A requirement for CD45 distinguishes Ly49D-mediated cytokine and chemokine production from killing in primary natural killer cells

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Engagement of receptors on the surface of natural killer (NK) cells initiates a biochemical cascade ultimately triggering cytokine production and cytotoxicity, although the interrelationship between these two outcomes is currently unclear. In this study we investigate the role of the cell surface phosphatase CD45 in NK cell development and intracellular signaling from activating receptors. Stimulation via the major histocompatibility complex I–binding receptor, Ly49D on CD45−/− primary NK cells resulted in the activation of phosphoinositide-3-kinase and normal cytotoxicity but failed to elicit a range of cytokines and chemokines. This blockage is associated with impaired phosphorylation of Syk, Vav1, JNK, and p38, which mimics data obtained using inhibitors of the src-family kinases (SFK). These data, supported by analogous findings after CD16 and NKG2D stimulation of CD45−/− primary NK cells, place CD45 upstream of SFK in NK cells after stimulation via immunoreceptor tyrosine-based activation motif-containing receptors. Thus we identify CD45 as a pivotal enzyme in eliciting a precise subset of NK cell responses.

The activation of NK cells forms part of the innate immune response to parasites, tumors, and virally infected cells and is important for their clearance (1). These responses are achieved via intracellular signaling cascades emanating from numerous activating receptors. For example, recognition of H-2d (2) or murine cytomegalovirus (MCMV) encoded m157 (3) by Ly49D and Ly49H respectively, results in cell-mediated cytotoxicity and large scale production of IFN-γ and, in response to MCMV infection, chemokines such as MIP-1α, MIP-1β, RANTES, and ATAC (4–7). Antibody bound to soluble or membrane antigens can bind CD16 on NK cells leading to antibody-dependent cell cytotoxicity (ADCC) and secretion of IFN-γ and GM-CSF (8, 9). These activating receptors do not contain intrinsic kinase activity, but instead associate with transmembrane adaptor molecules containing immunoreceptor tyrosine-based activation motifs (ITAMs). NK cells use three such ITAM adaptors; DAP12 homodimers required for Ly49D and Ly49H signaling (10) and either homo- or heterodimers of FceRIγ and CD3ζ for CD16 (11–13).

Whereas Ly49D, Ly49H, and CD16 only associate with ITAM-containing adaptors, NKG2D can signal through DAP12 (14–16) in mouse (17) and DAP10 in both mouse and human NK cells, which contains a YxxM binding motif for the p85 subunit of phosphoinositide-3-kinase (PI3K; 18). NKG2D is expressed on all NK cells and is capable of binding stress-induced ligands such as retinoid acid early inducible transcript (Rae-1; 19). The association of NKG2D with at least two distinct adaptor proteins suggests it may activate distinct signal pathways that may result in distinct cellular responses such cytokine/chemokine production versus cytotoxicity. Indeed, analysis of mice lacking key proximal signaling proteins indicates such a bifurcation in signaling may occur. Depending on their activation status, both Syk−/−/Zap70−/− and DAP12−/− NK cells are capable of killing target cells via NKG2D (20), yet have an abrogated IFN-γ response to NKG2D stimulation (14). Furthermore, DAP10 activation results in the phosphorylation of PLC-γ2 and Vav1 in NK...
cell lines whereas DAP12 recruitment leads to Syk, Zap70, SLP76, and LAT phosphorylation (21). Thus recruitment of either DAP12 or DAP10 appears to activate independent biochemical pathways leading to distinct cellular responses, although this has yet to be confirmed in primary cells.

The proximal signaling events leading to ITAM phosphorylation in NK cells are thought to involve the activation of src-family kinase (SFK) members, which in NK cells include Lck, Fyn, Lyn, Fgr, and Yes (22), however only limited evidence supports this role. Indeed, NK cells lacking Lck (23) and Fyn (23, 24) appear grossly normal, although a role for Fyn in the lysis of cells lacking MHC-I and NKG2D ligands has been reported (25). The transmembrane protein tyrosine phosphatase CD45 is expressed on all hematopoietic cells including NK cells and appears to function by dephosphorylating the COOH-terminal tyrosine of SFKs, leading to a conformation that favors autophosphorylation of the catalytic tyrosine (26–28). Indeed lymphocytes lacking CD45 show significant hyporesponsiveness to antigen receptor cross-linking attributed to a failure to appropriately activate SFK (29).

Early studies into the role of CD45 in NK cell function used various CD45-deficient NK cell lines or antibodies (30, 31). Early studies in the role of CD45 in NK cell function used various CD45-deficient NK cell lines or antibodies (30, 31). However, the generation CD45−/− mice demonstrated that in contrast to cell lines, primary CD45−/− NK cells are capable of normal natural killing (32). To date, studies in primary CD45−/− NK cells have not addressed the function of CD45 in the biochemical signal cascade emanating from activating NK cell receptors. Given the role of CD45 in regulating SFK activity and the proposed importance of SFK in NK cell receptor signaling, we investigated the regulation of primary NK cell signaling pathways in the absence of CD45. We find CD45 to be required for the full activation of Syk, Vav1, and calcium release, and in phosphorylation of JNK and p38 after ITAM activation. The failure to activate these signaling intermediates in CD45−/− NK cells is associated with dramatically impaired cytokine and chemokine production. In contrast, CD45 is dispensable for ITAM-mediated PI3K activity and DAP10-mediated cytotoxicity, establishing a potential dichotomy in signals required for primary NK cell cytotoxicity versus cytokine production.

RESULTS

CD45 is required for normal NK cell development

Mice with a targeted disruption of exon-6 in the CD45 gene have been previously shown to have enhanced numbers of splenic NK cells (32), however, it is unclear how this arises. We investigated the development and phenotype of NK cells from mice with a targeted disruption of CD45 exon-12 (33). These mice (hereafter referred to as CD45−/−) had a four- and fivefold increase in the percentage of NK1.1+CD49b+TCR-β− NK cells in the spleen and liver, respectively (Fig. 1 A). This increased percentage equates to significantly enhanced numbers of NK1.1+CD49b+TCR-β− cells in CD45−/− mice with 19 ± 2 × 106 and 1.3 ± 0.2 × 106 NK cells in the spleen and liver, respectively, compared with 2.4 ± 0.3 × 106 and 2 ± 0.4 × 105 (P < 0.05; n = 8) in control mice. This enhanced NK cellularity is unlikely to result from increased production in BM as the percentage of NK precursors (CD122+NK1.1−DX5−CD3−CD19−) (34) was unchanged (Fig. 1 A) and no difference was observed in bromodeoxyuridine (BrdU) uptake by BM NK1.1+CD49b+ cells (Fig. 1 B). We did observe however, greater turnover of NK cells in the spleen (P = 0.008), but normal turnover in the liver (Fig. 1 B). These findings indicate that the increased number of NK cells in the spleen of CD45−/− mice results from enhanced peripheral expansion. Given that enhanced

![Figure 1. The absence of CD45 alters NK cell development.](image-url)

(A) Spleen and liver from CD45−/− mice were analyzed for NK1.1+CD49b+ NK cells by flow cytometry. NK cell precursors from bone marrow were identified as CD122+NK1.1−DX5−CD3−CD19−. Data are representative of 12 mice or 6 mice for precursor analysis with percentages displayed next to each population. (B) Mice were given drinking water containing BrdU for 2 wk. Cells were stained with the same antibodies as in A with the addition anti-BrdU and analyzed by flow cytometry. Percentages of divided cells (BrdU+) are shown above each population, with CD45−/− spleen-derived NK cells having a greater percentage of dividing cells (P = 0.008). Data are representative of three independent experiments. (C) Hematopoietic chimeras were generated by injecting irradiated Ly5.1 mice with equal amounts of control (Ly5.2) and CD45−/− bone marrow. The resulting NK1.1+DX5+ population was analyzed for expression of Ly5.2 by flow cytometry to deduce donor origin. (D) The level of Ly49D, CD244, and CD16 protein expression on the surface of freshly isolated and IL-15-expanded NK1.1+CD49b+ cells was analyzed by flow cytometry. Data shown for C and D are representative of six independent experiments.

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**Figure 1. The absence of CD45 alters NK cell development.** (A) Spleen and liver from CD45−/− mice were analyzed for NK1.1+CD49b+ NK cells by flow cytometry. NK cell precursors from bone marrow were identified as CD122+NK1.1−DX5−CD3−CD19−. Data are representative of 12 mice or 6 mice for precursor analysis with percentages displayed next to each population. (B) Mice were given drinking water containing BrdU for 2 wk. Cells were stained with the same antibodies as in A with the addition anti-BrdU and analyzed by flow cytometry. Percentages of divided cells (BrdU+) are shown above each population, with CD45−/− spleen-derived NK cells having a greater percentage of dividing cells (P = 0.008). Data are representative of three independent experiments. (C) Hematopoietic chimeras were generated by injecting irradiated Ly5.1 mice with equal amounts of control (Ly5.2) and CD45−/− bone marrow. The resulting NK1.1+DX5+ population was analyzed for expression of Ly5.2 by flow cytometry to deduce donor origin. (D) The level of Ly49D, CD244, and CD16 protein expression on the surface of freshly isolated and IL-15-expanded NK1.1+CD49b+ cells was analyzed by flow cytometry. Data shown for C and D are representative of six independent experiments.
NK cell turnover in CD45<sup>−/−</sup> mice appears to occur only in the spleen and may reflect a consequence of the CD45-deficient environment, we prepared mixed hematopoietic chimeras. The NK cells in the spleen of the chimeras were mostly of CD45<sup>−/−</sup> origin (80% Ly5<sup>+</sup>) indicating the increased in vivo production is intrinsic to CD45<sup>−/−</sup> NK cells (Fig. 1 C), consistent with a previous report (32). We next examined whether CD45 was required for normal maturation by examining cells for the activation of activating and inhibitory receptors. We observed a significantly reduced proportion of NK1.1<sup>+</sup>DX5<sup>+</sup> cells expressing the activating receptor Ly49D (P = 0.043) in both the spleen and liver of CD45<sup>−/−</sup> mice (Table I; and unpublished data). The acquisition of other Ly49 receptors and CD94-NKG2A/C/E and NKG2D, thought to occur before the expansion stage in NK development (35), was not significantly affected by the absence of CD45. All receptors expressed on CD45<sup>−/−</sup> NK cells were at equivalent levels to controls except for a significant down-regulation of the activating receptor CD244 (P < 0.001), a phenotype that is stable in mixed chimeras (unpublished data). Interestingly, in vitro expansion of CD45<sup>−/−</sup> NK cells using IL-15 returned CD244 expression and the percentage of Ly49D<sup>+</sup> NK cells to levels equivalent to controls (Fig. 1 D and unpublished data).

**Normal growth of CD45<sup>−/−</sup> NK cells in response to IL15 and IL21**

CD45 and the SFK have been implicated as positive and negative regulators of cytokine responses in multiple cell types (27), prompting us to investigate the cytokine responsiveness of CD45<sup>−/−</sup> NK cells. We observed CD45<sup>−/−</sup> NK cells to proliferate normally across a range of IL-15 concentrations and different time points in tissue culture (Fig. 2, A and B). We recently showed that IL-21 functionally differentiates NK cells both in vivo and in vitro (36). IL-21 was able to slow IL-15–mediated division in both control and CD45<sup>−/−</sup> NK cells (Fig. 2 B) and further differentiate these cells as characterized by a down-regulation of NK1.1 (Fig. 2 C) and the acquisition of a larger, more granular phenotype and reduced viability (unpublished data). Taken together, these data show CD45 not to be required for IL-15 and IL-21 responsiveness, and that the cells derived from these cultures are indistinguishable by phenotype from controls.

**CD45 is essential for cytokine production after ITAM-containing receptor activation**

An important cellular response triggered by activating receptors on NK cells is the production of cytokines. To determine if CD45 is required for cytokine production, IL-15–

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**Table I. Phenotype of CD45<sup>−/−</sup> NK cells**

| Marker | CD45<sup>+/+</sup> % positive | CD45<sup>−/−</sup> % positive |
|--------|-----------------------------|-----------------------------|
| CD122  | 90.4 ± 0.9                  | 88.4 ± 1.4                  |
| Ly49A  | 13.0 ± 0.5                  | 11.6 ± 0.8                  |
| Ly49C/J| 42.2 ± 6.5                  | 37.2 ± 1.2                  |
| Ly49D  | 45.0 ± 3.0                  | 32.4 ± 4.3<sup>a</sup>      |
| Ly49G2 | 39.4 ± 1.1                  | 38.4 ± 2.7                  |
| Ly49H  | 47.6 ± 2.8                  | 44.6 ± 3.3                  |
| Ly49F  | 98.4 ± 0.8                  | 10.0 ± 1.3                  |
| CD49b  | 100 ± 0.0                   | 100 ± 0.0                   |
| FcγRII | 98.0 ± 1.3                  | 99.2 ± 0.6                  |
| CD43   | 83.4 ± 7.9                  | 93.7 ± 2.0                  |
| MAC-1  | 93.7 ± 1.5                  | 94.0 ± 6.7                  |
| CD94   | 43.2 ± 1.5                  | 57.4 ± 6.7                  |
| NKG2A/C/E | 41.8 ± 0.9             | 49.2 ± 3.4                  |
| NKG2D  | 100 ± 0.0                   | 100 ± 0.0                   |
| 2B4    | 96.4 ± 1.1                  | 61.4 ± 2.8<sup>a</sup>      |
| NK1.1  | 100 ± 0.0                   | 100 ± 0.0                   |

NK1.1<sup>+</sup>DX5<sup>+</sup> splenocytes from CD45<sup>+/+</sup> and CD45<sup>−/−</sup> mice were analyzed for the expression of surface markers. Values indicate the mean ± SEM percentages of NK cells deemed positive by flow cytometry from six mice per group.

<sup>a</sup>P < 0.045 by Student’s t test.
expanded NK cells were cultured overnight in the presence of plate-bound mAbs. We found CD45 to be critical for the production of IFN-γ after ligation of the ITAM-containing receptors Ly49D, NKG2D, and CD16 (P < 0.039) but not NK1.1 or the IL-12 and IL-18 receptors (Fig. 3 A). Interestingly, inhibitors of SFK (PP2 and SU6656) mimicked these results (P < 0.04; Fig. 3 B and unpublished data) suggesting a role for CD45 in the activation of these kinases in NK cells, whereas inhibitors of the PI3K pathway (Wortmannin and Ly294002) had little effect on IFN-γ production (unpublished data). Stimulation of mouse NK cells by coculture with CHO cells is predominantly through Ly49D recognition (37), and this also failed to elicit IFN-γ from CD45−/− NK cells at ratios of 1:1 and 1:10 for 24 h (Fig. 3 C). CD16 stimulation of control NK cells induced GM-CSF, which was significantly impaired in the absence of CD45 (P = 0.0004; Fig. 3 D) or after treatment of control cells with PP2 (P = 0.0003; Fig. 3 E). We also measured these parameters using freshly isolated NK1.1+CD49b+ TCR-β− cells and found that while cytokine production was reduced across all stimuli compared with in vitro expanded cells, CD45−/− NK cells still secreted vastly less cytokine compared with control cells (P < 0.04).

CD45 is essential for chemokine production via ITAM-containing receptors

NK cells are a potent source of inflammatory chemokines such as RANTES, MIP-1α, and MIP-1β (6, 38). These are critical for recruiting effector cells to the site of antigen challenge thus aiding in the clearance of pathogens. Ly49D liga-
The role of CD45 in this process appears to be the activation of the SFK because inhibition of SFK activity by PP2 induced the same phenotype, blocking the production of MIP-1α (P < 0.0005), MIP-1β (P = 0.0025), and RANTES (P < 0.01; Fig. 4 C). Taken together, these findings identify CD45 and the SFK as critical signaling molecules in the production of chemokines from NK cells.

**Natural cytotoxicity is independent of CD45**

CD45-exon 6–deficient mice were previously shown to have normal cytotoxicity against YAC-1 cells (32), which are recognized via NKG2D. To address whether CD45−/− NK cells could also kill non-NKG2D target cells, we tested their ability to lyse a range of targets. As expected, CD45−/− NK cells efficiently lysed NKG2D ligand+ target cells including YAC-1 and RMA-S Raeb1. Interestingly, control and CD45−/− NK cells were equally efficient at killing CHO cells, a process predominantly mediated through Ly49D (37; Fig. 5, A and B). These data suggest that unlike cytokine production, cytotoxicity triggered via Ly49D and NKG2D is CD45-independent. The MHC class I–deficient cell line RMA–S was killed more efficiently than MHC class I expressing RMA cells by NK cells from both knockout and control mice, indicating that NK inhibitory receptor signaling is intact in the absence of CD45. IL-21 enhanced control and CD45−/− NK cell killing against all targets to a similar degree, supporting the independence of IL-21 signaling and CD45 (Fig. 2).

As cytokine production after ITAM–containing receptor ligation was dependent on CD45, we investigated whether these same receptors could participate in redirected cytolysis of FcR+ P815 cells, a process known to be Syk/Zap70 dependent (20). IL-15–activated control and CD45−/− NK cells displayed equal basal killing and both responded to ITAM–dependent Ly49D and CD16 engagement with increased killing, most notably at an E/T ratio of 27:1 (Fig. 5 D) indicating that rejection of H-2d allografts by BALB/c BM (H-2d) was transferred into irradiated control or CD45−/− B6 (H-2b) mice. Antibody depletion studies have previously shown that H-2d rejection is dependent on Ly49D+ NK cells (39). We observed that both C57BL/6 and CD45−/− mice possessed significantly fewer splenic colonies when H-2d BM grafts were transferred compared with isogenic H-2b BM grafts (P = 0.01; Fig. 5 D) indicating that rejection of H-2d BM by Ly49D+ NK cells occurs independently of CD45. Similarly CHO killing is only slightly impaired when NK cells are treated with PP2 (Fig. 5 E), whereas PI3K inhibitors have a much greater effect on lysis supporting the notion that Ly49D–mediated lysis is independent of CD45 and SFKs. Taken together, these findings demonstrate that both

**Figure 4. **Ly49D–mediated chemokine production requires CD45. (A) IL-15–expanded CD45−/− and control NK1.1+CD49b+TCR-β− cells were cultured for 24 h in the presence of plate-bound anti-Ly49D. Culture supernatants were assayed for MIP-1α (P = 0.000027) and MIP-1β (P = 0.000014) by ELISA. Data from five independent experiments are shown. (B) NK cells were treated with a titration of PP2 and cultured for 24 h in the presence of plate-bound anti-Ly49D. Supernatants were assayed for MIP-1α (P = 0.000044) and MIP-1β (P = 0.0025) by ELISA. Statistics are for 10 μM PP2 treatment. Data shown are representative of three independent experiments. (C) IL-15–expanded CD45−/−, control and PP2–treated NK1.1+CD49b+TCR-β− cells were cultured for 24 h in the presence of plate-bound anti-Ly49D. Supernatants were assayed for RANTES (P = 0.000013; **, P < 0.01) by ELISA. Data are the average of four independent experiments.
in vivo and in vitro natural cytotoxicity of a variety of targets by NK cells occurs independently of CD45. The increased number of NK cells in CD45/−/− mice may alter the E/T ratio in vivo, masking potential differences in killing efficiency. However, since CD45-deficient mice have substantially greater spleen mass (unpublished data), target cell numbers may also be elevated, making accurate in vivo E/T calculations difficult.

**Optimal activation of Syk after ITAM-containing receptor triggering requires CD45**

As some NK cell functions were impaired after Ly49D and CD16 activation in the absence of CD45, we investigated signal propagation in these cells. The total tyrosine phosphorylation (Fig. 6 A) differed considerably between CD45+/− and control IL-15–expanded NK cells. We note the virtual absence of phosphoproteins of around 95 and 105 kD in size and a dramatic reduction of a phosphorylated band at 50-kD protein in the absence of CD45 (Fig. 6 A). Furthermore, in contrast to controls, Ly49D stimulation failed to increase phosphorylation of 97−, 68−, and 58-kD proteins in CD45+/− NK cells. As protein tyrosine phosphorylation was not enhanced in CD45+/− NK cells we assessed the activation status of Syk, it being the dominant Ly49D/DAP12-induced protein tyrosine kinase (PTK) in NK cells (21, 40). Consistent with earlier studies (40), Ly49D stimulation of control NK cells induced a large increase in phosphorylated Syk after 10 min (Fig. 6 B). However, very little phosphorylated Syk was detected in lysates from equivalently treated CD45+/− NK cells (Fig. 6 B). CD16 stimulation similarly failed to activate...
Syk in CD45\(^{-/-}\) NK cells (unpublished data) suggesting a general role for CD45 in activating Syk after ITAM activation in NK cells. Zap70 is also expressed in NK cells however its role in Ly49D signaling is largely unexplored. We found that unlike Syk, phosphorylation of Zap70 was not enhanced by Ly49D ligation in both control and CD45\(^{-/-}\) NK cells (Fig. 6 C). This was not due to inefficient stimulation, as tyrosine phosphorylation of a number of other proteins was induced when whole cell lysates were examined. Ly49D stimulation is known to activate PLC\(\gamma\) resulting in an increase of intracellular calcium (40). We observed such calcium flux in control NK cells after Ly49D stimulation but saw no change in the intracellular calcium level in identically treated CD45\(^{-/-}\) NK cells (Fig. 6 D). CD45\(^{-/-}\) NK cells were capable of releasing calcium after ionomycin treatment, indicating that the defect in CD45\(^{-/-}\) NK cells was specific for the Ly49D pathway (Fig. 6 E). These finding show CD45 is essential for full Syk phosphorylation and calcium flux after activation of ITAM-containing receptors.

The absence of CD45 impairs MAPK activation in primary NK cells

CD16 stimulation of human NK cells results in SFK- and Syk-dependent p38 phosphorylation and enhances cytotox-
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icity and IFN-γ mRNA accumulation, processes that are sensitive to inhibition of MAPK p38 (41, 42). Furthermore, c-Jun NH2-terminal kinase (JNK) is phosphorylated after NK cell interaction with immune complexes (42) and is also strongly implicated in coupling surface receptor induced signals to IFN-γ production in T cells (43, 44). To investigate whether the impaired ITAM-mediated IFN-γ production in CD45−/− NK cells was associated with reduced activity of the MAPKs, we assessed the phosphorylation state of p38 and JNK after Ly49D ligation. Indeed, CD45−/− NK cells showed reduced phosphorylation of p38 after a 2- and 10-min stimulation with impaired JNK phosphorylation observed at 10 min, confirming that Syk is important in coupling surface receptor signals to the activation of JNK and p38 (Fig. 7 A). Interestingly, despite inducing IFN-γ and augmenting lysis of P815 cells, NK1.1 stimulation did not activate p38, raising the possibility that p38 is specifically downstream of ITAM-containing receptors in primary NK cells. Analysis of MAPK-Erk1/2 phosphorylation was also performed and whereas total Erk1/2 was readily observed in primary NK cells, we were unable to detect phosphorylation of these proteins after Ly49D stimulation (unpublished data).

Ly49D activates PI3K but not Vav1 in CD45−/− primary NK cells

The ability of Ly49D on CD45−/− NK cells to recognize and lyse CHO cells implies that Ly49D/DAP12 stimulation activates signaling proteins required for cytolyis. Pharmacological studies have highlighted the importance of the PI3K pathway in natural killing (14, 20, 45) and that this pathway can be activated in an ITAM-independent manner (10, 18). To assess whether the PI3K pathway is activated after Ly49D/DAP12 stimulation, we measured the level of phospho-Akt in lysates of Ly49D-stimulated NK cells. We observed a striking increase in phospho-Akt after 10 min of Ly49D stimulation in both control and CD45−/− NK cells (Fig. 7 B). This finding suggests that, like the NKG2D pathway, Ly49D may also activate the PI3K pathway and that Akt is less reliant than MAPK-Erk1/2 phosphorylation after Ly49D stimulation. The guanine nucleotide exchange factor Vav1 is also required for the optimal activity of signaling molecules downstream of Ly49D, with normal Akt phosphorylation being observed in the presence of impaired Syk, Vav1, and p38 and JNK phosphorylation in CD45-deficient NK cells.

DISCUSSION

Differential requirement of CD45 in NK cell effector functions

This study has identified CD45 as pivotal in signal transduction from a prototypic ITAM-containing receptor on NK cells, determining distinct functional outcomes from similar ligand interactions. We show for the first time in NK cells that modulation of a cell surface molecule can determine the nature of the response. CD45−/− NK cells fail to produce IFN-γ, GM-CSF, MIP-1α/β, and RANTES when ITAM-containing surface receptors are stimulated, a process that normally results in robust production of these soluble factors. Another cellular consequence from engagement of Ly49D on NK cells is the release of lytic granules capable of killing engaged cells. In contrast to cytokine production, NK cells devoid of CD45 efficiently killed cells expressing ligands for Ly49D. The ability of SFK inhibitors to block cytokine/chemokine production from CD16-, NKG2D-, or Ly49D-stimulated NK cells but only minimally effect cytotoxicity mimics the CD45-null phenotype and strongly suggests that CD45 lies upstream of the SFK and that its activity is differentially required for Ly49D responses. Furthermore, the impaired Ly49D- and CD16-mediated phosphorylation of Syk in CD45−/− NK cells suggests that CD45-mediated SFK activity is important for the recruitment of Syk to phosphorylated ITAMs and/or the phosphorylation of recruited Syk. An important question from our findings is how does engagement of the same receptor lead to a bifurcation of signaling pathways that differ in the requirement for CD45?

Signaling requirements for cytokine production

The failure of CD45−/− NK cells to secrete IFN-γ after CD16, NKG2D, or Ly49D stimulation is consistent with the phenotype of Syk−/−/Zap70−/−/− mice (14, 20) and with the pharmacological inhibitors of Syk being able to impair Ly49D-mediated IFN-γ production (38). Impaired Syk activity in Ly49D-stimulated CD45−/− NK cells coincides with hypophosphorylation of Vav1 and JNK and a failure to release intracellular calcium. The involvement of Vav1 in this process, however, is unclear as CD16-mediated IFN-γ production has previously been shown to occur independently of Vav1 (46). In contrast, calcium and JNK are likely to be involved in this process. Augmented IFN-γ production by T cells after CD158j ligation strongly correlated with increased phospho-JNK (44), while exogenous HIV-1 Tat, which blocks calcium influx can inhibit the production of IFN-γ after interactions with dendritic cells (48). These findings define a signal transduction pathway showing absolute dependence on CD45-SFK-Syk activity for cytokine production from ITAM-containing receptors (Fig. 8 A), raising the question of how cytotoxicity is differentially regulated.
Same receptor, different pathway—signaling for cytotoxicity

Signal transduction pathways leading to cytotoxicity have been defined in NK cells to some extent. It is clear for example that killing through the Ly49D receptor requires activation of Syk/Zap70 as mice deficient in both fail to lyse CHO cells (20). Similarly, DAP12-deficient NK cells fail to induce cytotoxicity after Ly49H-mediated recognition of m157-expressing cells (3) and analysis of NK cell lines has shown DAP12 to activate the PI3K pathway in a Syk-dependent manner (49). Although there is some evidence that Zap70 can compensate for an absent Syk (50), the presence of a nonfunctional Syk is dominant, suggesting that Zap70 normally has little role in the induction of cytotoxicity (21, 40). We noted an equal increase in phospho-Akt, a direct product of PI3K activation, in Ly49D-stimulated CD45<sup>−/−</sup> H11002 NK cell lysates compared with control lysates. This finding identifies for the first time that Ly49D activates the PI3K pathway in primary NK cells and that this signaling pathway can function independently of CD45 and in the absence of normal levels of Syk activity (Fig. 8 A). Interestingly, Ly49D-mediated IFN-γ production has been shown to be sensitive to pharmacological inhibitors of PI3K, Syk, and SFK (38) suggesting that multiple pathways contribute to IFN-γ production after Ly49D stimulation. Even though our findings support roles for Syk and SFK activity in IFN-γ production, we failed to find any evidence for PI3K involvement in this process. The basis of this difference is currently unclear but may reflect differences in the derivation of the NK cells or the assay conditions.

Whereas it is apparent that cytotoxicity after NKG2D ligation requires PI3K recruitment to the YxxM motif of DAP10 (21) and activation, processes which occur independently of Syk and Zap70 (21) and calcium release (15), DAP10 is not recruited to Ly49D after its ligation (10, 18, 51) and is therefore an unlikely mediator of the resultant cytotoxicity. It is, however, interesting to note that both NKG2D/DAP10 (18) and Ly49D/DAP12 activate PI3K, placing this lipid kinase centrally to both cytotoxic signaling pathways.

Recently, DAP12-mediated cytotoxicity in contrast to NKG2D/DAP10-mediated killing was shown to be largely Vav independent (47). Our data support the notion that Vav1 is not required for DAP12-mediated CHO lysis, and suggest that Syk lies upstream of Vav1. We have not addressed the phosphorylation state of Vav1 after NKG2D ligation, although recent findings (47) would predict it to be normal since CD45<sup>−/−</sup> NK cells efficiently kill YAC-1 and RMA-S Rae1<sup>−/−</sup> cells.

It is puzzling to note that although both DAP12 and DAP10 appear to require tyrosine phosphorylation for the recruitment and activation of kinases (21, 40, 52), only DAP12-mediated pathways appear affected in CD45<sup>−/−</sup> NK cells with DAP10-mediated killing intact, a phenotype also seen in the Syk/Zap70<sup>−/−</sup> mice. Furthermore, the intact NKG2D-mediated killing by primary CD45<sup>−/−</sup> and Syk/Zap70<sup>−/−</sup> NK cells contrasts with their failure to secrete IFN-γ via NKG2D stimulation and suggests that the requirement of SFKs and Syk in DAP10 phosphorylation is less stringent than that of DAP12. The finding that PP2 treatment only mildly affects YAC-1 killing (20) supports this. Furthermore, it appears that cytotoxicity is only completely blocked if PI3K, SFK, and Syk are inactivated in

Figure 8. A model of CD45-dependent and -independent NK cell signaling. (A) Engagement of the ITAM-recruiting receptor, Ly49D results in various responses that are differentially regulated by CD45. Cytokine signaling and cytotoxicity are functional in CD45<sup>−/−</sup> NK cells. In contrast cytokine and chemokine production are absolutely dependent on CD45; most likely through its activation of SFKs and full activation of Syk, ultimately leading to calcium release and the activation of MAPK family members. (B) The level of Ly49D-mediated Syk/Zap70 activity controls NK cell responses with full activity (WT) required for cytokine production and cytotoxicity via this receptor. Reduced activity (CD45<sup>−/−</sup>) correlates with blocked cytokine production, whereas no activity (Syk/Zap70<sup>−/−</sup>) also blocks Ly49D-mediated killing.
combination (20), highlighting the importance of natural killing and thus the numerous mechanisms leading to its triggering. Collectively this evidence suggests a signaling cascade after Ly49D ligation that involves PI3K but is distinct from the DAP10-mediated pathway, which can function independently of Syk/Zap70 (20).

The level of Syk activity differentially activates distinct signaling pathways

Our observation that CD45−/− NK cells differentially activate cytotoxicity and cytokine production after stimulation through prototypical ITAM-containing receptors adds significantly to the understanding of these phenomenon by associating this dichotomy with discrete changes in the activity of signaling molecules. These results suggest two possible models for the induction of effector functions after ITAM receptor stimulation. In one there is a bifurcation of signaling with one pathway running through PI3K to cytotoxicity and the second through Vav1/Ca2+/JNK to cytokine production. The alternative proposal is that cytotoxicity and cytokine production are triggered at different signaling thresholds.

Ly49D can stimulate the phosphorylation of Akt, a critical substrate in cytotoxicity (16, 20, 45), with this pathway showing an absolute requirement for Syk/Zap70 (20). Reduced Ly49D-mediated phospho-Syk and basal phospho-Zap70 in CD45−/− NK cells does not affect the efficiency of CHO killing, supporting the notion that the small amount of Syk activity in the context of cell–cell interactions that is largely CD45 dependent, but have yet to examine the activation of Syk in the context of cell–cell interactions.

CD45 in NK cell development and cytokine signaling

CD45 is essential for B and T cell antigen receptor signaling, development and maturation (26). However this is not the case for NK cells. Peripheral expansion of CD45−/− NK cells was enhanced in vivo, a phenomenon specific to the spleen, cell intrinsic and independent of IL-15 responsiveness. Furthermore, CD45−/− NK cells express all the surface receptors characteristic of full maturation (35). The reduced percentage of Ly49D+ NK cells in vivo was particularly interesting since this occurs in Syk−/−/Zap70−/− mice (20) and suggests that CD45 activation of SFK and Syk may be involved in positive signaling through Ly49D influencing the survival or selection of Ly49D+ cells in vivo. CD45 has also been identified as a Jak phosphatase implicated in the negative regulation of cytokine signaling (54). The normal response of CD45−/− NK cells to IL-15, −12, −18, and −21 in vitro excludes cytokine hyperresponsiveness as an contributing factor in the enhanced NK cellularity in CD45−/− mice.

Our findings suggest a model of the biochemical pathways activated in NK cells after interactions between ligands such as H-2d, m157, Rae1β, and Ig-Fc fragments and ITAM-containing receptors such as Ly49D, Ly49H, NKG2D, and CD16 (Fig. 8 A). Upon ligand binding, receptors are drawn together recruiting ITAM-containing adaptor molecules such as DAP12. CD45 then activates the SFKs allowing phosphorylation of the ITAM and the recruitment of PTKs (Syk/Zap-70) via their SH2 domain binding to phosphorylated ITAM, where they may be phosphorylated by the SFKs. Syk can now activate Vav1 and signaling pathways required for the production of cytokines/chemokines including the release of calcium and the activation of the MAPKs, JNK, and p38. In contrast, PI3K activation after ITAM phosphorylation requires only a small enhancement of Syk/Zap70 activity and is responsible for NK cell cytotoxicity. This model postulates segregated signaling pathways dictated by their dependence on CD45–SFK–mediated activation of Syk with the production of cytokines/chemokines absolutely dependent on CD45. In contrast cytotoxicity is functional in the absence of CD45. This finding has important implication for NK cell effector functions during development and differentiation, as CD45 expression on NK cells appears to change depending on maturation and activation state of the cell (32). Furthermore, it may be possible to regulate these functions specifically by targeting CD45 on the surface of NK cells.

MATERIALS AND METHODS

Mice. C57BL/6 and CD45−/− (gift from Dr. V. Tybulewicz (National Institute for Medical Research, Mill Hill, UK; reference 33) mice were bred and maintained at The Walter and Eliza Hall Institute of Medical Research. CD45 mutation was backcrossed at least eight times onto the C57BL/6 background before being made homozygous and maintained by brother–sister mating. All mice were used between 8 and 20 wk of age. Competitive BM chimeras were generated by i.v. transfer of 2 × 106 CD45−/− and C57BL/6 bone marrow cells to C57BL/6 recipients after two doses of irradiation at 5.5 Gy. Animal experimentation was in strict accordance with protocols approved by the Royal Melbourne Hospital Animal Ethics Committee.

Antibodies, flow cytometry, and cell sorting. Antibodies specific for NK1.1 (PK136), Ly49A (YE132), Ly49C/1 (SW566), Ly49D (4E5/E1), Ly49G2 (4D11), CD16 (24G2), B220 (RA3–6B2), Ly5.2 (A1042), Ly5.1 (A20.1), CD3 (KT3–1-1), CD11b (Mac-1; M1/70), Gr-1 (B6.8C5), TCR-β (H57-5921), IFN-γ (BB170 and XM1.2), GM-CSF (MP1–22E9 and MP1–316G6), and IL-10 (JESS-2AS, 1) were prepared as described previously (36). Antibodies against CD94 (18d3), DX5, NKG2A/C/E (20d5), NKG2D (CX5), CD244 (SW2B4), Ly49F (HBF-719), CD8 (56–7.3), BrdU (B44), CD122 (TM-1), IL-10 (SXC-1), and CD49b (HM2) were purchased from (BD Biosciences). Anti-Ly49H (3D10) (55) was a gift from Dr. A. Scalzo (University of Western Australia, Nedlands, Australia). Antibodies used in immunoblot analysis and immunoprecipitation were: anti-phosphotyrosine and anti-Akt (Upstate Biotechnology); anti-Vav1 (blotting), anti-actin, and anti-Syk (Santa Cruz Biotechnology, Inc.); anti-
phospho-P44/42 MAPK (Thr202/Tyr204), anti-phospho-Akt (Ser473), anti-Vav1 (IP), anti-phospho-P38 (Thr180/Tyr182), anti-P38, anti-Zap-70 (blotting), anti-phospho-Jnk (Thr183/Tyr185), and anti-JNK (Cell Signaling Technology); anti-phospho-Src (Tyr416; Biosource); anti-Zap-70 (IP; Calgene Laboratories); anti-rabbit-HRP, anti-goat HRP (Silenus); anti-mouse IgG1, -2b, and -k (Southern Biotechnology Associates, Inc.).


cell suspensions were prepared by forcing organs through metal sieves. Liver lymphocytes were isolated from a 40–80% Percoll gradient (Amersham Biosciences) centrifuged for 20 min at 2,500 rpm. For flow cytometry, single-cell suspensions were stained with the appropriate mAb in PBS containing 2% FCS. Biotinylated mAb were revealed by Cy5- or PE-streptavidin (Southern Biotechnology Associates, Inc.). Cells were analyzed on an LSR (BD Biosciences) with dead cells being excluded by propidium iodide staining.

Cell sorting was performed using FacsDIVA (BD Biosciences) or MO-FLUX (Cytomation) to 98% purity.

**NK cell in vitro function.** Sorted NK cells were cultured in Nunclon™ six-well flat bottom tissue culture plates (Nunc) at 10⁶ cells/ml in IMDM supplemented with 10% FCS and 50 ng/ml Gentamycin (Sigma-Aldrich).

Cells were typically expanded for 5–7 d in 50 ng/ml recombinant human IL-15 (R&D Systems). For cytokine/chemokine production, MULTI-WELL™ 48-well tissue culture plates (BD Labware) were coated with 20 μg/ml of the indicated mAb overnight in PBS at 4°C. NK cells were washed extensively and cultured for 24 h at 10⁶ cells/well in 0.5 ml of IMDM with 10% FCS. Supernatants from 5 × 10⁶ CHO cells culture alone were used as negative controls. Cytokines used included: IL-12 (2 ng/ml), IL-18 (10 ng/ml), and IL-21 (100 ng/ml) all from R&D Systems. IFN-γ and IL-10 production was determined by ELISA as described previously (36). GM-CSF was detected by sandwich ELISA using MP1-22E9 as a capture Ab and MP1-31G6-biotin as a detection Ab. RANTES, MIP-1α, and -β Quantum kits (R&D Systems) were used according to the manufacturer’s instructions. Cell proliferation was performed by culturing 5 × 10⁶ cells/well in 96-well, flat-bottom plates with a titration of IL-15 (0.5–50 ng/ml) for 48 h with or without IL-21. Plates were then pulsed with 1 μCi of [³H]thymidine (NEN Life Science Products) and harvested after 8 h onto glass fiber filters (Packard instrument Co.) and incorporation was determined by scintillation counting. The cytotoxicity of freshly isolated and in vitro expanded NK cells was measured in vivo. The YAC-1, CHO, P185, RMA, RMA-S, and RMA-S-Rae1 tumor cell lines were loaded with 200 μCi of [³H]thymidine (NEN Life Science Products) and harvested after 8 h onto glass fiber filters (Packard instrument Co.) and incorporation was determined by scintillation counting. The cytotoxicity of freshly isolated and in vitro expanded NK cells was measured using a 4-h ⁵¹Cr-release assay described previously (56). The YAC-1, CHO, P185, RMA, RMA-S, and RMA-S-Rae1 tumor cell lines were loaded with 200 μCi of [³H]thymidine (ICN Pharmaceutical). Reverse ADCC assays were performed exactly as described previously (20). The pharmacological inhibitors PP2, Ly294002, and SU6656 (Calbiochem) were solubilized in DMSO and incubated with NK cells for 30 min at 37°C before culturing. PP3 and DMSO were used as controls.

**NK functions measured in vivo.** Injection of mismatched BM were performed by i.v. injection of 2 × 10⁶ BALB/c (H-2b) BM cells in PBS into recipients after two doses of 5.5 Gy. Before 8 d, spleens were harvested and colonies counted using a dissecting microscope.

NK cell turnover was measured by addition of 0.5 mg/ml of BrdU (Sigma). Liver lymphocytes were isolated from 40–80% Percoll gradient (Amersham Biosciences) centrifuged for 20 min at 2,500 rpm. For flow cytometry, single-cell suspensions were stained with the appropriate mAb in PBS containing 2% FCS. Biotinylated mAb were revealed by Cy5- or PE-streptavidin (Southern Biotechnology Associates, Inc.). Cells were analyzed on an LSR (BD Biosciences) with dead cells being excluded by propidium iodide staining.

Cell sorting was performed using FacsDIVA (BD Biosciences) or MO-FLUX (Cytomation) to 98% purity.

**Statistical analysis.** Data were analyzed with Microsoft Excel software applying the two-tailed Student’s t test. The null hypothesis was rejected and differences deemed significant when P < 0.05.

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