cells were maintained in an incubator at 37 °C and 5% CO2.

Tumor models. Radiation induced thymic lymphomas. Young mice (4–6 weeks old) received low doses of γ radiation (1.6 Gy) once a week for 4 weeks as previously described13. In these experiments we used ten mice per group, and experiments were repeated twice.

B16 melanoma. Mice received 105 B16 clone F10 (B16) cells i.v or s.c and survival was monitored weekly. Mice were sacrificed and tumors were used for flow cytometry and RNA sequencing. Summary statistics were calculated as previously described14,15.

In vitro analysis of NK cells. For NK killer assay, B16 cells were labeled with PK136. In these experiments we used ten mice per group and experiments were repeated two times.

In vitro analysis of NK cells. For NK killer assay, B16 cells were labeled with PK136. In these experiments we used ten mice per group and experiments were repeated two times.

Purification of NK cells and RNA isolation for qPCR. NK cells were enriched from spleens using biotinylated DX5 antibodies, streptavidin-coated beads and magnetic cell sorting (Miltenyi). Next, NK cells (CD3−NK1.1−) were sorted to high (>99% purity) on a FACs Artia II (BD Biosciences). RNA was isolated via the Trizol method, and cDNA was generated with a reverse transcriptase core kit (Eurogentec). The expression of mRNA was examined by quantitative PCR analysis with a 7500 Fast Real Time PCR machine. Taqman assays were used to quantify the expression of Ifng (IFN-γ, Mm00485148_m1). The relative mRNA expression was normalized by quantification of Rn18S (18S, Mm03928990_g1) RNA in each sample.

Flow cytometry. Cells were pretreated with Fc block (clone 2.4G2, produced in-house). To-pro-3 (Life Technologies) was used to label dead cells. Cells were stained and analyzed in PBS containing 1% BSA and NaN3 with antibodies listed below. Intracellular staining, permeabilization and fixation of cells was done with the Fix/Perm kit (BD Biosciences). The cells were measured on a FACSVerse or FACSari flow cytometry system (BD Biosciences), and data were analyzed using FlowJo v10 software (Tree Star, Ashland, OR). To analyze ligands expression tumor cells were stained with fusion proteins (NK2D-Fc or NKG2D-Fc) or irrelevant fusion protein and secondary FITC labeled anti-human antibody. For flow cytometry, we used monoclonal antibodies to mouse CD3e (145-2C11), CD62L (M104), ST-HSC (CD117+), PreProNK (CD117dimSca1+), LT-HSC (CD117+CD127+CD48−Sca1+),ship-1 (1D6) from MBL, CD247 (CD3−NK1.1−), Biotinylated Abs against CD4 (GK1.5), CD8 (53-6.7), Ly49H (3D10), and Ly49D (4e4) were kind gift from W. Yokoyama (Washington University in St. Louis, USA). For hematopoietic stem cell staining, bone marrow cells were stained with Abs against CD34 (RAM34), Flt3L (A2F10.1), CD127 (A7R34), CD16/32 (93), Sac1 (D7), and CD117 (2B8). Populations were defined according to previous publication8 as following CLP (CD117+Sac1+Flt3L+), CMP (CD117−Sac1−Flt3L−), MP (CD117−Sac1−Flt3L+), ST-HSC (CD117−Sac1−Flt3L−), CMP (CD117−Sac1−Flt3146,147), CMP (CD117−Sac1−Flt3148), CMP (CD117−Sac1−Flt3149), CMP (CD117−Sac1−Flt3150), CMP (CD117−Sac1−Flt3151), CMP (CD117−Sac1−Flt3152), CMP (CD117−Sac1−Flt3153), Biotinylated Abs against CD4 (GK1.5), CD8 (33), B220 (RA3-68), Gr-1 (RB6-8C5), CD11b (M1/70) and NKG2D-Fc and hPVR-Fc were produced by our in-house facility.

MCMV infection. The tissue culture grown mCMV MW97.01 and MCMV infection. The tissue culture grown mCMV MW97.01 and MCMV infection. The tissue culture grown mCMV MW97.01 and MCMV infection.
In vivo NKG2D blocking. Rag1<sup>−/−</sup>EYFP<sup>+/−</sup> Nkg2D<sup>−/−</sup>DTR mice were injected i.p. on two consecutive days with 0.8 mg diphtheria toxin. From the second day onwards, mice received once every 3 d 200 μg anti-NKG2D (BioXcell, clone HMG2D) or isotype control antibodies. After 15 d, spleens and bone marrow were analyzed.

**ImmunoBlot.** NK cells were enriched from splenocytes using MACS and then sorted to high (>99% purity) on a FACS Aria II (BD Biosciences). Cell extracts were generated using laemmli sample buffer (Syk, FcR1γ and Zap 70) or RIPA buffer (CD3ζ) in the presence of protease inhibitors (complete Ultra, Roche). Equal amounts of total lysate were analyzed by 12% SDS–PAGE. Proteins were transferred to Immobilon-P and incubated with blocking buffer (Tris buffered saline/Tween-20) containing 2% low-fat milk for 1 h before incubating with an antibody against Syk (Cell Signaling), FcR1γ (Merck Millipore), Zap-70 (Cell Signaling) and CD3ζ (Sigma), Akt (Cell signaling) or β-actin (Santa Cruz). Bands were visualized with ECL Prime Immuno Blotting Detection Reagent (GE Healthcare) using ImageQuant LAS 4000mini (GE Healthcare, Life Science).

**Quantitation and statistical analysis.** To analyze statistical significance, we used Student’s t-test, Mann–Whitney, Kruskal–Wallis and ANOVA, with Bonferroni’s post-test correction for multiple comparisons. To assess survival rates, the Kaplan–Meier model was used followed by log-rank (Mantel–Cox) test for pairwise group comparisons. Statistical significance is defined as: *P < 0.05; **P < 0.01; ***P < 0.001.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The data that support the findings of this study are available from the corresponding author upon request. The NCBI SRA accession codes are: SRX4548789, SRX4548788, SRX4548787, SRX4548786, SRX4548785, SRX4548784, SRX4548783, SRX4548782, SRX4548781.
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  State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection N.A.

Data analysis

Flow Cytometry data analysis was performed using FlowJo software (Tree Star, version 10)
For qualified cluster analysis we used String (www.String-db.org)
Clustering of differentially expressed genes was visualized using Cytoscape 3.5.0 software
Statistical analysis between groups was performed with GraphPad Prism 5 and 7
Differential expression analysis was done using EdgeR 3.14.2 and Limma 3.28.1
In data analysis MS Excel was also used

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The data that support the findings of this study are available from the corresponding author upon request. The accession codes are: SRX4548789, SRX4548788, SRX4548787, SRX4548786, SRX4548785, SRX4548784, SRX4548783, SRX4548782, SRX4548781

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size was determined by power analysis based on pilot experiments and previous findings (Zafirova et al., Immunity 2009.) |
|-------------|-------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data was excluded                                                                                                           |
| Replication | All data was successfully replicated at least two times. How many times each experiment was performed and which statistical analysis was used is indicated in the figure legends. |
| Randomization | Mice were age and sex matched. Mice were allocated to groups by an independent animal caretaker. |
| Blinding | In our experiments we used mostly genetically modified mice which were genotyped before start of each experiment to determine appropriate number of animals per group to ensure statistical power of results for each experiment. However, individual mice and from them derived samples were numbered regardless of genotype, which finally allowed blind analysis of samples though. The samples were matched with their genotype only after the analysis. |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| Involved in the study           | Involved in the study |
| - Unique biological materials   | - ChIP-seq |
| - Antibodies                    | - Flow cytometry |
| - Eukaryotic cell lines         | - MRI-based neuroimaging |
| - Palaeontology                 |         |
| - Animals and other organisms   |         |
| - Human research participants   |         |

Antibodies

| Antibodies used |
|-----------------|
| Flow Cytometry: |
| Name / Clone name / Catalog no. / (most used) Lot no. / dilution factor / manufacturer |
| CD3e/145-2C11 / 45003182 / 4304569 / 1:300 / eBioscience |
| NK1.1/PK136 / 45594182 / 4331619 / 1:400 / eBioscience |
| CD49b/DX5 / 13597183 / E03102-1631 / 1:100 / eBioscience |
| FNγ/XMG1.2 / 17731182 / 4332526 / 1:200 / eBioscience |
| TNFα/MP6-XT22 / 11732181 / 1927449 / 1:100 / eBioscience |
| IL-6/8C9 / 21670064 / LO144 / 1:100 / Immuno tools |
| GM-CSF/MP1-22E9 / 12-7331-41 / E031118 / 1:100 / eBioscience |
NKG2D (CD314)/CX5/25-5882-82/4275095/1:50/eBioscience
CD247 (CD3ζ)/6B10.2/12-2479-80/4291757/1:50/eBioscience
ZAP-70/IE7.2/11-6695-83/4308883/1:400/eBioscience
CD122 /TM-b1/17-1222-80/E00481633/1:100/eBioscience
CD11b/M170/ 25011281/4289817/1:400/eBioscience
c-kit (CD117)/ACK2/11-1171-82/E004741631/1:100/eBioscience
FcrRIy /1D6/M191-4/ 1:100/MBL
Ly49H (3D10) and Ly49D (4e4) were kind gift from W. Yokoyama (Washington University in St. Louis, USA)/1:100
CD34 /RAM34/14-0341-81/E02497-1631/1:100/eBioscience
Flt3L /A2F10.1/17-1351-80/E02731-1633/1:100/eBioscience
CD127 /A7R34/14-1271-82/E014711633/1:100/eBioscience
CD16/32 /93/14-0161-81/E06356-1631/1:100/eBioscience
Sca1/D7/17598181/E073531635/1:100/eBioscience
pSyk(Y348)/moch1ct/12901442/4325047
pPLCγ(Y786M9S/14008/1:400/Cell Signaling
Sla/rabbit polyclonal/PA5-22356/TA2506553/1:50/Invitrogen
Fusion proteins (NCR1-Fc, NKG2D-Fc and hPVR-Fc) were produced by our in-house facility and used 10µg/sample

Western blot:
Name / Clone name / Catalog no. / (most used) Lot no. / dilution factor / manufacturer
Syk/D3Z1E/3198/2/1:1000/Cell signaling
FcR1γ/rabbit polyclonal/06-727/2882662/1:500/ Merckmillipore
Zap70/99F2/2705/10/1:500/Cell signaling
CD3ζ/rabbit polyclonal/SA6535580/210468/1:500/Sigma
Akt /11E7/4685/6/1:80000/Cell signaling
β-actin/C4/MAB/2665057/1:80000/Santa Cruz

Validation
Validation of purchased antibodies was done by the suppliers. Data on the validation of in-house generated reagents (fusion proteins), has been provided in the supplementary figures.

Eukaryotic cell lines
Policy information about cell lines
Cell line source(s) The B16-F10 cell line was purchased from ATCC (CRL-6475)
Authentication Authentication was provided by ATCC
Mycoplasma contamination The cell line was confirmed to be negative for mycoplasma contamination by PCR.
Commonly misidentified lines (See iCLAC register) No commonly misidentified cell lines were used.

Animals and other organisms
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research
Laboratory animals Mice used in experiments were 6-12 weeks old. Both male and female mice were used but they were strictly age- and sex-matched within experiments.

Klrk1-/- Zafirova et al., 2009.
Klrk1flox/flox Lenartić et al., 2017.
Klrk1Δ/Δ Zafirova et al., 2011.
NCR1gfp/gfp Gazit et al., 2006.
IFNγ-/- Jackson Laboratories (2287)
C57BL/6 Jackson Laboratories (B6; strain 664)
Hcst-/- Laboratory of M. Colonna
Tyrobp-/- Laboratory of M. Colonna
Rag1cre/+ Laboratory of M. Busslinger
Rosa26−foxed STOP YFP Laboratory of Ari Wisman
NCR1cre Laboratory of V. Sexl
CD4cre Laboratory of D. Litman
CD3ζ− CNRS, Orleans (B6-Cd3ζ tm1Mal)
Deleter-cre (B6.C-Tg(CMV-cre)1Cgn/J) Jackson Laboratories (6054)
Balb/c Jackson Laboratories (strain 00651)
Sla-/- Laboratory of Jane McGlade4)

Wild animals Study did not involve wild animals

Field-collected samples Study did not involve samples collected from the field
Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Samples were prepared as described in the methods section. Briefly, animals were sacrificed according to European guidelines and spleens and bone marrow were removed. Single cell suspensions were generated by mashing organs through a 70μM sieve (spleen) or by smashing bones with a mortar, after which suspensions were run through a sieve. Erythrocytes were lysed using a hypotonic solution. Before staining with specific antibodies, cells were pretreated with Fc block (clone 2.4G2, produced in-house). To-pro3 (Life Technologies) or Fixable Viability Dye (eBioscience) was used to exclude dead cells.

Instrument

BD FACS ARIA and BD FACS VERSE

Software

FlowJo software (Tree Star, version 10)

Cell population abundance

After sorting we purity was determined to be 97-99%.

Gating strategy

Specific gating strategies are specified in the figure legends and/or methods section. Briefly, doublets were excluded using FSC-H vs. FSC-A gating, followed by SSC-H vs. SSC-A gating. Dead cells were excluded by viability dye. Based on FSC/SSC properties gating for lymphocytes was performed. Next, markers for specific cell populations were used to define populations. In most experiments, murine NK cells were investigated, which were defined as CD3-NK1.1+ or CD3-NKp46+, depending on the experiment and/or genotype of the mice under investigation. For cytokine production or for specific marker expression, positive gates were set based on isotype control, FMO, or on cells genetically deficient for the specified marker (e.g. NKG2D, NCR1, CD3zeta). For analysis of haematopoietic precursors in the bone marrow, a lineage channel was used, as well as specific markers to define precursor populations.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.