Notch Signaling at Later Stages of NK Cell Development Enhances KIR Expression and Functional Maturation

Martin Felices, Dave E. M. Ankarlo, Todd R. Lenvik, Heather H. Nelson, Bruce R. Blazar, Michael R. Verneris and Jeffrey S. Miller

*J Immunol* 2014; 193:3344-3354; Prepublished online 29 August 2014;
doi: 10.4049/jimmunol.1400534
http://www.jimmunol.org/content/193/7/3344
Notch Signaling at Later Stages of NK Cell Development Enhances KIR Expression and Functional Maturation

Martin Felices,1 Dave E. M. Ankarlo,1 Todd R. Lenvik, Heather H. Nelson, Bruce R. Blazar, Michael R. Verneris, and Jeffrey S. Miller

The Notch signaling pathway plays a substantial role in human NK cell development. However, the role of Notch on killer Ig–like receptor (KIR) upregulation and acquisition of effector function has not been explored. To evaluate how Notch influences terminal differentiation, cord blood–derived NK cells or sorted peripheral blood NK cells were cultured with IL-15 for 7 d with inhibitory or activating Notch signals. Inhibition of Notch signaling significantly decreased KIR expression, whereas activation enhanced it. Overexpression of activated Notch on cord blood–derived NK cells resulted in a 2-fold increase in KIR expression, indicating that Notch signaling plays a direct, cell-intrinsic role in KIR regulation. Moreover, Notch-mediated KIR expression on NK cells is regulated through cis inhibition by delta-like ligand 1. Notch signaling also enhances CD16 upregulation that precedes KIR expression. Concomitantly with the upregulation of KIR and CD16, Notch signaling induces increased cytolytic effector capacity and cytokine secretion, even in posttransplant samples in which NK cell function is inherently defective. Given these attributes of Notch signaling, we propose that Notch agonists may enhance NK cell maturation and tumor killing in a posttransplant setting. The Journal of Immunology, 2014, 193: 3344–3354.

Natural killer cells are a critical component of the immune system, where they play a role in viral responses and tumor control. Human NK cells develop from CD34+ hematopoietic stem cells (HSCs) and must traverse through a number of developmental stages prior to acquisition of CD56 expression and functional competence (1). NK cell commitment is marked by CD56 expression that can be divided further into two populations of NK cells based on CD56 intensity, with the CD56bright NK cells preceding, and giving rise to, the CD56dim NK cells (2, 3). CD56dim NK cells are thought to be more functionally mature with greater cytotoxic capacity and cytokine production after target cell recognition (4–6). Acquisition of inhibitory killer Ig–like receptor (KIR) expression occurs progressively during development within the CD56dim NK cell subset and results in increased function, driving NK cell education or licensing (7–10). Although some of the components necessary for KIR expression on NK cells have been elucidated (11–13), many questions remain concerning which signaling pathways are involved in KIR expression and functional maturation of NK cells.

The Notch signaling pathway has been shown to have a role in the development and function of the innate and adaptive immune system (14). Early mouse experiments showed that Jagged2, a Notch ligand, was capable of inducing NK cell development in vitro from murine HSCs (15). However, subsequent murine studies indicated that in vivo NK cells develop independently of Notch signaling (16). In humans, our group and others have demonstrated that activation of the Notch pathway at early points in NK cell development leads to accelerated NK cell appearance in the cultures, but also results in a developmental block at the CD56bright stage, thus preventing NK cells from achieving KIR expression and full maturation (17–19). Notch activation early in development abrogates the need for stroma or IL-15 to drive NK cell commitment (acquisition of CD56). More importantly, ablation of Notch signaling early on through use of γ-secretase inhibitor (gSI) or Notch-blocking Abs resulted in almost complete loss of NK cell development, indicating that Notch signals critically influence NK cell development in humans.

Little is known about the role of Notch at later stages of NK cell maturation. One study showed that Notch activation itself can enhance IFN-γ secretion by decidual and peripheral blood NK (PBNK) cells, suggesting that Notch signaling may influence function on mature NK cells (20). Our group has demonstrated that a pair of microRNAs (miR-181a/b) that target a negative regulator of Notch signaling, nemo-like kinase, is expressed at their highest levels in the more mature CD56+ NK cells, illustrating the potential need for Notch signaling at later stages of NK cell development (21). Taken together, the data imply that Notch signaling in humans has a prominent role during early NK cell differentiation, but might also play a separate role for more mature NK cells. The present study shows that Notch signaling at later stages of NK cell development results in enhanced KIR expression, CD16 expression, and NK cell functionality. Additionally, we provide a mechanism for regulation of Notch-mediated KIR expression.

Materials and Methods

Cell culture

Peripheral blood NK (PBNK) cells were magnetically isolated from peripheral blood through negative selection (StemCell Technologies), whereas umbilical cord blood (UCB) CD34-derived NK cells were differentiated from CD34+ hematopoietic progenitor cells isolated from UCB by double-
Prior to magnetic separation, a Histopaque gradient (Sigma-Aldrich) was used to obtain mononuclear cells. Where noted, PBNC cells were further sorted into CD56^brightKIR^-, CD56<sup>bright</sup>^KIR<sup>+</sup>, or CD56<sup>dim</sup>^KIR<sup>-</sup> NK cells using a FACSAria II cell sorter (BD Biosciences) and used for cell culture or processed for RNA or protein. Depending upon the experiment, UCB CD34<sup>-</sup> cells were maintained and plated, as described, prior to coculture (23) after irradiation with 2000 rad. All studies used the following media with or without gSI (Calbiochem), where noted: complete DMEM (Cellgro) with 10 ng/ml IL-15 (R&D Systems), supplemented with 10% human AB serum (Valley Biomedical), 24 g/L sodium selenate, 20 mg/L ascorbic acid, and 50 g/L sodium selenate.

**Patient samples**

Transplant patient samples used for functional studies have been described previously (24). Briefly, 28-d posttransplant samples were harvested and cryopreserved from acute myelogenous leukemia patients that received adult donor HLA partially matched T cell–depleted (CD34<sup>-</sup>-selected) grafts with no posttransplant immunosuppression. Cells were then incubated with the human erythroleukemia cell line K562 (2:1 [E:T] ratio) for 5 h, and NK cells were analyzed for function. Samples were obtained after informed consent and approval from the University of Minnesota Institutional Review Board in compliance with the Declaration of Helsinki.

**KIR-ligand typing**

HLA-C group dimorphism is characterized by polymorphism at codons 77 (AGC versus AAC) and 80 (AAC versus AAA). A custom TaqMan single nucleotide polymorphism genotyping assay (Life Technologies, Carlsbad, CA) for codon 77 was tested using a LightCycler 480 instrument (Roche). HLA-B typing was performed in two amplification steps, followed by pyrosequencing. Initial amplification step (PCR1) was as described by Pozzi et al. (25). This HLA-B–specific initial amplification product (PCR1) was then used for a second amplification step, as described by Yun et al. (26). HLA-C1, C2, or Bw4 ligands were assigned based on this sequence data.

**Abs and flow cytometry**

The Abs used in this study were CD56 PE-Cy7 and allophycocyanin-Cy7, CD158a/CD158b/CD158e1 PE (used in experiments where KIR were pooled), CD158e1 BV421, TNF-α AF647, IFN-γ Pacific blue, delta-like ligand (DLL)1 and DLL4 PE, purified mouse IgM isotype control (BioLegend), CD158b FITC, CD107a FITC, purified mouse anti-human CD16 (BD Biosciences), CD3 ECD, CD158b allophycocyanin (Beckman Coulter), and CD158a PE-Cy7 (eBioscience). For CD16 activation studies, cells were cultured with anti-CD16 or isotype control for 30 min and then cross-linked with goat anti-mouse IgG for 5 h. Staining, acquisition, and analysis were performed, as previously described (27). Finally, cells were run on a LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Treestar).

**Quantitative RT-PCR and Western blot analysis**

For evaluation of transcripts and protein, NK cells were enriched magnetically and (where noted) further separated magnetically into KIR<sup>+</sup> populations. RNA was processed, and quantitative RT-PCR on GAPDH<sup>-</sup>-normalised transcript expression (normalized to GAPDH) was determined at different stages of NK cell development (stage 1, CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>+</sup>; stage 2, CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>–</sup>; stage 3, CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>+</sup>CD94/CD16<sup>+</sup>; and stage 4/5, CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>+</sup>CD94/CD16<sup>+</sup>). PBNC cells were first enriched utilizing magnetic beads and then sorted based on CD56 expression and presence of KIR. Notch-1 transcript expression (normalized to GAPDH) was determined in CD56<sup>bright</sup>KIR<sup>–</sup>, CD56<sup>bright</sup>^KIR<sup>+</sup>, and CD56<sup>dim</sup>^KIR<sup>+</sup> NK cells (n = 4). Representative Western blot of full-length (FL) and ICN protein in enriched adult NK cells after overnight culture with no cytokine or 10 ng/ml IL-15 in the presence of either DMSO or 20 μg/ml gSI (n = 6). c-Myc transcript expression (normalized to 18S) in adult NK cells after 3 d of stimulation with 10 ng/ml IL-15 in the presence of either DMSO or 20 μg/ml gSI (n = 6). Statistical significance is indicated as *p ≤ 0.05.

**FIGURE 1.** Regulation of Notch expression and signaling on NK cells. (A) Pooled UCB (n = 5) was used as a source of hematopoietic progenitors, and Notch-1 transcript expression (normalized to GAPDH) was determined at different stages of NK cell development (stage 1, CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>+</sup>; stage 2, CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>+</sup>; stage 3, CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>+</sup>CD94/CD16<sup>+</sup>; and stage 4/5, CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>+</sup>CD94/CD16<sup>+</sup>). (B) PBNC cells were first enriched utilizing magnetic beads and then sorted based on CD56 expression and presence of KIR. Notch-1 transcript expression (normalized to GAPDH) was determined in CD56<sup>bright</sup>KIR<sup>–</sup>, CD56<sup>bright</sup>^KIR<sup>+</sup>, and CD56<sup>dim</sup>^KIR<sup>+</sup> NK cells (n = 4). (C) Representative Western blot of full-length (FL) and ICN protein in enriched adult NK cells after overnight culture with no cytokine or 10 ng/ml IL-15 in the presence of either DMSO or 20 μg/ml gSI (n = 6). (D) c-Myc transcript expression (normalized to 18S) in adult NK cells after 3 d of stimulation with 10 ng/ml IL-15 in the presence of either DMSO or 20 μg/ml gSI (n = 6). Statistical significance is indicated as *p ≤ 0.05.
FIGURE 2. Notch signaling blockade results in reduced KIR expression. KIR expression was evaluated on developing NK cells or adult NK cells after inhibition of the Notch pathway. (A and B) UCB progenitors were differentiated into NK cells by coculture with EL08-1D2 stroma and cytokines for 21 d, harvested, and cultured for an additional 7 d in 10 ng/ml IL-15 with DMSO or 20 μM gSI. Flow plots (A) and aggregate data (B) showing KIR expression on CD56+CD3− NK cells (n = 14). (C) Representative dot plot and (D) aggregate data of fresh sorted adult donor CD56−KIR− NK cells cultured for 7 d in 10 ng/ml IL-15 with DMSO or 20 μM gSI showing KIR expression on CD56−CD3− NK cells (n = 4). (E) Representative dot plot and (F) aggregate data of sorted CD56brightKIR− (top panels and open bars) and CD56dim KIR− (bottom panels and filled bars) NK cells cultured for 7 d in 10 ng/ml IL-15 with DMSO or 20 μM gSI showing KIR expression on CD56−CD3− NK cells (n = 5). (F) CD56bright and CD56dim paired comparison were done separately, as these represent different experiments. Statistical significance is indicated as **p < 0.01, ***p < 0.001.
cells (No IL-15). Treatment with gSI resulted in diminished Notch cleavage, indicating that IL-15 specifically induces Notch signaling on NK cells. As c-Myc is downstream of Notch, we asked whether IL-15–induced Notch signaling can amplify c-Myc. NK cells were treated with IL-15 in the presence or absence of gSI for 3 d, and c-Myc transcripts were evaluated (Fig. 1D). Blocking Notch cleavage reduced the c-Myc upregulation, indicating that Notch signals downstream of IL-15 are needed for optimal c-Myc expression, and perhaps also for subsequent KIR expression.

**Notch signaling modulates KIR expression**

To determine whether Notch signaling can influence KIR expression at stage 4/5 of development, CD34+ cells were differentiated into NK cells for 21 d in culture to avoid the effects of Notch early during development, and then treated with or without gSI and IL-15 for 7 d (Fig. 2A, 2B). Blocking Notch signaling late in development consistently resulted in decreased induction of KIR expression. These results were confirmed on sorted CD56bright PBNKs that had been treated with IL-15 with or without gSI for 7 d (Fig. 2C, 2D). In all conditions, blockade of Notch signaling similarly reduced KIR induction on NK cells. In additional studies, PBNK cells were further sorted into either CD56bright or CD56dim KIR− NK cells to investigate whether Notch signaling in both NK cell subsets is capable of enhancing KIR expression (Fig. 2E, 2F). Interruption of Notch signaling in CD56bright and CD56dim NK cells resulted in decreased KIR expression, which has been associated with decreased function (24).

Although blockade of Notch signaling decreased induction of KIR expression, complimentary and more direct approaches were investigated to definitively show the linkage between Notch and KIR expression. Notch activation was triggered on day 21 CD34-derived NK cells (predominantly stage 4/5) by coculture for 7 d with control OP9 cells (OP9-N) or OP9 cells expressing Notch ligands, OP9-DLL1 or OP9-DLL4, in the presence of IL-15 (Fig. 3A). Coculture with OP9 cells containing Notch ligands resulted in an increased proportion of KIR-positive cells. However, the magnitude of the increase was most likely impaired by rapid NK cell–directed cytotoxicity toward the stroma, which limited the sustained delivery of Notch signaling. When the same experiment was repeated with PBNKs (Fig. 3B), coculture with OP9-DLL4 cells, but not with OP9-DLL1 cells, resulted in an increased proportion of KIR-positive cells. Although this finding is intriguing, subtle differences have been noted in the past between DLL1 and DLL4 signaling, perhaps explaining why PBNKs and UCB CD34-derived NKs display distinct sensitivities to the DLLs (30). To test whether the effect of Notch on KIR expression is cell intrinsic, day 28 CD34-derived NK cells were transduced with the active ICN and GFP (to track transduced cells) or with GFP alone (Supplemental Fig. 1A). Cells were then cultured for 7 d with IL-15, and KIR expression was evaluated on the GFP low and GFP high populations or ICN low and ICN high populations based on the level of GFP expression in either group (Fig. 3C). No differences in KIR expression were seen in the control group that had been transduced with just a GFP-expressing minicircle based on either the high or low GFP expression. However, cotransduction with ICN and GFP DNA minicircles resulted in a significant induction of KIR expression in cells that showed high transduction (as evidenced by high GFP expression) compared with cells that showed low-level transduction, in which levels of KIR did not differ from controls. Taken together, these data indicate that Notch signaling modulates KIR expression, and it does so in a cell-intrinsic manner.

A role for Notch signaling in NK cell education mediated by KIR interactions with cognate ligand (self-KIR) was evaluated next. Donors were KIR-ligand typed, and self-KIR acquisition on CD56dim-sorted NK cells (Supplemental Fig. 1B) and cord blood–derived NK cells (data not shown) was measured after 7 d of culture with IL-15 and gSI or DMSO control. Although more single self-KIR NK cells were present after 7 d of culture, interruption of Notch signaling by gSI resulted in a similar decrease in KIR irrespective of the presence or absence of cognate HLA ligand (Supplemental Fig. 1B, left panel). As NKG2A can also mediate NK cell education, we analyzed the data by excluding NKG2A (Supplemental Fig. 1B, center panel) and found no differences either. In aggregate, Notch signaling had no effect on overall or individual KIR expression patterns when cognate ligand was included in the analysis (Supplemental Fig. 1B, right panel).
FIGURE 4. Notch signaling enhances CD16 expression prior to KIR expression. (A) Day 21 UCB CD34-derived (n = 4), (B) PBMC-sorted CD56bright CD3 KIR− (n = 8), and (C) PBMC-sorted CD56bright CD3 KIR+ (n = 8) NK cells were cocultured with 10 ng/ml IL-15 and OP9-native, OP9-DLL1, or OP9-DLL4 cells for 7 d and harvested to assess CD16 expression. (D and G) CD16 expression on KIR− or KIR+ NK cell populations from UCB NK (n = 4), (E and H) CD56bright NK (n = 8), and (F and I) CD56bright NK (n = 8) in the same setting described above. (J) KIR and (K) CD16 expression was tested at day 24 (3 d after start of coculture) of UCB NK culture (instead of day 28) to assess the role of Notch signaling on expression of these molecules at an earlier time point; coinoculation with OP9 cells was started at day 21, as previously described. Statistical significance is indicated as *p ≤ 0.05, **p < 0.01, ***p < 0.001.
Notch signaling enhances CD16 expression prior to KIR upregulation

Expression of CD16 on NK cells occurs after the CD56bright stage of development as NK cells become CD56dim (1). To investigate whether Notch signals can influence CD16 expression, day 21 differentiated CD34-derived NK cells were cultured with OP9 cells expressing or lacking Notch ligands. Notch signaling at later intervals of development resulted in an increased proportion of NK cells expressing CD16 (Fig. 4A). Although less so, Notch ligands also enhanced CD16 expression on CD56bright (Fig. 4B) and had a minimal effect on CD56dim (Fig. 4C) NK cells sorted from peripheral blood. Notch signaling increased CD16 expression (mean fluorescence intensity) on a per cell basis on the CD34-derived UCB NK and the sorted CD56dim subsets (Supplemental Fig. 1C). We next analyzed CD16 expression on NK cells without or with KIR expression. This analysis showed that Notch-mediated CD16 upregulation was more significant in the less mature KIRNK cells (Fig. 4D) than for KIRNK cells (Fig. 4G). The differences were reduced on sorted CD56bright NK cells (Fig. 4E, 4H) and further decreased on the CD56dim (Fig. 4F, 4I) KIR-negative PBNK cells as maturation progressed, consistent with the Notch-1 expression seen in these subsets (Fig. 1B). Taken together, these data indicate that Notch signaling after NK cell commitment promotes CD16 expression at a stage prior to KIR expression. To further explore this sequencing, CD34-derived UCB NK cells were incubated with OP9 cells containing Notch ligands at day 21, but in this study they were harvested earlier (after 3 d at day 24) compared with the 7 d used in other experiments) to better separate the kinetics of Notch-mediated KIR versus CD16 upregulation. At day 24, Notch has not yet induced KIR expression (Fig. 4I), but DLL1 has already enhanced CD16 upregulation (Fig. 4K). This kinetic analysis supports the premise that Notch signals upregulate CD16 prior to KIR expression.

Notch signaling pushes NK cells to terminal differentiation

Because Notch signaling mediates KIR and CD16 expression, we next tested its effect on NKG2A and CD57, which are well characterized in expression as NK cells terminally differentiate. NKG2A is expressed at high levels on CD56bright NK cells and intermediate levels on the CD56dim NK cells, and is further decreased with KIR expression, whereas CD57 is expressed in an opposite pattern (1, 7). To address the effect of Notch signaling on expression of these markers, CD56dimKIRNK cells were sorted from PBMCs and placed in culture with IL-15 and Notch inhibitors or with Notch-ligand–bearing cells. Inhibition of Notch signaling with gSI lowered the proportion of NK cells expressing CD57 by 25%, but did not have an effect on NKG2A expression (Fig. 5C, 5D). These data indicate that Notch signaling is involved in either maintenance or induction of CD57 NK cells, known to be a marker of terminally differentiated and functional competent NK cells.

Notching signaling at later stages of development results in enhanced NK cell function

Notch-mediated induction of maturation markers (KIR, CD16, and CD57) suggests a phenotype of enhanced function. To test this hypothesis, CD34-derived NK cells or sorted PBNK cells (CD56dimKIR− or CD56dimKIR+) were cocultured with IL-15 and OP9 cells for 7 d and then activated through CD16 cross-linking to assess NK cell activation (Fig. 6A, 6C, 6E, respectively). Basal levels of CD107a expression, possibly mediated through the killing of the OP9 cells during coculture, could be seen in the IgG control, but this was not changed by Notch ligands. In contrast, CD16 cross-linking was enhanced by Notch signaling and resulted in increased CD107a expression (Fig. 6A, 6C, 6E, respectively). Basal levels of CD107a expression, possibly mediated through the killing of the OP9 cells during coculture, could be seen in the IgG control, but this was not changed by Notch ligands. In contrast, CD16 cross-linking was enhanced by Notch signaling and resulted in increased CD107a expression (Fig. 6A, 6C, 6E, respectively). Basal levels of CD107a expression, possibly mediated through the killing of the OP9 cells during coculture, could be seen in the IgG control, but this was not changed by Notch ligands. In contrast, CD16 cross-linking was enhanced by Notch signaling and resulted in increased CD107a expression (Fig. 6A, 6C, 6E, respectively). Basal levels of CD107a expression, possibly mediated through the killing of the OP9 cells during coculture, could be seen in the IgG control, but this was not changed by Notch ligands. In contrast, CD16 cross-linking was enhanced by Notch signaling and resulted in increased CD107a expression (Fig. 6A, 6C, 6E, respectively).

**FIGURE 5.** Notch signaling can also alter expression of other differentiation markers. PBMC-sorted CD56dimCD3− NK cells were cocultured with 10 ng/ml IL-15 and DMSO or 20 μM gSI (A and B) or OP9-native, OP9-DLL1, or OP9-DLL4 cells (C and D) for 7 d and harvested to assess CD57 (A and C) and NKG2A (B and D) expression (n = 7). Statistical significance is indicated as *p ≤ 0.05, ***p < 0.001.
increasing CD16-mediated signals and by increasing the proportion of more functional KIR+ NK cells.

**Notch signaling can rescue the function of defective NK cells after transplantation**

The data presented above indicate that Notch signaling at later points of NK cell development can result in enhanced function. We next chose to explore an in vivo setting of developing hematopoiesis in which NK cell function is defective, as we have seen early after T cell–depleted allogeneic transplantation (24). To investigate whether Notch signaling can rescue these defects, cells were collected at day 28 after transplant and cryopreserved, and thawed PBMCs were cultured for 1 wk with IL-15 and OP9 cells with or without Notch ligands and then activated with K562 target cells. A modest but consistent increase in degranulation was noted when the NK cells were cocultured with Notch ligands and activated by targets (Fig. 7A). A more significant increase was seen in IFN-γ (Fig. 7B) and TNF-α expression (Fig. 7C) upon target-mediated activation of effectors that had received Notch signals. At this time point after transplant, CD16 was low, consistent with increased CD56bright NK cells seen early after transplantation (31). CD16 was even lower when NK cells were cultured with targets, but this is explained by ADAM17 clipping induced by NK cell activation (32). Notch signaling increased basal CD16 and countered some of the downmodulation seen by target cell exposure (Fig. 7D). Collectively, these findings indicate that inducing Notch signaling increases NK cell function and CD16 expression posttransplant, which could be important for inducing a NK cell graft versus tumor effect alone or in combination with Ab-mediated cellular cytotoxicity through CD16.

**Notch-mediated KIR expression on NK cells is limited by DLL1 cis inhibition**

To date, this study has focused on the role of Notch signaling in late stages of NK cell maturation and how it impacts NK cell function.

**FIGURE 6.** Notch signaling can enhance NK cell function through CD16 cross-linking. UCB CD34-derived NK cells (A and B) (n = 15), sorted CD56brightKIR+ PBNK cells (C and D) (n = 7), or sorted CD56dimKIR+ PBNK cells (E and F) (n = 11) were cultured with 10 ng/ml IL-15 and OP9-N, OP9-DLL1, or OP9-DLL4 cells for 7 d. Cells were then cross-linked with (control) mouse anti-human IgG (open bars) or mouse anti-human CD16 (filled bars). (A, C, and E) CD107a expression on total CD56+CD3− NK cells or (B, D, and F) subpopulations based on KIR expression is shown. Repeated measures ANOVA with Tukey’s multiple comparison test was used for statistical analysis. For KIR breakup panels, separate tests were carried out for KIR+ and KIR− groups to diminish statistical clutter. Statistical significance is indicated as *p ≤ 0.05, **p < 0.01, ***p < 0.001.
However, the underlying factors that regulate Notch signaling on NK cells remain unknown. Although DLL expression in trans results in Notch cleavage and activation, delta expression in cis has been shown to inhibit Notch activation in different cell systems (33). Given these findings, because Notch signaling influences KIR expression, DLL expression was studied in magnetically isolated KIR⁺ and KIR⁻ NK cells. DLL1 transcript and protein were observed to be higher on KIR⁺ NK cells (Fig. 8A, 8B), whereas there were no differences in DLL4 transcript or protein expression. These results are consistent with the possibility that DLL1 cis inhibition might be responsible for control of KIR expression on NK cells. To explore this further, NK cells were sorted into subsets and analyzed for DLL expression. DLL1 transcripts and protein (Fig. 8C, 8D) were found to be highly expressed in CD56bright NK cells and expressed at the lowest levels in the most mature CD56dimKIR⁺ NK cell subset. No significant changes were seen in DLL4 expression (data not shown). This supports the hypothesis that cis inhibition might be strongest in the CD56bright developmental stage, which precedes expression of CD16 and KIR. Therefore, downmodulation of DLL1 expression would be needed to generate CD16⁺ and KIR⁺ NK cells. We have previously shown that IL-15 can mediate KIR expression through c-Myc (13). To investigate whether exposure to this cytokine could be responsible for DLL1 downmodulation, CD56bright NK cells, which show maximal levels of DLL1, were cultured with IL-15, and DLL1 transcript expression was evaluated after 72 h (Fig. 8E). DLL1 expression was decreased 4-fold after 72 h of IL-15 treatment, indicating that IL-15 signaling is involved in downmodulation of DLL1. This finding suggests that, among other mechanisms, IL-15-induced KIR expression occurs through downregulation of inhibitory cis-DLL1. To test directly whether cis inhibition is responsible for controlling KIR expression, we transduced UCB-derived NK cells with a siRNA specific for DLL1 (to knockdown DLL1 in cis) and then cocultured the cells with OP9-DLL1 cells to provide DLL in trans (Fig. 8F). Knockdown of DLL1 in cis nearly doubled the expression of KIR (compared with the siRNA controls) when the cells were exposed to DLL in trans, indicating that cis expression of DLL1 is partially responsible for restricting KIR expression on CD56bright NK cells.

Discussion
In combination with previous data, our results suggest a temporally specific need for Notch signaling during NK cell development. Blockade of Notch signaling at an early stage in human NK cell differentiation cultures stops emergence of NK cells, indicating that Notch signaling is needed early for HSC differentiation to the NK lineage (18). Conversely, this and other studies show that activation of Notch signaling early results in accelerated NK cell development, even in the absence of stroma or IL-15, demonstrating that Notch signals comprise one of the key elements for early NK cell differentiation (17, 19). However, constitutive activation of the Notch pathway early on through transduction with the active portion of Notch results in a developmental and functional block at the CD56bright stage, thus suggesting that, even though Notch signaling is necessary for NK cell commitment, it becomes det-

FIGURE 7. Notch signaling can enhance function of NK cells early after hematopoietic cell transplantation. Frozen PBMCs from adult donor T cell-depleted grafts (day 28 posttransplant) were thawed, rested overnight, and placed in culture with 10 ng/ml IL-15 and OP9-N, OP9-DLL1, or OP9-DLL4 cells for 7 d. NK cell effectors alone (open bars/Eff) or after 5-h coculture with K562 target cells (filled bars/Eff + Targ) were assessed for (A) CD107a, (B) IFN-γ, and (C) TNF-α (n = 10). (D) CD16 was also measured on these cells to determine whether Notch modulates expression on clinical samples (n = 10). Given the stark differences between controls (Eff) and activation group (Eff + Targ), repeated measures ANOVA with Tukey’s multiple comparison test was carried out individually within those groups. Statistical significance is indicated as *p ≤ 0.05, **p < 0.01, ***p < 0.001.
rimental at later stages of development by inhibiting full matu-
ration. Furthermore, a stage-specific modular presence of micro-
RNAs that control expression of nemo-like kinase, a negative
regulator of Notch signaling, can be seen during NK cell devel-
opment, thereby demonstrating a need for tuning of Notch signals
during differentiation (21). Our present study shows that Notch
also signals at later stages of development to induce NK cell
terminal maturation by increasing CD16 and KIR expression and
that this enhances NK cell function. Taken together, these data
form a developmental human NK cell model in which, to attain
full functional maturation, Notch signals are required initially,
detrimental during intermediate stages and important during final
stages, including acquisition of function.

It is important to understand the mechanisms by which Notch
signaling is regulated and modulates NK cell maturation. As might
be expected, IL-15, which has been shown to have a role in
mature NK cells and in KIR expression (11–13, 34), also has
a role in this process. We show that IL-15 induces downregulation
of DLL1 on CD56\textsuperscript{bright} NK cells, which express high levels of
DLL1, by releasing them from cis inhibition by DLL, similar to
what has been described in other cell systems (33), thus facilitat-
ing functional Notch signaling in trans. The DLL signals in trans
result in Notch-mediated cell-intrinsic upregulation of KIR,
that is, in part through c-Myc transcription. c-Myc transcription is
downstream of both IL-15 and Notch and has been shown to bind
to the distal KIR promoter and enhance its transcription (13). Our
findings indicate that the effect is additive, as inhibition of Notch
signaling through gSI or Notch-blocking Abs (data not shown)
culminated in a decrease, but not complete ablation, of KIR ex-
pression in the cultures when IL-15 was present. Of importance,
Notch signaling was capable of inducing KIR, CD16, and CD57
expression on mature PBNK cells, showing that terminal NK cell
maturation can be enhanced in the periphery given the proper
stimulus. IL-15 is produced by several cell types, including
monocytes, macrophages, dendritic cells (DCs), keratinocytes,
muscle cells, renal epithelial cells, and endothelial cells (35–38),

FIGURE 8. Cis-delta-like 1 expression on NK cells limits Notch signaling and acquisition of KIR. (A) A two-step magnetic enrichment was used to obtain CD56\textsuperscript{CD3}\textsuperscript{KIR\textsuperscript{+} or KIR\textsuperscript{−}} NK cells, and delta-like 1 (DLL1, top) and delta-like 4 (DLL4, bottom) transcripts were assessed and normalized to 18S
(n = 6). (B) DLL1 (top) and DLL4 (bottom) protein expression was assessed (in terms of mean fluorescence intensity) on CD56\textsuperscript{CD3} NK cells from PBMCs of healthy human donors (n = 4). (C) DLL1 transcript (normalized to GAPDH) and (D) protein (in terms of mean fluorescence intensity) expression were analyzed from CD56\textsuperscript{CD3} NK cells sorted from PBMCs (n = 4). (E) CD56\textsuperscript{CD3} NK cells were sorted from PBMCs and treated for 0 h (no treatment) or 72 h with 10 ng/ml IL-15. Cells were then harvested and assessed for DLL1 transcript expression and normalized to
GAPDH (n = 4). (F) Day 28 CD34-derived NK cells were harvested and transduced with siRNA controls (siC) or siRNAs specific for DLL1 (siDLL1) and
then rested for 1 d. Cells were next cultured for 6 d with 10 ng/ml IL-15 and OP9-DLL1 cells to provide Notch signals in trans and harvested at day 35. KIR
expression was then assessed on the CD56\textsuperscript{CD3} NK cells (n = 4). Statistical significance is indicated as *p ≤ 0.05, **p < 0.01, ***p < 0.001.
and can be upregulated through GM-CSF, TLR agonists, and type I IFNs (37, 39). On this basis, one could surmise that pathogenic infections might be capable of inducing NK cell maturation through Notch signaling downstream of IL-15 in the periphery. In fact, our group has previously shown that CMV reactivation post-transplant enhances KIR expression and functional maturity on NK cells (27, 40), perhaps in part through this Notch-mediated mechanism. Besides possibly driving Notch signaling downstream of IL-15, pathogenic infections can also drive expression of Notch ligands on DCs (41–44), perhaps creating a more direct source for Notch signaling–mediated functional maturation of NK cells because not all Notch ligand–driven DC effects are controlled directly by Th differentiation (45, 46).

NK cell–based immunotherapies are currently being tested in the clinic (47–49). Accordingly, the possibility of enhancing NK cell maturation and function is of great translational interest. As previously noted, CMV infection induces NK cell maturation and is associated with less myeloid leukemia relapse post-HSCT transplant (50–52). However, the mortality rate associated with CMV infection might outweigh the tumor control benefits that it provides (50–52). Therefore, understanding the mechanisms by which this particular infection drives NK cell functional maturation is critical to development of novel therapeutic strategies. Our data indicate that Notch signaling might provide one of these mechanisms. Notch signaling induces functional maturation of NK cells through both KIR and CD16 expression. We have shown that KIR+ NK cells have higher cytotoxic capabilities than KIR− NK cells, most likely due to their ability to undergo education or licensing (8–10). Although we did not see a direct role for Notch signaling in specifically inducing expression of KIR for self-ligands, increasing the proportion of KIR+ NK cells (through Notch) increases the functional potential of the NK cell population by generating a larger pool of cells capable of becoming educated and functional. Our data indicate that Notch signaling also induces CD16 expression prior to KIR upregulation, possibly making NK cells more responsive to Fc-mediated stimuli independent of KIR expression. It should be noted that our coculture system does not provide continuous Notch signaling, as the NK cells kill the OP9 cells quickly, necessitating transfer of NK cells onto new OP9 cells days after initiation of the culture to maintain Notch signals. Despite these limitations, we were still able to demonstrate Notch-mediated rescue of cytotoxicity, cytokine production, and CD16 expression on patient samples 28 d after transplantation. These results suggest increasing Notch signaling may be an approach to increasing NK cell maturation and function after transplantation to enhance clearance of minimal residual disease. However, Notch signals have also been shown to result in detrimental effects due to increased graft-versus-host disease, so ex vivo activation of Notch signaling might be the safer route to induce NK cell maturation (53–55). An added benefit of targeting the Notch pathway in the acute myeloid leukemia setting is that Notch activation has recently been shown to directly inhibit acute myeloid leukemia growth and survival (56). Therefore, future use of clinical grade Notch agonists might enhance clearance of tumors in at least two ways, including direct inhibition of tumor growth and survival and increase of the immunotherapeutic value of the NK cells that target the tumors and prevent relapse.

Acknowledgments

We thank the Züniga-Pflücker laboratory for the kind gift of the OP9-DLL1 and OP9-DLL4 cells.

Disclosures

The authors have no financial conflicts of interest.

References


17. Bachanova, V., V. McCullar, T. Lenvik, R. Wangen, K. A. Peterson, D. E. Ankarlo, A. P. Fren, and W. M. Yokoyama. 2009. Notch signals may be an approach to increasing NK cell maturation and function after transplantation to enhance clearance of minimal residual disease. However, Notch signals have also been shown to result in detrimental effects due to increased graft-versus-host disease, so ex vivo activation of Notch signaling might be the safer route to induce NK cell maturation (53–55). An added benefit of targeting the Notch pathway in the acute myeloid leukemia setting is that Notch activation has recently been shown to directly inhibit acute myeloid leukemia growth and survival (56). Therefore, future use of clinical grade Notch agonists might enhance clearance of tumors in at least two ways, including direct inhibition of tumor growth and survival and increase of the immunotherapeutic value of the NK cells that target the tumors and prevent relapse.

Acknowledgments

We thank the Züniga-Pflücker laboratory for the kind gift of the OP9-DLL1 and OP9-DLL4 cells.

Disclosures

The authors have no financial conflicts of interest.


42. Skokos, D., and M. C. Nussenzweig. 2007. CD8⁻ DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. J. Exp. Med. 204: 1525–1531.


Supplementary Figures:

(A) UCB CD34-derived NK cells were harvested at day 28, transduced with minicircles expressing GFP alone or GFP plus the active intracellular portion of Notch (ICN), and cultured for 7 days with 10 ng/ml IL-15. Representative histograms of GFP gating strategy on GFP alone (left) or GFP plus ICN (right) transduced samples at the time of harvest. (B) Donors were HLA typed and then CD56 \text{dim} NK cells were sorted from PBMCs and placed in culture for 7 days with IL-15 and DMSO (white bars) or 20 uM gSI (grey bars). Single KIR expression for presence (Edu) or absence (unEdu) of self-HLA is displayed for all single KIR (left panel) or single KIR excluding NKG2A (center panel).

Supplementary Figure 1
panel). Only donors containing at least one uneducated KIR were included in analysis (n = 6). Individual KIR expression (right panel) is also displayed (n = 8). (C) CD16 MFI was assessed on the CD16+ population of NK cells from (left) day 21 UCB CD34-derived NK cells (n = 4), (center) PBMC sorted CD56brightCD3KIR− NK cells (n = 8), and (right) PBMC sorted CD56dimCD3KIR− NK cells (n = 8) after co-culture with 10 ng/ml IL-15 and OP9-Native, OP9-DLL1, or OP9-DLL4 cells for 7 days. (D) UCB CD34-derived NK cells (white bars (n = 15), sorted CD56brightKIR− PBNK cells (grey bars (n = 7)), and sorted CD56dimKIR− PBNK cells (black bars (n = 11)) were cultured with 10 ng/ml IL-15 and OP9-N, OP9-DL1, or OP9-DL4 cells for 7 days. Cells were then cross-linked with mouse anti-human CD16 and analyzed for intracellular IFNγ (left panel) or TNFα (right panel) five hours after cross-linking.