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Natural killer cell differentiation driven by Tyro3 receptor tyrosine kinases

Anouk Caraux[1,5], Qingxian Lu[2,5], Nadine Fernandez[3], Sylvain Riou[4], James P Di Santo[1], David H Raulet[3], Greg Lemke[2] & Claude Roth[4]

Although understanding of the function and specificity of many natural killer (NK) cell receptors is increasing, the molecular mechanisms regulating their expression during late development of NK cells remain unclear. Here we use representational difference analysis to identify molecules required for late NK cell differentiation. Axl protein tyrosine kinase, together with the structurally related receptors Tyro3 and Mer, were essential for NK cell functional maturation and normal expression of inhibitory and activating NK cell receptors. Also, all three receptors were expressed in maturing NK cells, the ligands of these receptors were produced by bone marrow stromal cells, and recombinant versions of these ligands drove NK cell differentiation *in vitro*. These results collectively suggest that Axl, Tyro3 and Mer transmit signals that are essential for the generation of a functional NK cell repertoire.

Natural killer (NK) cells are bone marrow–derived lymphocytes capable of recognizing and eliminating virus-infected and transformed cells. NK cells tend to attack target cells with reduced expression of major histocompatibility complex (MHC) class I molecules, a process mediated by the integration of signals from both inhibitory and activating cell surface receptors. In the mouse, there are two families of MHC class I–specific inhibitory receptors, Ly49 and CD94-NKG2, both of which are expressed on overlapping subsets of mature NK cells[1]. Although these receptors can discriminate between self and nonself by monitoring the expression of MHC class I molecules, studies have indicated the existence of MHC class I–independent inhibitory receptors that are also important inhibitors of NK cell activity[2–4]. Activating NK cell receptors in the mouse include NKRP1A and NKRP1C (NK1.1), DX5, CD2, 2B4, CD69, Ly49D, Ly49H, Lag3, NKp46 and NKG2D[5,6]. Despite increasing knowledge about the specificity and function of some of these receptors[7], the molecular events that govern their regulation during NK cell development remain unclear.

Understanding of NK cell lineage commitment has grown through analysis of *in vitro* culture systems and mutant mice with NK cell deficiencies[8]. *In vivo* studies have shown that fetal and neonatal mouse NK cells express CD94-NKG2A but lack most inhibitory Ly49 receptors[9–11], with the exception of Ly49E, which is expressed on fetal NK cells[12]. As mice mature, the proportion of NK cells expressing each Ly49 receptor increases gradually and sequentially, and adult expression profiles are reached several weeks after birth[10–13]. In addition to the Ly49 and CD94-NKG2 receptors, developing NK cells acquire the expression of several integrins: integrin α_v, DX5 and Mac-1 appear sequentially, and integrin α_v is ultimately downregulated during a late stage of the NK cell maturation process[14]. The acquisition of CD94-NKG2 and Ly49 receptors has also been examined using *in vitro* models of NK cell development. Whereas CD94-NKG2 receptors are expressed by NK cells derived from precursor cells cultured in interleukin 15 (IL-15)[13,15], the initiation of Ly49 expression requires additional signals, which are provided by bone marrow stromal cells. In the presence of those signals, Ly49 receptor expression is induced in an ordered and cumulative way, although the precise order varies as a function of the culture system and the detection method used[16–18]. Those results have collectively led to models in which acquisition of CD94-NKG2 and Ly49 receptors are early and relatively late events, respectively, in NK cell development. In general, expression of the full spectrum of receptors is correlated with the acquisition of killing activity.

Here we identify bone marrow stroma–derived signals that regulate the expression of most inhibitory and activating receptors on NK precursor cells. Axl, a member of the Tyro3 family of protein tyrosine kinase receptors, was differentially expressed by two bone marrow–derived mouse NK clones with differential functional capabilities. Moreover, Tyro3 receptors (Tyro3, Mer and Axl) and their ligands (Gas6 and protein S) were expressed by NK cells and bone marrow stromal cells, respectively. These protein tyrosine kinase receptors were critical in the formation of the NK cell receptor repertoire in the bone marrow and in the functional maturation of NK cells in the spleen. Finally, Gas6 and protein S promoted the growth and maturation of NK cell precursors *in vitro*.

[1]Laboratoire Cytokines et développement Lymphoïde, Département d'Immunologie, Institut Pasteur, 75724 Paris Cedex 15, France. [2]Molecular Neurobiology Laboratory, The Salk Institute, La Jolla, California 92037, USA. [3]Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, California 94720-3200, USA. [4]Laboratoire Immunité Cellulaire Antivirale, Département d'Immunologie, Institut Pasteur, 75724 Paris Cedex 15, France. [5]These authors contributed equally to this work. Correspondence should be addressed to C.R. (clroth@pasteur.fr).

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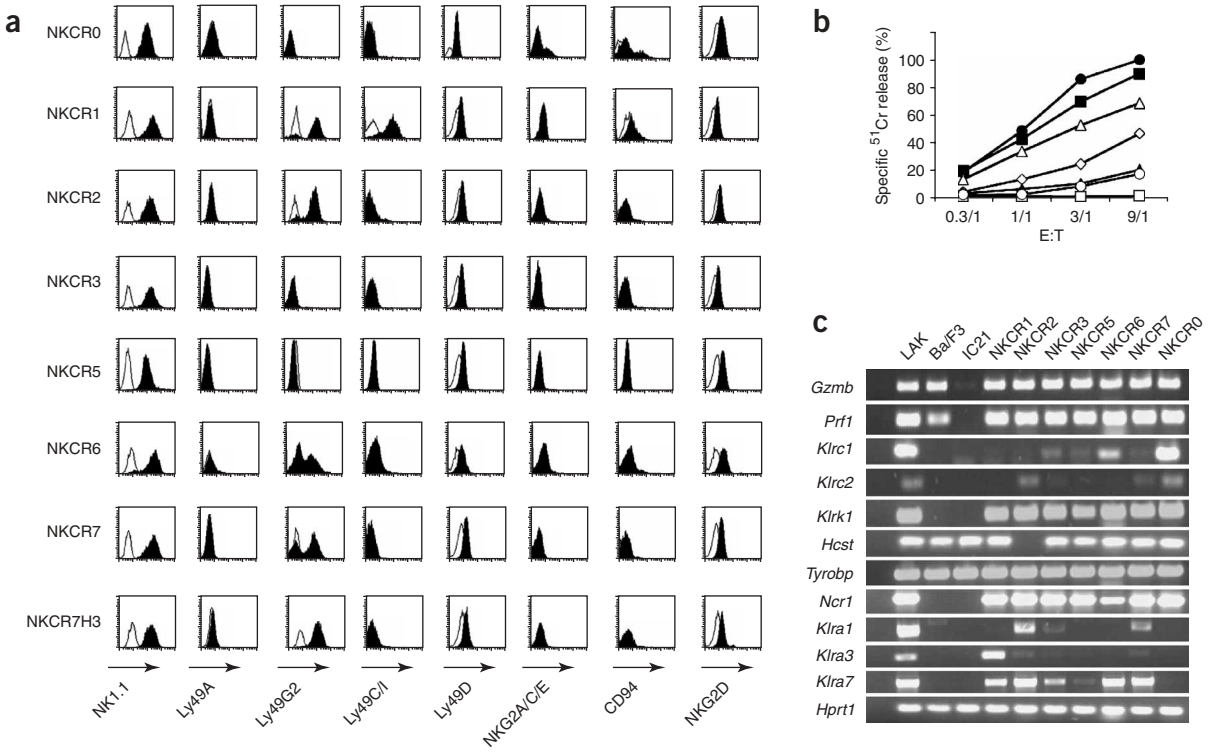

Figure 1 Phenotypic and functional characterization of NK clones. (**a**) Flow cytometry of NK clones of various maturational status. NKCR7H3 is a subclone of NKCR7. Data are from one experiment representative of three. (**b**) Killing of YAC-1 cells by IL-2-activated polyclonal NK cells (●), and NKCR5 (■), NKCR7 (△), NKCR1 (◇), NKCR3 (▲), NKCR2 (○) and NKCR0 (□) NK cell clones, assessed by 4-hour ^{51}Cr-release assay. E:T, effector/target. Data are means ± s.e.m. of triplicates from one experiment representative of three with similar results. (**c**) RT-PCR of transcript expression in NK cell clones. *Gzmb* encodes granzyme B; *Prf1*, perforin; *Klrc1*, NKG2A; *Klrc2*, NKG2C; *Klrk1*, NKG2D; *Hcst*, DAP10; *Tyrobp*, DAP12; *Ncr1*, NKp46; *Klra1*, Ly49A; *Klra3*, Ly49C/I; *Klra7*, Ly49G1/2/3; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase. Ba/F3, mouse pro–B cell line; IC21, macrophage cell line. Data are from one experiment representative of two.

RESULTS

Phenotype and function of NK clones

We used an *in vitro* stroma-dependent system[17] to derive stable NK cell lines and clones from early (c-Kit$^+$Sca2$^+$Lin$^-$) or late (NK1.1$^+$Ly49s$^-$) bone marrow precursor cells from mice deficient in the tumor suppressor p53. Flow cytometry showed that all of the NK clones were NK1.1$^+$CD3$^-$ and had similar expression of NK1.1 but different expression of NKG2 and Ly49 receptors (**Fig. 1a**). A fraction of the cells of clone NKCR0, derived from early precursor cells, expressed CD94-NKG2 heterodimers, whereas clones derived from late precursors lacked surface CD94-NKG2 heterodimers. In contrast, Ly49G2 and Ly49C/I were expressed on the surface of some of the NK clones derived from late precursors (NKCR1, NKCR2, NKCR6 and NKCR7) but were not expressed on the NKCR0 clone. NKCR0 cells showed no cytotoxicity against YAC-1 target cells, but NKCR5 and NKCR7 cells efficiently killed YAC-1 target cells (**Fig. 1b**). The clones NKCR1, NKCR3, NKCR2 and NKCR6 had low cytolytic activity (**Fig. 1b** and data not shown).

To determine whether differences in the expression of known NK cell receptors or their signaling adaptors accounted for the differences in cytotoxicity among these NK clones, we analyzed the expression of transcripts encoding activating receptors and their adaptors (**Fig. 1c**). All clones expressed transcripts encoding NKG2D and NKp46 receptors, and all expressed transcripts encoding DAP12 and DAP10 adaptors, with the exception of NKCR2, which expressed only DAP12. That is consistent with an association between NKG2D and KARAP-DAP12 in this clone, as has been demonstrated in activated NK cells[19,20]. Clone NKCR0 expressed NKG2A and NKG2C transcripts but lacked transcripts encoding Ly49 receptors. Conversely, each of the NK clones derived from late precursors expressed transcripts encoding one or more inhibitory Ly49 receptors, and in some cases, receptor protein was detected on the cell surface (**Fig. 1a,c**). Transcripts encoding perforin and granzyme B were detected in all of the NK clones. Notably, cytotoxicity was not correlated with NK cell receptor expression, suggesting that NK cell function may be regulated in part by unknown receptors or at the level of NK cell receptor signaling.

Expression of Tyro3 receptors and ligands

To identify putative receptors that might regulate NK cell cytotoxicity, we used representational difference analysis. We prepared cDNA libraries from clones NKCR5 and NKCR0, which differed considerably in cytolytic activity and in the type of precursor cell from which they were derived but had similar expression of most of the NK cell transcripts tested (**Fig. 1b,c**). 'Tester' cDNA pools derived from clone NKCR5 or NKCR0 were subtracted with an excess of 'driver' cDNA derived from clone NKCR5 or NKCR0. After one round of enrichment for cDNA fragments expressed in NKCR5 but not NKCR0 cells (**Fig. 2a**, lane 1) or in NKCR0 but not NKCR5 cells (**Fig. 2a**, lane 2), we subcloned the differential products and sequenced individual cDNA fragments. One of these, a differential product 'preferentially' expressed in NKCR5, contained an open reading frame encoding Axl[21–23].

Semiquantitative RT-PCR confirmed that there was much more expression of *Axl* in clone NKCR5 than in clone NKCR0 and demonstrated differences in *Axl* expression among the remaining clones

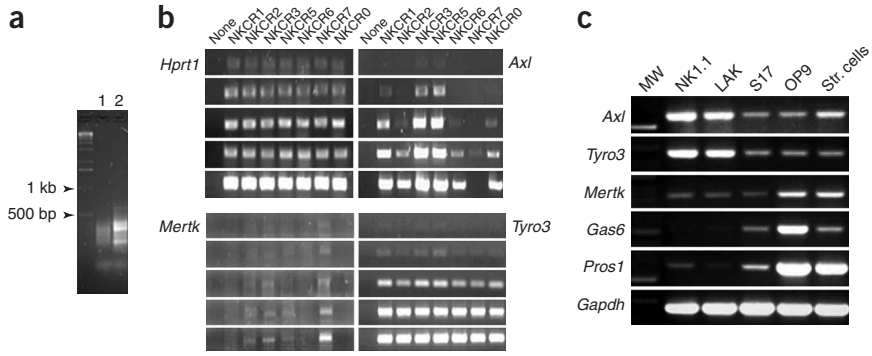

Figure 2 Expression of Tyro3 receptors on NK cells and Tyro3 ligands on bone marrow stromal cells. (**a**) Differential products correspond to cDNA fragments over-represented in the NKCR5 but not the NKCR0 clone (lane 1) and in the NKCR0 but not the NKCR5 clone (lane 2). Left lane, molecular size marker. (**b**) Semiquantitative RT-PCR of the expression of transcripts encoding Tyro3 receptors (right and left margins) in NK clones (above lanes). For comparison of the amount of receptor transcripts, PCR included varying numbers of amplification cycles, from low numbers (top gels) to high numbers (bottom gels), with equivalent amounts *Hprt1* transcript as an internal control. (**c**) RT-PCR of the expression of transcripts (left margin) encoding Tyro3 receptors and their ligands Gas6 and protein S (*Pros1*) in purified NK cells (NK1.1), IL-2-activated polyclonal NK cells (LAK) and bone marrow–derived stromal cells (Str. cells) or stromal cell lines (S17 and OP9). MW, molecular size marker. *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase. Data in **b**,**c** are from one experiment representative of three.

(**Fig. 2b**, top right). Because *Axl* was not expressed by the cytotoxic NKCR7 clone yet was expressed by the poorly cytotoxic NKCR3 and NKCR1 clones, we used semiquantitative RT-PCR to assess expression of the closely related receptors Mer (encoded by *Mertk*) and Tyro3 in each of the NK clones (**Fig. 2b**, bottom left and right). These analyses showed clonal differences in *Mertk* but not *Tyro3* expression and indicated that the clones with higher expression of *Axl* and/or *Mertk* transcripts (NKCR1, NKCR5 and NKCR7) were generally more cytotoxic than clones with very low expression of both receptors (NKCR2 and NKCR0). The exceptions were NKCR5 and NKCR3, which differed in their cytotoxicity but not in *Axl* and *Mertk* expression.

Tyro3 receptors are activated by two secreted ligands, Gas6 and protein S (ref. 24), and this receptor-ligand signaling system is essential for the homeostatic maintenance of diverse cell populations in the nervous, reproductive and immune systems[24–26]. The findings that Gas6 is expressed by bone marrow stromal cells, that this expression correlates with the ability of these cells to support hematopoiesis in culture[27] and that Gas6-Axl interactions induce activation of the TCF-Lef transcription factors[28–30] are all consistent with the possibility that Tyro3 receptors and their ligands might regulate NK cell development. We therefore analyzed expression of the three receptors and their ligands Gas6 and protein S in NK cells and in bone marrow stromal cells. RT-PCR analysis showed that both highly purified splenic NK cells and NK cells activated with IL-2 *in vitro* (LAK cells) had relatively high expression of *Axl* and *Tyro3* transcripts as well as detectable expression of *Mertk* transcripts (**Fig. 2c**). These results confirm published studies showing Mer expression on cells of the innate immune system[31]. Transcripts encoding protein S were present in small amounts in these cells, in agreement with their broad expression in various cell types[24–26], and Gas6 transcripts were undetectable in these cells (**Fig. 2c**). In contrast, bone marrow stromal cells and the stromal cell lines S17 and OP9 had relatively high expression of transcripts encoding protein S and lower expression of transcripts encoding Gas6.

Tyro3$^{-/-}$, *Axl*$^{-/-}$ and *Mertk*$^{-/-}$ NK cell function

To determine whether receptors of the Tyro3 family are actually required for NK cell function, we tested the cytotoxic activity of NK cells from mice lacking Tyro3 (*Tyro3*$^{-/-}$), Axl (*Axl*$^{-/-}$) or Mer (*Mertk*$^{-/-}$). We initially back-crossed each mutant mouse to the C57BL/6 (B6) strain for five generations before interbreeding; genotyping by locus-specific PCR[32] showed that all mice tested, including the wild-type control littermates, were homozygous for the B6 NK cell gene complex (data not shown). Compared with wild-type poly(I)·poly(C)-induced NK cells and LAK cells, *Axl*$^{-/-}$, *Axl*$^{-/-}$*Mertk*$^{-/-}$ and *Axl*$^{-/-}$*Tyro3*$^{-/-}$ NK cells showed a 90% reduction in killing activity (**Fig. 3a**). In contrast, the cytotoxic activity of *Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells was as low as that of *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells. Although these observations did not allow us to definitively conclude that Tyro3 and Mer receptors have nonredundant functions in NK cell differentiation, they suggest that all three receptors contribute to the development of cytotoxic potential, a finding consistent with the additive action of the three receptors reported before in other cell types[25,26].

We next addressed whether a lack of Tyro3 receptors affected other NK cell effector functions, such as interferon-γ (IFN-γ) production. Because interaction with either NKR-P1 or NKG2D activating receptors can induce IFN-γ production through the immunoreceptor tyrosine-based activation motif–containing Fc receptor-γ subunit[33] or DAP12 adaptor[19], respectively, we stimulated NK cells by directly crosslinking NKR-P1 or NKG2D with plate-bound specific antibodies. IFN-γ production was substantially lower in *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells (**Fig. 3b**). As NK cells have also been shown to produce IFN-γ in response to cytokines such as IL-12 and IL-18 (refs. 34,35), we tested the ability of combinations of cytokines to induce IFN-γ secretion from *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells. Compared with wild-type NK cells, *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells produced significantly less IFN-γ in response to cytokine stimulation (**Fig. 3c**). These data indicate that neither cytokines nor activating NK cell receptors are capable of inducing normal IFN-γ production in NK cells lacking Tyro3 receptors.

We next determined whether the functional defects of *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells might be associated with small amounts of transcripts encoding activating receptors or transcription factors known to control cytotoxicity and IFN-γ production in NK cells[36]. Purified *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells expressed normal quantities of transcripts encoding perforin, granzyme B, NKp46, FcRγ, DAP10 and DAP12 (**Fig. 3d**). Furthermore, expression of the transcription factors GATA-3, T-bet, Eomes and MEF, which are important in NK cell development, was similar in NK cells from *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice and wild-type littermates (**Fig. 3d**). These data suggest that deficiencies in these receptors, adaptors, cytotoxic molecules and transcription factors do not account for the defects noted in *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells.

Tyro3$^{-/-}$, *Axl*$^{-/-}$ and *Mer*$^{-/-}$ NK cell repertoire

Next we determined whether the impaired function of *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells was associated with an altered NK cell receptor repertoire. Published studies have shown an aberrant

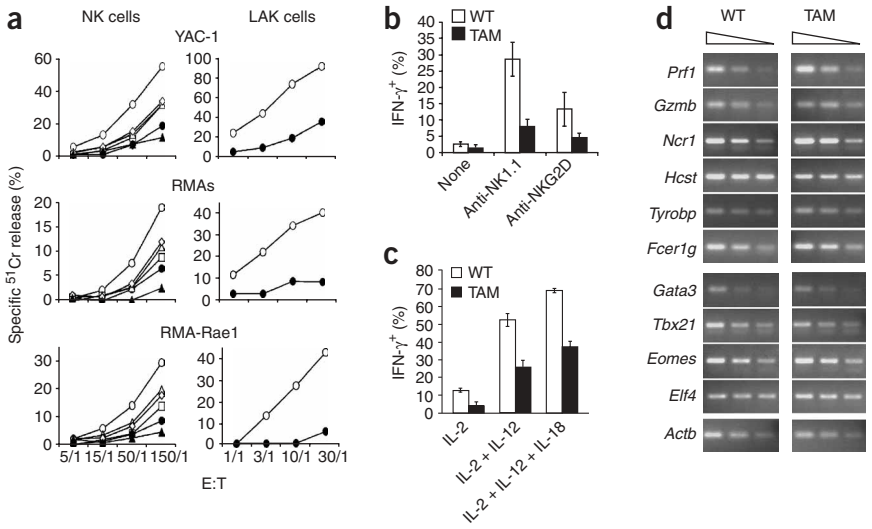

Figure 3 Impaired function of NK cells lacking Tyro3 receptors. (**a**) Killing of YAC-1, RMAs and RMA-Rae1γ target cells by wild-type (○), *Axl*$^{-/-}$ (□), *Axl*$^{-/-}$*Mertk*$^{-/-}$ (◇), *Tyro3*$^{-/-}$*Axl*$^{-/-}$ (△), *Tyro3*$^{-/-}$*Mertk*$^{-/-}$ (▲) and *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ (●) NK cells, assessed by 4-hour ^{51}Cr-release assay. Data are the mean ± s.e.m. of triplicates from one experiment representative of three with similar results. Intracellular staining to measure the proportion of NK1.1^{+}CD3^{-} wild-type (WT) or *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ (TAM) NK cells producing IFN-γ after 4 h of *in vitro* stimulation with anti-NK1.1 or anti-NKG2D (**b**) or after 18 h of *in vitro* stimulation with various cytokines (horizontal axis; **c**). Four wild-type mice and five *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ mice were analyzed individually. $P = 0.002$, anti-NK1.1; $P = 0.03$, anti-NKG2D; $P = 0.018$, IL-2; $P = 0.0011$, IL-2 plus IL-12; $P = 0.0012$, IL-2 plus IL-12 and IL-18 (two-tailed Student's *t*-test). (**d**) Semiquantitative RT-PCR analysis of expression of various transcripts (left margin) in splenic NK cells (CD3^{-}NK1.1^{+}) sorted from wild-type or *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ mice. Wedges indicate threefold dilutions of cDNA. *Actb* encodes β-actin. Data in **b**–**d** are from one experiment representative of three.

non–lymphocyte-autonomous proliferation and activation of T cells and B cells in *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice that is mainly due to the constitutive activation of macrophages and dendritic cells[26]. The number of NK cells in the spleens and bone marrow and NKT cells in the thymi of *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice was identical to that of wild-type littermates. However, flow cytometry showed a significant reduction (compared with that of wild-type littermates) in the percentage of *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK1.1^{+}CD3^{-} NK cells expressing most inhibitory and activating receptors (**Fig. 4a**,**b**). One third to one half as many bone marrow NK cells expressed the inhibitory Ly49A and Ly49G2 receptors, activating Ly49D and NKG2D receptors or DX5 (**Fig. 4a** and **Supplementary Fig. 1** online). Expression of Ly49C/I and Ly49F was more similar in wild-type and mutant mice. There was also a reduction in the percentage of NK cells expressing most inhibitory and activating receptors in the mature NK1.1^{+}DX5^{+} subpopulation (data not shown), suggesting that Tyro3 receptors regulate the transition from an immature NK1.1^{+}DX5^{-} precursor to a mature NK1.1^{+}DX5^{+} phenotype and also upregulate Ly49 receptors independently of DX5 expression. Despite their reduced expression of many NK cell receptors, *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells expressed wild-type amounts of CD94-NKG2 heterodimers (**Fig. 4a**,**b**). This finding is notable given that expression of NKG2A but not Ly49 receptors is induced after culture of bone marrow precursor cells in cytokines in the absence of stromal cells[15]. Overall, compared with wild-type NK cells, splenic *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells showed a smaller reduction in usage of Ly49D, NKG2D and DX5 receptors and no significant modification of the repertoire of inhibitory Ly49 receptors (**Fig. 4b**).

We also analyzed the NK cell repertoire in the bone marrow of *Axl*$^{-/-}$, *Axl*$^{-/-}$*Mertk*$^{-/-}$, *Axl*$^{-/-}$*Tyro3*$^{-/-}$and *Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice. We found no alteration in receptor expression in *Axl*$^{-/-}$ mice, and none of the 'double-mutant' mice showed a reduction in the expression of inhibitory Ly49 receptors (Ly49A, Ly49G or Ly49C/I). However, Ly49D expression was reduced in *Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice and DX5

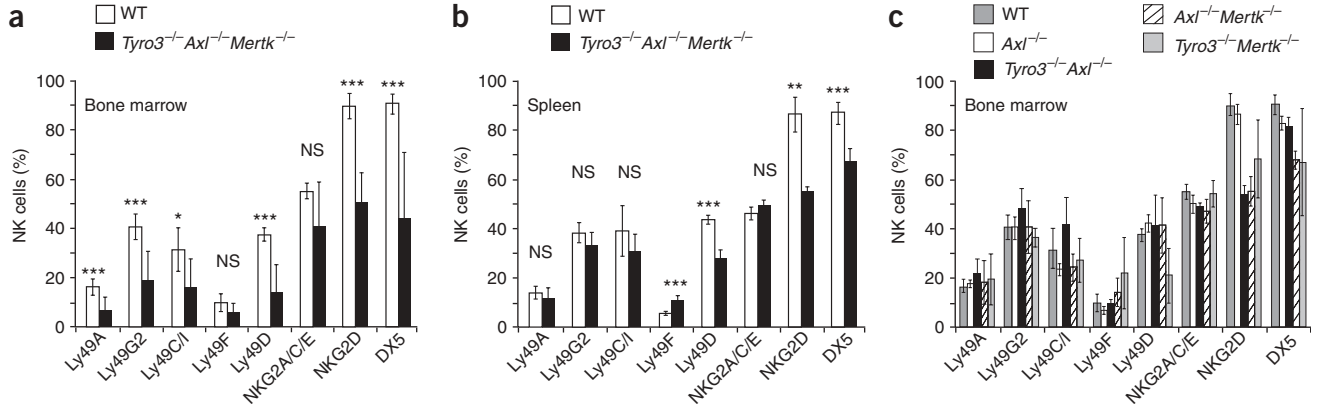

Figure 4 Tyro3 receptors in late differentiation of bone marrow and splenic NK cells. Percentage of NK1.1^{+}CD3^{-} cells from bone marrow (**a**,**c**) and spleens (**b**) of adult mice (genotypes, keys; $n = 3$–5 mice per group) expressing various NK cell receptors (horizontal axes). (**a**,**b**) Wild-type mice, $2.77 \times 10^5 \pm 0.64 \times 10^5$ and $10.4 \times 10^5 \pm 2.74 \times 10^5$ NK cells in the bone marrow and spleen, respectively; *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ mice, $1.35 \times 10^5 \pm 1.19 \times 10^5$ and $9.18 \times 10^5 \pm 6.9 \times 10^5$ NK cells in the bone marrow and spleen, respectively. Mean fluorescence intensity of NKG2D expression: 67 ± 11 and 44.3 ± 3, wild-type bone marrow and splenic NK cells, respectively; 27 ± 7 and 26.3 ± 1.5, *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ bone marrow and splenic NK cells, respectively. ***, $P < 0.01$; **, $P < 0.02$; *, $P < 0.05$; N.S., not significant (two-tailed Student's *t*-test). (**c**) *Axl*$^{-/-}$: $P = 0.039$, Ly49D; $P = 0.0082$, DX5. *Tyro3*$^{-/-}$*Axl*$^{-/-}$: $P = 0.0083$, NKG2A/C/E; $P = 0.0016$, NKG2D; $P = 0.023$, DX5. *Mertk*$^{-/-}$*Axl*$^{-/-}$: $P = 0.0021$, NKG2D; $P = 0.00082$, DX5 (all compared with wild-type mice). Data are representative of three experiments.

expression was reduced in *Axl*$^{-/-}$*Mertk*$^{-/-}$ and *Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice, whereas NKG2D expression was significantly reduced in *Axl*$^{-/-}$*Mertk*$^{-/-}$ and *Axl*$^{-/-}$*Mertk*$^{-/-}$ mice, albeit to a lesser extent than in *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells (**Fig. 4c**). These data are again consistent with the idea that Tyro3 receptors have overlapping functions in developing NK cells.

To identify the developmental stage during which the defect in *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells occurs, we analyzed the expression of CD122, CD11b, CD43 and integrin α_v, which are differentially regulated during NK cell maturation[14]. We noted a 50% reduction in the expression of CD11b and CD43 and a more modest decrease in CD122 expression in the bone marrow and spleens of *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice (**Fig. 5**). In contrast, we detected a substantial increase in the expression of integrin c_v, suggesting a requirement for Tyro3 receptors between stages II and III of NK cell development[14] (**Fig. 5**).

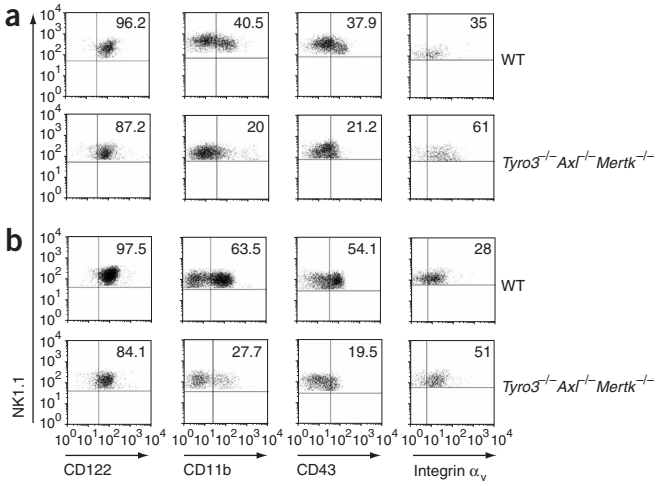

Figure 5 Impaired maturation of NK cells lacking all three Tyro3 receptors. Flow cytometry of marker expression (along margins) on the surface of NK1.1$^+$CD3$^-$ cells isolated from the bone marrow (**a**) and spleens (**b**) of wild-type and *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ littermates. Numbers in top right corners indicate percent NK1.1$^+$ cells positive for marker along bottom margin. Data are representative of at least five experiments.

Tyro3 receptors essential for NK cell differentiation

Given the broad expression pattern of Tyro3 receptors and their ligands, as well as the non–cell autonomous lymphoproliferative phenotype noted in *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice[26], it was possible that defects in other cell types might indirectly modulate NK cell maturation. To address that issue, we transferred sorted c-Kit$^+$Sca2$^+$Lin$^-$ bone marrow precursor cells from either wild-type or *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice into irradiated *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ or wild-type recipients. Flow cytometry of cells from the bone marrow and spleens of reconstituted mice showed a substantial reduction in the expression of Ly49G2, Ly49D, DX5 and CD11b and a substantial increase in integrin α_v expression on *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ donor NK cells compared with that of wild-type donor NK cells (**Fig. 6a,b**). However, *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ donor NK cells expressed wild-type amounts of NKG2D, suggesting that the expression of this receptor on developing NK cells may depend on other cell types. Conversely, NK cells from wild-type donors differentiated equally well after transfer into either wild-type or *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice. These results collectively indicate that the defect in the *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cell repertoire, at least for most NK cell receptors, is cell autonomous. *In vitro* stimulation of donor NK cells with IL-2 and IL-12 confirmed that only precursors expressing Tyro3 receptors can give rise to mature and functional NK cells (**Fig. 6c**).

Tyro3 receptors drive NK differentiation *in vitro*

Our results suggested that interactions between Tyro3 receptors and their ligands may constitute most or all of the stromal cell–derived signal that drives late NK cell differentiation. To determine definitively whether Tyro3 ligands expressed by stromal cells stimulate NK cell differentiation, we did a set of cell culture experiments in a controlled setting. Specifically, we evaluated the ability of 3T3 cells transfected with cDNA expression vectors encoding Gas6 and protein S (called '3T3-Gas6' and '3T3–protein S', respectively) to support NK cell growth and differentiation. In limiting-dilution conditions, sorted wild-type c-Kit$^+$Sca2$^+$Lin$^-$NK1.1$^-$ bone marrow precursor cells yielded robust numbers of NK clones with similar frequencies when grown on OP9 stromal cells, 3T3-Gas6 cells or 3T3–protein S cells, whereas we detected only a few NK colonies after culture with untransfected 3T3 cells (**Fig. 7a**). In contrast, we detected no NK

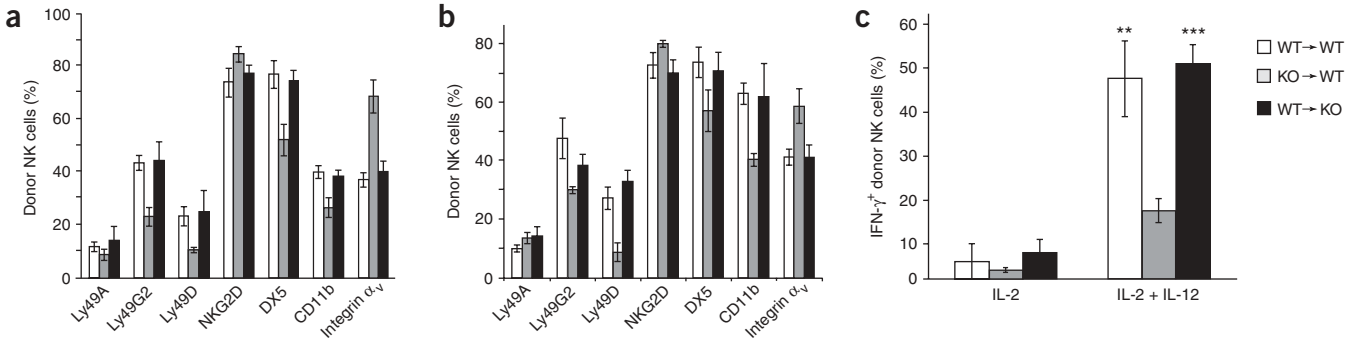

Figure 6 Cell-autonomous defect of NK cells lacking Tyro3 receptors. Flow cytometry of wild-type (WT) or *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ (KO) donor-derived NK cells in the bone marrow (**a**) and spleens (**b**) of wild-type or *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ recipient mice (n = 3–5 mice per group). *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ donor NK cells are CD45.2$^+$, wild-type donor NK cells are CD45.1$^+$ and recipient mice are CD45.2$^+$. (**a**) $P < 0.02$, WT→WT versus KO→WT. $P < 0.05$, Ly49G2, DX5, CD11b and integrin α_v, and P = not significant, Ly49D (KO→WT versus WT→KO). (**b**) $P < 0.05$, WT→WT versus KO→WT. $P < 0.02$, Ly49D and integrin α_v, and P = not significant, Ly49G2, DX5 and CD11b (KO→WT versus WT→KO). (**c**) Functional characterization of donor NK cells. Intracellular staining to measure the percentage of donor-derived NK1.1$^+$CD3$^-$ cells producing IFN-γ after *in vitro* stimulation with various cytokines (horizontal axis); n = 3–4 mice per group. ***, $P < 0.01$, and **, $P < 0.02$. Data are from one experiment.

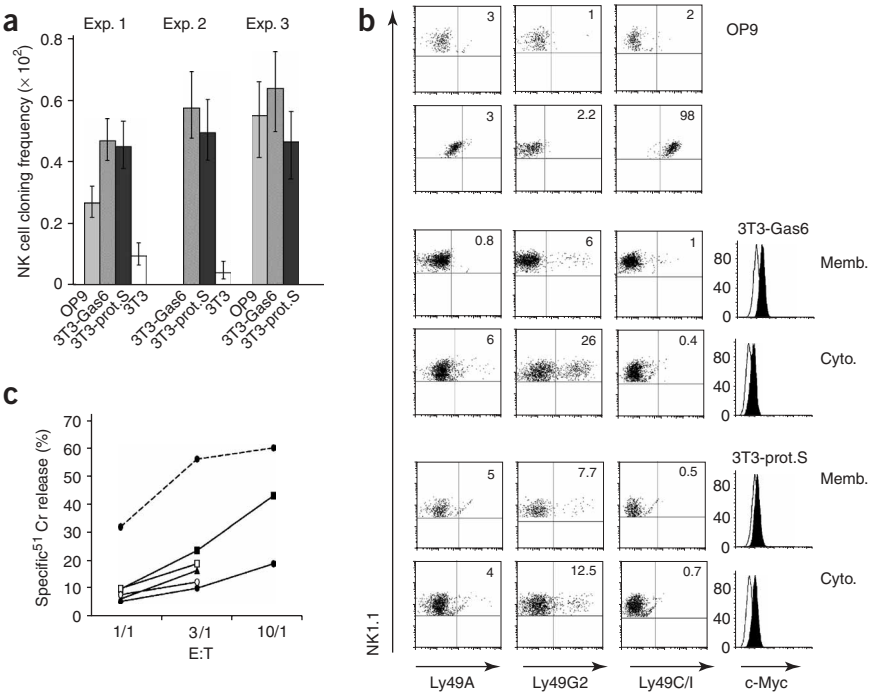

Figure 7 Interaction of Tyro3 receptors with their ligands induces NK cell differentiation *in vitro*. (**a**) Cloning frequencies of sorted c-Kit$^+$Sca2$^+$Lin$^-$ wild-type bone marrow precursors on feeder cells (horizontal axis). 3T3-prot.S, 3T3–protein S. (**b**) Ly49 receptor expression on NK clones derived *in vitro* from c-Kit$^+$Sca2$^+$Lin$^-$ precursor cells. Dot plots gated on NK1.1$^+$CD3$^-$ cells show representative clones derived at 64 and 32 cells/well on OP9 stromal cells (top two rows), at 16 cells/well on 3T3-Gas6 feeder cells (middle two rows), or at 32 and 16 cells/well on 3T3–protein S feeder cells (bottom two rows). Numbers in top right corners indicate percent NK1.1$^+$ cells positive for receptor along bottom margin. **Supplementary Table 1** online presents data from three independent experiments. Histograms (far right), surface and intracellular staining of 3T3 cells (open) and 3T3-Gas6 or 3T3–protein S cells (filled) with biotinylated anti-c-Myc. (**c**) Functional activity of five representative NK clones derived *in vitro* from c-Kit$^+$Sca2$^+$Lin$^-$NK1.1$^-$ bone marrow precursor cells. Killing of YAC-1 target cells by individual NK colonies (solid lines) derived at 32 cells/well on 3T3-Gas6 cells (filled symbols) or 3T3–protein S cells (open symbols), assessed by 4-hour ^{51}Cr-release assay. LAK cells serve as a positive control (dashed line). The number of NK cells in LAK cell sample was determined by flow cytometry and was adjusted to ensure that equal numbers of NK cells were used in each sample. Data are representative of three experiments.

colonies after incubation of sorted *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ c-Kit$^+$Sca2$^+$Lin$^-$NK1.1$^-$ bone marrow precursor cells on either OP9, 3T3-Gas6 or 3T3–protein S cells. These results demonstrate that an interaction between Tyro3 receptors expressed on NK precursor cells and their ligands expressed by bone marrow stromal cells is sufficient to induce NK cell growth *in vitro* (**Fig. 7a** and **Supplementary Table 1** online). A fraction of clones growing on OP9, 3T3-Gas6 and 3T3–protein S cells contained cells expressing Ly49A, Ly49G2 or Ly49C/I (**Fig. 7b**), and some representative NK colonies showed cytotoxic activity against YAC-1 target cells (**Fig. 7c**), demonstrating that Gas6 and protein S can also induce the functional maturation of NK cells *in vitro*.

DISCUSSION

In this study we have shown that functional maturation and expression of inhibitory and activating NK cell receptors requires stimulation of Tyro3 receptors on committed NK cell precursors. *In vivo*, this stimulation almost certainly occurs in the bone marrow, where stromal cells have high expression of both Gas6 and protein S. Gas6-induced activation of Axl has been shown to activate β-catenin and TCF-1–Lef, which regulates Ly49A receptor expression[28,29]. However, whereas TCF-1 positively regulates the expression of Ly49A and Ly49D and negatively regulates the expression of Ly49G and Ly49I[37], Tyro3 receptors are necessary both for the acquisition of nearly all inhibitory and activating receptors on NK precursor cells in the bone marrow and for the functional maturation of NK cell precursors in the spleen. In addition to reduced expression of Ly49 receptors, *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ bone marrow NK cells showed reduced expression of Mac-1 and DX5 and a substantial increase expression of integrin α_v but normal expression of NKG2-CD94 heterodimers. Given the phenotypic characteristics of NK cell developmental intermediates[14], these results indicate that Tyro3 receptors are required between stages II and III of NK cell development. During this transition, immature NK cells already express NKG2-CD94 heterodimers but do not yet express Ly49 receptors and do not yet regulate the expression of α_v, DX5 and Mac-1 integrins. The apparently smaller reduction in expression of inhibitory and activating receptors on *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ splenic NK cells suggests several hypotheses that should be tested. It is possible that the few Ly49$^+$ NK cells that arise in the bone marrow of *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice selectively accumulate or proliferate in the spleen over time and independently of Tyro3 receptors. Another possibility is that the splenic microenvironment provides alternative differentiation signals that induce Ly49 expression. Although those hypotheses are not mutually exclusive, the fact that expression of NKG2-CD94 heterodimers and Ly49 receptors precedes the main proliferative stage of NK cell development[14] supports the first model. Kinetic analyses of Ly49 repertoire expression in the bone marrow and spleens of young *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ mice should discriminate between those two different possibilities.

Although 50% of *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Mertk*$^{-/-}$ NK cells expressed the NKG2D activating receptor, these cells were nonetheless mostly incapable of lysing target cells expressing NKG2D ligands. Furthermore, although *Axl*$^{-/-}$ NK cells expressed a wild-type NK cell receptor repertoire, they were nonetheless unable to kill target cells. Those data indicate that the impaired function of NK cells lacking Tyro3 receptors is not simply the result of a reduction in NKG2D or DX5 expression and suggest that unknown activating receptors or signaling molecules, whose expression and function are also dependent on Tyro3 receptor signaling, might modulate NK cell function. Besides those unknown molecules, interaction of the Tyro3 receptor with its ligand protein S recruits the p85 subunit of phosphatidylinositol-3-OH kinase, resulting in phosphorylation of the kinase Akt[38]. As activation of phosphatidylinositol-3-OH kinase is important in the induction of NK cell killing[39–41], it may also be involved in cytokine production by NK cells.

Our *in vivo* reconstitution experiments demonstrated an NK cell–autonomous function for Tyro3 receptors and identified a function for Tyro3 receptor-ligand interactions during late NK cell differentiation. Our results have shown that both Gas6 and protein S ligands

support clonal growth and differentiation of early c-Kit$^+$Sca2$^+$Lin$^-$ NK1.1$^-$ NK precursor cells. Although other signals provided by the bone marrow microenvironment[42] or by other cell types[43–47] may participate in the NK cell maturation process *in vivo*, the clonal growth and differentiation of NK cells *in vitro* after interaction with either Gas6 or protein S challenges the idea of direct involvement of these ligands in NK cell development.

In summary, we have demonstrated that the three receptors of the Tyro3 family are essential in the terminal differentiation and functional maturation of NK cells. In the absence of those receptors, the repertoire of inhibitory and activating NK cell receptors was altered considerably, NK cells showed substantially diminished cytolytic activity against target cells, and NK cells were incapable of initiating cytokine production in response to immune stimuli. We have also shown that Tyro3 receptors are expressed by NK cells and NK cell precursor cells. Gas6 and protein S Tyro3 receptor ligands were expressed on bone marrow stromal cells, on which NK differentiation depends, and recombinant versions of these ligands promoted the growth and maturation of isolated NK precursor cells *in vitro*. Further investigation of the 'downstream' signals and cell surface receptors induced after activation of the Tyro3 receptors will be essential for full understanding of the molecular mechanisms through which NK cells reach their full potential as immune effectors.

METHODS

Preparation of NK cell clones. Using an *in vitro* stroma-dependent system for the clonal growth and differentiation of bone marrow precursor cells[17], we derived stable NK cell lines and clones from p53-deficient mice. Purified early (c-Kit$^+$Sca2$^+$Lin$^-$) or late (NK1.1$^+$ Ly49s$^-$) bone marrow cells were sorted as described[17] and were seeded at a density of 1 cell/well onto irradiated stromal cells in 96-well flat-bottomed plates (Falcon 3077) in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 2 mM glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES and antibiotics. The following cytokines were added at the initiation of culture: recombinant mouse c-Kit ligand (100 U/ml; a gift from F. Melchers, Basel Institute for Immunology, Basel, Switzerland), recombinant mouse IL-7 (100 U/ml; a gift from A. Cumano, Institut Pasteur, Paris, France), recombinant mouse Flt3 ligand (100 U/ml; a gift from DNAX, Palo Alto, California) and recombinant human IL-15 (100 ng/ml; a gift from Immunex, Seattle, Washington). Cultures were incubated at 37 °C in humidified air with 7% CO_2. Most clones were tested at days 7–11 by flow cytometry for expression of CD19, CD3, NK1.1, and Ly49A, Ly49G2, Ly49C/I, Ly49D, NKG2A/C/E, CD94 and NKG2D. Selected NK cell lines were cloned at a density of 1 cell/well on irradiated OP9 stromal cells in complete medium containing recombinant mouse IL-2 (100 U/ml; a gift from A. Cumano, Institut Pasteur, Paris, France). Single-cell suspensions were stained and then analyzed on a FASCalibur (Beckton Dickinson). Typically, 1×10^6 cells were incubated with fluorescence-labeled or biotinylated antibodies and then were washed three times with PBS containing 0.1% BSA.

Antibodies. Phycoerythrin-, fluorescein isothiocyanate– and allophycocyanin-conjugated antibodies specific for CD3, CD19, CD11b, CD43, NK1.1, Ly49A, Ly49C/I and Ly49G2, as well as biotinylated antibodies specific for CD122 and integrin α_v, were purchased from BD Pharmingen. Biotinylated antibody to c-Myc (anti-c-Myc) was purchased from Covance Research Products. Anti-Ly49F (HBF-719), anti-Ly49D (SED-85)[48], anti-CD94 (18d3), anti-NKG2A/C/E (20d5)[49] and anti-NKG2D (MI-6)[50] were produced by D.H.R. Antibodies were conjugated to fluorescein isothiocyanate (Boehringer Mannheim) or biotin (Pierce).

RT-PCR. Highly enriched NK cells were prepared from the spleens of ten B6 mice after depletion with biotinylated antibodies specific for CD5, CD19 and Gr-1 and anti-biotin magnetic beads and further purification of DX5$^+$CD3$^-$ cells on a Moflo cell sorter (Dako Cytomation). After enrichment, NK cells were 98.9% pure. NK cells activated with IL-2 *in vitro* were prepared after 5 d of culture with recombinant mouse IL-2 (100 U/ml). Bone marrow stromal cells were prepared as described[17]. Total RNA was extracted with TRIzol solution (Invitrogen). Reverse-transcription reagents from Invitrogen were used for reverse transcription. Hotstar Taq polymerase (Qiagen) was used for PCR. Primer sequences used to detect transcripts for NK cell receptors, adaptors and effector molecules in NK clones are in **Supplementary Table 2** online. Primers used to detect Tyro3 receptor transcripts in NK cell clones were as follows: *Axl* sense (5′-ATCGGAGGAAGAAGGAGACTCG-3′) and antisense (5′-CTCCA TACCATGGCAATGTCG 3′); *Tyro3* sense (5′-CATTCCAGAGCAGCAGTTC AC-3′) and antisense (5′-CCACACACACTGTCATGTCCT-3′); and *Mertk* sense (5′-ACCTCCACACCTTCCTGTTA-3′) and antisense (5′-CGTGGAGAAGGTA GTCGTACATCT-3′). The amount of cDNA was adjusted according to PCR with primers for hypoxanthine guanine phosphoribosyl transferase at 23, 25, 27, 30 and 33 cycles. *Axl* was detected at 27, 30, 33, 35 and 38 cycles, *Tyro3* was detected at 23, 25, 30, 35 and 40 cycles and *Mertk* was detected at 28, 31, 34, 37 and 40 cycles. Primer sequences used to detect transcripts for NK cell receptors, adaptors, effector molecules, transcription factors, Tyro3 receptors and ligands in NK cells and bone marrow stromal cells are in **Supplementary Table 3** online. PCR cycling conditions were one cycle of 95 °C for 15 min, 35 cycles of 94 °C for 30 s and 56 °C for 30 s, one cycle at 72 °C for 1 min and one extension step at 72 °C for 10 min.

Representational difference analysis. The 'cDNA subtraction' was done by representational difference analysis[51] with poly(A)$^+$ RNA isolated from NKCR5 and NKCR0 cells, which were cultured in the presence of recombinant mouse IL-2. For isolation of transcripts expressed in NKCR5 but not NKCR0 cells, NKCR5 cells were used as the 'tester' population that was subtracted with an excess of 'driver' cDNA prepared from NKCR0 cells. The reverse procedure was done to identify transcripts 'preferentially' expressed in NKCR0 cells. After one round of representational difference analysis subtraction, the differential products (DPs), corresponding either to cDNA expressed in NKCR5 but not in NKCR0 (DP_A) or to cDNA expressed in NKCR0 but not in NKCR5 (DP_B) were subcloned into pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen). The inserts were checked for differential expression against DP_A and DP_B probes. For cDNA expressed in NKCR5, clones hybridizing only with DP_A were identified by sequencing. One clone contained an open reading frame of 320 base pairs corresponding to Axl receptor tyrosine kinase cDNA.

Transfection of 3T3 cells. Full-length cDNA sequences encoding Gas6 and protein S were cloned into pcDNA6 (Invitrogen) and transfection of 3T3 cells was done using calcium phosphate precipitation[52]. Clones of blasticidin-resistant cells were isolated in limiting-dilution conditions and were stained with anti-c-Myc. Stable transfectants with high expression of c-Myc were selected as feeder cells for *in vitro* growth of NK clones.

Limiting-dilution culture of bone marrow precursor cells. Bone marrow cells were isolated from 8- to 10-week-old B6 mice by irrigation of femurs and tibias, followed by purification over stepwise Percoll (Parmacia) gradients as described[17]. Precursor cells from *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ mice or wild-type littermates were stained with the following combination of antibodies: fluorescein isothiocyanate–anti-Sca2, phycoerythrin–anti-c-Kit and biotin-conjugated antibodies specific for 'lineage' markers (NK1.1, CD3ε, CD19, Gr-1, CD11b and Ter119). Cells were then washed twice, were stained with CyChrome-streptavidin and were sorted with a Moflo cell sorter. OP9 cells and parental or transfected 3T3 cells were irradiated with 25 Gy and were seeded at a density of 5×10^4 cells/well in 96-well flat-bottomed plates. Sorted NK bone marrow precursor cells were seeded at various densities, onto either irradiated stromal cells or fibroblasts with or without expression of Gas6 and protein S, in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 50 μM 2-mercaptoethanol, recombinant mouse c-Kit ligand (100 U/ml), recombinant mouse IL-7 (100 U/ml), recombinant mouse Flt3 ligand (100 U/ml) and recombinant human IL-2 (100 U/ml). After 15–17 d of culture, growing colonies were tested individually for expression of NK1.1, Ly49A, Ly49G2 and Ly49C/I. The frequency of NK colonies was estimated by fitting of a generalized linear model and finding the maximum likelihood with the Newton-Raphson method. The significance of the reported differences between populations was calculated with L-Calc software (StemSoft Software).

^{51}Cr-release assay. NK cell effectors were isolated 24 h after injection of poly(I)·poly(C) or were activated *in vitro* for 5 d with recombinant mouse IL-2

(100 U/ml). To ensure that comparable numbers of effector cells were included in each sample, input was normalized to the percentage of $NK1.1^+CD3^-$ cells (determined by flow cytometry) in each sample.

Statistical analysis. For statistical analysis of the data, mean comparison and distributions were analyzed with an unpaired Student's *t*-test and the two-tailed chi-squared (χ^2) test, respectively (Excel software; Microsoft). *P* values greater than 0.05 were considered nonsignificant.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

A.C. did the representational difference analysis; Q.L. collaborated in the analysis of wild-type and knockout mice; N.F., S.R. and J.P.D. participated in critical revision of the paper; D.H.R. participated in study design and contributed to writing of the manuscript; G.L. maintained the allelic series of *Tyro3, Axl* and *Mertk* mouse mutants and contributed to writing of the manuscript; and C.R. designed and conceptualized the research, analyzed data and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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