Bone marrow-derived stromal cells and Notch ligands support the sequential acquisition of CD94 and NKp80 during human NK cell development

Ansel P. Nalin[1,5], Blaire K. Schumacher[5], Kiran V. Vedantam[5], Alexander C. Sprague[5], Bethany L. Mundy-Bosse[2,5], Michael A. Caligiuri[4], Aharon G. Freud[3,5]

[1]Medical Scientist Training Program, Biomedical Sciences Graduate Program; [2]Division of Hematology, Department of Internal Medicine; [3]Department of Pathology; [4]City of Hope National Medical Center; [5]The Comprehensive Cancer Center, The Ohio State University

Abstract

Human natural killer (NK) cells develop in secondary lymphoid tissues (SLTs) from precursor cells that differentiate into NK and other innate lymphoid cell (ILC) subsets. We investigated the role of Notch signaling in NK cell maturation. We used a stromal feeder cell development assay together with activation of Notch signaling to generate mature NK cells *in vitro* from SLT-derived ILC precursor cells (ILCPs). NK cell developmental stages express Notch receptors at the surface. Activation of Notch in immature NK cells promoted the developmental transition wherein NK cells attain functional maturity. *In vitro*-derived NK cells in the presence of Notch activation expressed the surface activating receptor NKp80, expressed transcription factors T-BET and EOMES, and produced perforin, granzymes, and interferon-γ. These data identify Notch signaling as a cellular pathway regulating NK cell functional maturation.

Introduction

Natural killer (NK) cells comprise a subset of cytotoxic innate lymphoid cells (ILCs) that are involved in the immune response to viruses and also participate in tumor immune surveillance[1]. NK cells develop from hematopoietic progenitor cells that originate in the bone marrow and migrate to secondary lymphoid tissues (SLTs). Research by our group and others has identified precursor cell populations, selectively enriched in the tonsils and lymph nodes, which can develop into mature NK cells[2, 3]. Previous work by our group has characterized a pathway of human NK cell development according to distinct stages that are found naturally in SLTs[4]. The stages of NK cell development are defined based on changes in surface marker expression. A five-stage model for NK cell development was proposed by our group that includes classification of SLT-resident intermediate NK cell populations based on surface expression patterns of CD34, CD117, CD94, NKp80, and CD16[5]. Stage 1 (CD34+CD117-CD94-NKp80-CD16-) and stage 2 cells (CD34+CD117+CD94-NKp80-CD16-) are the earliest progenitor cells that have developmental potential to become T cells or dendritic cells in addition to NK cells. Stage 3 cells (CD34-CD117+CD94-NKp80-CD16-) include a subset of ILC precursor cells that lack T/DC developmental potential but can become NK cells and all other ILC subsets. Stage 4 cells (CD34-CD117+/-CD94+CD16-) can be further classified based on surface expression of NKp80. Stage "4A" cells (NKp80-) express some NK-associated surface markers but lack NK cell function. Stage "4B" cells (NKp80+) express a greater functional phenotype, including their ability to produce interferon (IFN)-γ and mediate cytotoxicity. Stage 5 cells (CD34-CD117+/-CD94+/-NKp80+CD16+) comprise mature NK cells with the highest cytotoxicity.

The exact mechanisms that regulate human NK cell development are not fully described. Understanding the normal processes regulating NK cell development will not only address this

knowledge gap in immunology, but also provide the basis for translational applications of NK cell biology. In this report we describe the role of the Notch signaling pathway in NK cell development. Notch is an evolutionarily conserved signal pathway involved in numerous cellular processes, and has been previously shown to influence lymphocyte development. There are five known canonical Notch ligands and four Notch receptors that exists in mammals. The Notch signaling pathway becomes activated when a Notch ligand binds to the extracellular domain of the transmembrane Notch receptor. The receptor undergoes proteolysis by an associated gamma-secretase enzyme, releasing Notch intracellular domain (NICD) to the nucleus. NICD binds to the RBPJ-κ transcription factor and recruits co-activator proteins to form a transcriptionally-active complex that controls the expression of target genes[6, 7, 8, 9]. In this report we show that Notch ligands expressed by stromal feeder cells promote the formation of mature NKp80+ ("4B") NK cells. In the presence of Notch ligands, mature NK cells were produced from immature NK cells and ILC precursor cells. Culture-derived NKp80+ cells displayed a similar transcriptional and functional phenotype as stage 4B cells that naturally exist in the tonsil.

Experimental Procedures

Tissue Collection

All tissues were obtained and collected under active protocols approved by The Ohio State University Institutional Review Board. Human pediatric tonsil tissue samples were obtained through the Cooperative Human Tissue Network from Nationwide Children's Hospital, Columbus, Ohio. Leukocyte-depleted red blood cells were obtained from the American Red Cross.

Cell Isolation

ILCs were enriched from SLTs as previously described[10, 11]. Briefly, single cell suspensions from fresh human pediatric tonsils were generated by dissociation via a GentleMACS Dissociator (Miltenyi Biotec) according to manufacturer's instructions. Cells were resuspended in fetal bovine serum (FBS, Sigma-Aldrich) with leukocyte-depleted red blood cells (RBCs) and a custom human NK cell enrichment RosetteSep reagent containing a cocktail of bivalent antibodies against glycophorin A and CD3, CD4, CD19, CD20, CD36, CD66b, and CD123 (STEMCELL Technologies). Cells were mixed with RBCs and RosetteSep reagent, incubated on a nutator for 30 min at room temperature, diluted in PBS (Thermo Fisher Scientific), layered over Ficoll-Paque PLUS (GE Healthcare), and centrifuged at 1800 rpm for 30 min at room temperature with the brake off. The monolayers were harvested and residual RBCs were lysed using eBioscience 1x RBC Lysis Buffer (Thermo Fisher Scientific). From the tonsillar ILC-enriched fractions, NK cell developmental stages were sorted (>99% purity) using a FACSAriaII sorter (BD Biosciences). ILCPs were sorted as Lineage (CD3, CD4, CD5, CD14, CD19, CD20, CD123, FcεR1α)-CD34-CD294-KLRG1-NKp44-CD94-NKp80-CD16-CD117+ lymphocytes. Stage 4A NK cells were sorted as Lineage-CD34-CD294-KLRG1-CD94+NKp80-CD16-KIR2D-KIR3DL1/2-NKG2C-lymphocytes. Stage 4B NK cells were sorted as Lineage-CD94+NKp80+CD16+ lymphocytes. Stage 5 NK cells were sorted as Lineage-NKp80+CD16+CD57- lymphocytes.

Flow Cytometry

Immunophenotyping via flow cytometry was performed using an LSRII cytometer (BD Biosciences). Antibodies directed against surface or intracellular proteins were used according to manufacturer's instructions. Where appropriate, isotype-matched or unstimulated controls were

used to determine nonspecific staining. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit and SYTOX Blue Dead Cell Stain (Thermo Fisher Scientific) viability dyes were used to exclude non-viable cells in the analyses. Data were acquired using FACSDiva (BD Biosciences) and analyzed using FlowJo (TreeStar) software. Intracellular staining was performed using Cytofix and Cytoperm Fixation and Permeabilization Solution Kit (BD Biosciences) for cytokine analysis, or Foxp3 Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) for transcription factor analysis.

Real-Time RT-PCR

From FACS-purified primary tonsil-derived NK cell populations, mRNA was isolated and purified using the Total RNA Purification Kit (Norgen Biotek) according to manufacturer's instructions, and cDNA was synthesized using Superscript IV VILO Master Mix (Thermo Fisher Scientific). Standard quantitative real-time RT-PCR reactions were performed on a Viia7 Real-Time PCR System (Life Technologies) using primer sequences obtained from published reports[12]. Gene expression was normalized to the *18S* mRNA internal control: ΔCt = Ct(gene of interest) – Ct(*18S*). Relative mRNA expression for each gene was calculated as 2^(- ΔCt).

Cell Culture

All *in vitro* cell culture experiments were incubated at 37ºC in 5% CO_2 atmosphere. FACS-purified tonsil-derived primary NK cells were cultured for 14 days in 200 µl culture media per well in 96-well flat-bottom plates (TrueLine). The culture media for *in vitro* development experiments contained DMEM and F12 (2:1 ratio) supplemented with 1% antibiotic/antimycotic (Thermo Fisher Scientific), 20 mg/mL ascorbic acid, 24 µM 2-mercaptoethanol, 0.05 mg/mL sodium

selenite (Sigma), and 10% heat-inactivated human AB serum (Valley Biomedical). For experiments with stromal-feeder cells, OP9 or OP9-DL1 cells were obtained from the lab of Dr. Juan Carlos Zuniga-Pflucker[13]. Stromal cells were maintained in MEM-α + Glutamax media (Thermo Fisher Scientific) with 10% fetal bovine serum and 1% antibiotic/antimycotic (Thermo Fisher Scientific). One day prior to culture of NK cells, OP9 or OP9-DL1 cells were seeded at a density of 2000 cells per well (in a 96 well flat-bottom plate) and incubated at 37ºC for cell adhesion. Stromal cell media was removed immediately prior to addition of FACS-sorted NK cells. Fresh media and cytokines were replenished twice per week. For cytokine production assays, cells were harvested following culture and resuspended in 250 µl RPMI-1640 media supplemented with 10 ng/ml each of IL-12, IL-15, and IL-18 and brefeldin A (GolgiPlug Protein Transport Inhibitor, BD Biosciences) for 4 hrs with prior to analysis by intracellular flow cytometry.

Statistical Analysis

One-way ANOVA and paired t-tests were used to compare means within groups, and p values < 0.05 were considered significant.

Results

Co-culture of ILC precursor cells with stromal feeder cells supports development into NK cells.

ILC precursor cells (ILCPs), enriched from primary samples of human pediatric tonsils and isolated by FACS, were cultured with human interleukin (IL)-7 and in the presence or absence of a murine bone marrow-derived cell line, OP9. ILCPs incubated in media with IL-7 alone did not

develop into NK cells nor show significant proliferation. When co-cultured with OP9 cells, ILCPs acquired surface expression of CD94, consistent with a stage 4A NK cell phenotype (Fig. 1A). Next, ILCPs were co-cultured with a modified cell line derived from OP9 cells and genetically engineered to constitutively overexpress the human Notch ligand, delta-like-1 (OP9-DL1)[13]. ILCPs co-cultured with OP9-DL1 cells generated CD94+ cells, a majority of which also expressed NKp80 (stage 4B), compared to those derived with OP9 cells. Treatment with Notch inhibitor DAPT did not inhibit the generation of CD94+ cells from ILCPs, but did decrease the percentage of these cells that co-expressed NKp80 (Fig. 1B). Taken together, these data show that stromal feeder cells support the differentiation of CD94+ NK cells from ILC precursor cells, and the presence of Notch ligands promotes expression of NKp80.

NK cell developmental intermediates express Notch receptors.

Our observations described above suggested that Notch regulates the stage 4A to 4B transition during NK cell development. Next we determined which Notch receptors are expressed by NK cell developmental intermediates (NKDIs). We FACS-purified populations of NKDIs and used quantitative real-time RT-PCR to determine the relative mRNA expression levels of each of the four mammalian Notch receptors in different stages of development. *NOTCH1* and *NOTCH2* transcripts were significantly expressed in each of the isolated populations in contrast to *NOTCH3* and *NOTCH4* (Fig. 2A). Moreover *NOTCH1* transcript levels were higher in the mature stages of development, and *NOTCH2* transcripts were more highly expressed in earlier stages. Next we measured Notch expression at the surface using flow cytometry. Interestingly, enriched ILCs freshly isolated from the tonsil did not have detectable expression at the surface of either NOTCH1 or NOTCH2 by flow cytometry. However, after 3 days *in vitro*, Notch receptors were detectable

by flow cytometry at the cell surface. Similar to the trends shown by qPCR, NOTCH1 expression increased as NK cells proceeded through stages of maturation. NOTCH2 was more highly expressed in the ILC precursor population and was decreased in mature NK cells (Fig. 2B). The presence of Notch receptors at the surface of NKDIs suggested that Notch may be important in regulating NK cell development.

Notch activation *in vitro* promotes the 4A to 4B transition.

To determine the role of Notch in NKp80 acquisition, we FACS-purified tonsillar stage 4A (CD94+NKp80-) NK cells and cultured them *in vitro* with IL-7 alone or with OP9 or OP9-DL1 cells. Stage 4A cells cultured with OP9-DL1 cells had a greater percentage of CD94(+) NK cells that co-expressed NKp80 compared to those cultured with plain OP9 cells in the absence of DL1. To test the effects of modulating the Notch pathway, stage 4A cells were similarly co-cultured with OP9 or OP9-DL1 cells and in the presence of Notch inhibitor DAPT or vehicle control. Treatment with DAPT inhibited NKp80 acquisition in stage 4A NKDIs cultured with OP9-DL1 cells (Fig. 3A-B). These results provided evidence that activation of Notch signaling promotes the stage 4A to 4B transition.

Culture-derived NKp80+ cells display a mature NK cell phenotype.

Surface expression of NKp80 in *ex vivo* tonsillar NK cells correlates with functional maturity. We next sought to determine whether NKp80+ cells generated *in vitro* shared similar characteristics as *ex vivo* stage 4B cells based on cytokine production and transcriptional profile. To test for NK cell functionality, stage 4A cells were cultured with IL-7 on OP9-DL1 cells for 14 days followed by stimulation with IL-12, IL-15, and IL-18. IFN-γ was specifically produced by culture-derived

NKp80+ cells (Fig. 4A). Next we measured the production of specific proteins involved in mediating cytotoxicity. In stage 4A cells cultured in the presence of OP9-DL1 stroma, expression of perforin, granzyme A, and granzyme M was highest among NKp80+ cells (Fig. 4B). To determine the transcriptional profile of culture-derived NK cells, we used intracellular flow cytometry to measure the relative expression of transcription factors T-BET and EOMES, which are expressed at stage 4B *ex vivo*[5]. Culture-derived CD94+NKp80+ ("*in vitro* stage 4B") cells had greater relative expression of T-BET and EOMES compared to the CD94+NKp80- or CD94-NKp80- populations (Fig. 4C). Taken together, these data demonstrate that NKp80+ NK cells produced *in vitro* in the presence of OP9-DL1 cells have a functional phenotype that matches *ex vivo* tonsillar stage 4B NK cells.

Discussion

In this study, we show that a murine bone marrow-derived stromal feeder cell *in vitro* development system is capable of supporting the differentiation of NK cells from an ILC precursor cell. In these conditions, no other exogenous cytokines were added besides IL-7, which was primarily added for cell survival as ILCPs did not differentiate into NK cells nor significantly proliferate when IL-7 was added in the absence of stroma. Furthermore, the presence of human Notch ligand delta-like-1, constitutively overexpressed by the OP9-DL1 stromal cells, promoted the functional maturation of NK cells, as shown by surface expression of activating receptor NKp80 and production of IFN-γ. ILCPs co-cultured with OP9 cells, without the overexpressed Notch ligand, generated CD94+ NK cells that remained predominantly NKp80-. The stromal-intrinsic factor responsible for differentiation to NK cells in this condition is yet unknown. Interestingly, ILCPs co-cultured with OP9-DL1 cells gave rise to a lower overall percentage of CD94+ cells compared to those cultured

on OP9 cells. However, the majority of OP9-DL1-derived CD94+ cells were NKp80+, whereas the OP9-derived CD94+ cells were mostly NKp80-. One reason for this is that ILCPs have the capacity to develop into other ILC populations besides NK cells, especially in the presence of Notch[14]. For example, Notch activation may concurrently promote ILC2 and ILC3 differentiation from a proportion of ILCPs. In the absence of Notch, ILCPs may be more likely to develop into NK cells instead of ILC2s or ILC3s, which would explain the higher proportion of CD94+ cells derived on OP9 cells compared to OP9-DL1. However, among the CD94+ cells that are produced when co-cultured with stromal feeder cells, the presence of overexpressed Notch ligand supported NKp80 acquisition and functional development.

Notch is an evolutionarily conserved signal pathway responsible for multiple processes of cell development and physiology. Binding of a Notch ligand to one of the four Notch receptors initiates a signaling cascade resulting in localization of Notch intracellular domain to the nucleus for activation of transcription[7]. In general, canonical signaling downstream of each receptor is a conserved process; however, some differential effects may exist when comparing signaling through one specific Notch receptor versus another[15]. This raises the question of whether a specific Notch receptor is primarily involved in the mechanism of NK cell maturation. Based on our real-time PCR data, NOTCH1 and NOTCH2 are the likely receptors involved in NK cell biology, as NOTCH3 and NOTCH4 transcripts were not significantly detected in ILCPs or mature NK cells. Interestingly we observed opposing trends when comparing the relative expression levels of NOTCH1 and NOTCH2 in stages of NK cells. Both NOTCH1 and NOTCH2 are expressed in ILCPs, as shown by surface flow cytometry. NOTCH1 expression increases with maturation, with stage 4A cells demonstrating higher average expression compared to ILCPs and stage 4B and 5

cells showing the highest expression of NOTCH1. Conversely, NOTCH2 is most significantly expressed in ILCPs, minimally expressed in stage 4A cells, and not expressed at the surface of stage 4B and 5 cells. These patterns are similar to those seen based on analysis of Notch receptor transcripts by qRT-PCR. It is yet unknown how each of these receptors are specifically involved in the pathways of ILC development; however, it is not unreasonable that NOTCH1 and NOTCH2 have differing roles. Based on the expression patterns of the individual receptors, NOTCH1 may be involved in the later stages of NK cell functional maturation by regulating the transcription of target genes needed for mature NK cell function and cytotoxicity. In contrast, NOTCH2, primarily expressed in ILCPs, may be involved in the differentiation to ILC2s or ILC3s. A possible explanation for modulation of Notch signaling downstream of receptor activation may be through the requisite co-activator proteins. When the Notch intracellular domain migrates to the nucleus, it binds other proteins to form a transcriptionally-active complex. NOTCH1 and NOTCH2 may interact with a different set of proteins that allows for binding to different target genes. Thus NOTCH1 and NOTCH2 may have certain non-overlapping binding sites that allow for regulation of different ILC differentiation pathways. Further study is needed to determine how NOTCH1 and NOTCH2 are involved in the mechanisms of NK, ILC2, and ILC3 differentiation.

Our results showed that Notch activation in the presence of stromal cells promoted the acquisition of NKp80+ cells from ILCPs and stage 4A NK cells. Culture-derived NKp80+ cells were similar to *ex vivo* stage 4B cells based on their phenotypic and functional properties. Notably, *in vitro*-derived stage 4B cells expressed the transcription factors T-BET and EOMES which have been implicated in the latter stages of both human and mouse NK cell maturation[16, 17, 18]. Additionally, perforin, granzymes, and IFN-γ were produced by NKp80+ cells. It is yet unknown the mechanism

by which Notch promotes functional maturation. One possible explanation is that each of the genes for these transcription factors and functional mediators are direct targets of NOTCH1 and/or NOTCH2 in NK cells, and become upregulated by activated Notch. Alternatively, Notch signaling may upregulate a global transcription factor that is responsible for promoting multiple genes involved in NK cell maturation. Since Notch is widely involved in many cellular processes, it is likely that the signaling cascade downstream of Notch is closely regulated in NK cells. The binding of NOTCH1 and NOTCH2 to specific gene targets important for ILC differentiation may be modulated by the activity of co-factors or other transcription factors. This would allow for Notch to regulate different gene targets in different conditions. Future directions of this work will identify how specific Notch target genes regulate the intracellular mechanism for NK and ILC differentiation.

Figure 1

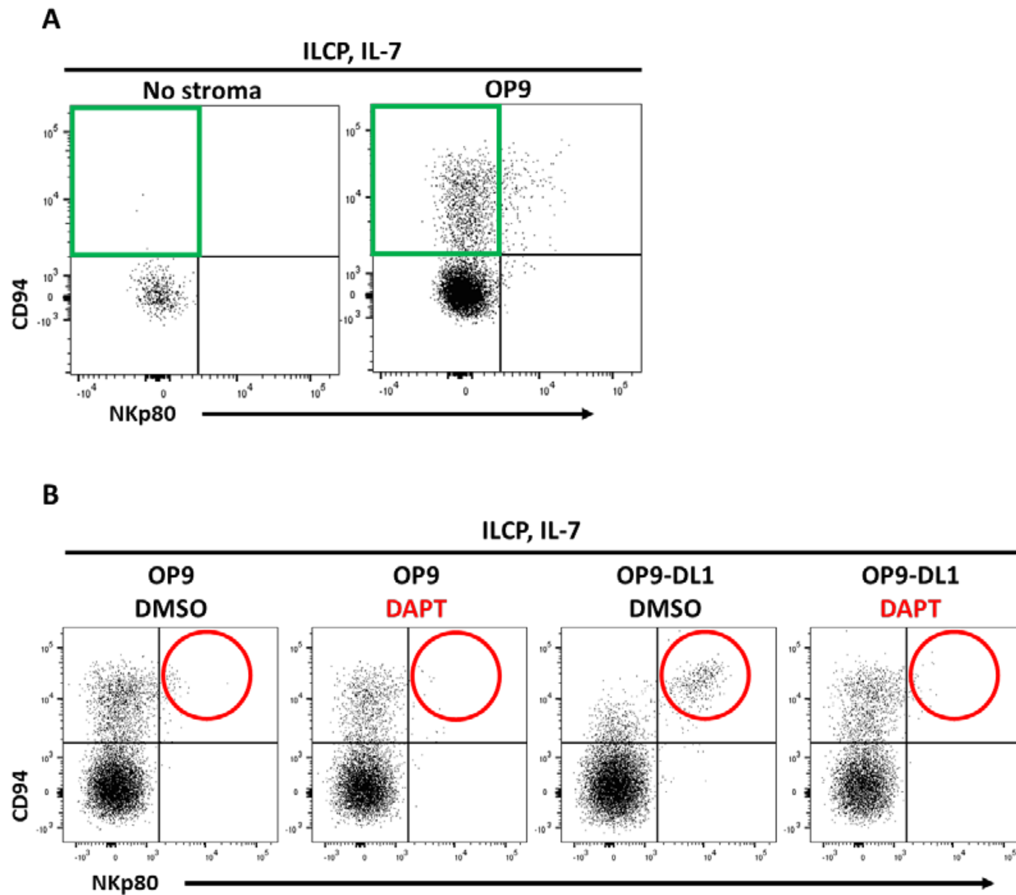

Figure 1. Notch activation in the presence of stromal feeder cells promotes NK cell differentiation. (A) FACS-purified tonsillar ILCPs were cultured with IL-7 (10 ng/ml) and in the presence or absence of OP9 stromal cells for 14 days prior to analysis by flow cytometry. Green boxes indicate culture-derived CD94+ NK cells. (B) FACS-purified tonsillar ILCPs were cultured with IL-7 (10 ng/ml) in the presence of OP9 or OP9-DL1 cells and the addition of either DMSO or DAPT (10 µM). Red circles indicate culture-derived CD94+NKp80+ cells. Representative flow cytometry plots shown for similar results, n=3.

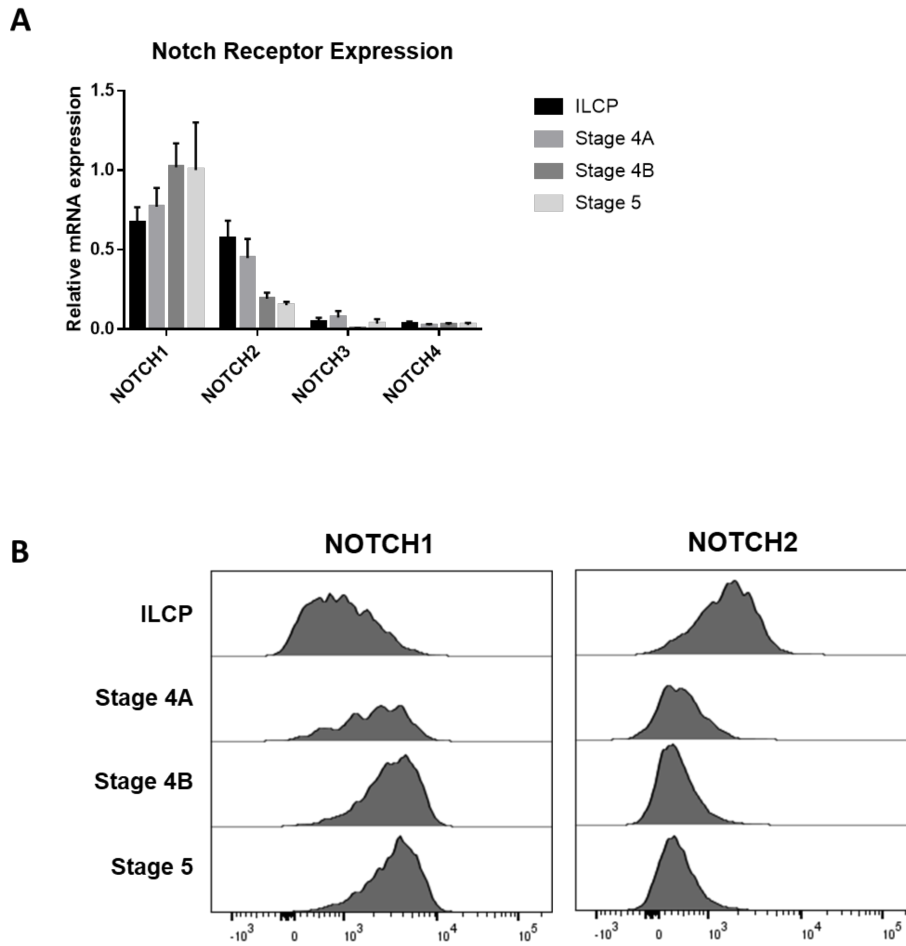

Figure 2. Human NK cells express Notch receptors. (A) Relative mRNA transcript expression levels for *NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4* were measured by quantitative real-time RT-PCR. Gene expression was normalized to the *18S* mRNA internal control: ΔCt = Ct(gene of interest) – Ct(*18S*). Relative mRNA expression for each gene was calculated as 2^(- ΔCt). N=5. (B) Expression of NOTCH1 and NOTCH2 in NK cell developmental stages was measured by surface flow cytometry. Representative flow cytometry plots shown for similar results, n=3.

Figure 3

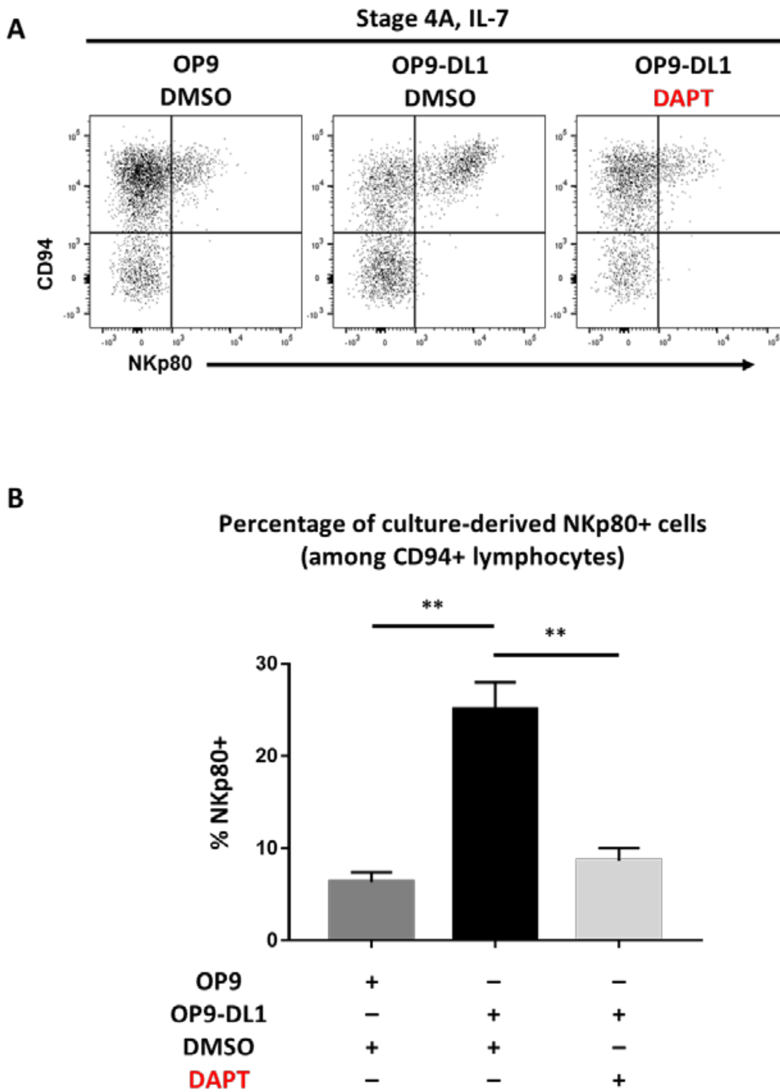

Figure 3. Notch activation promotes the stage 4A to 4B transition during NK cell development. (A) Representative flow cytometry and (B) summary statistics of FACS-purified tonsillar stage 4A cells cultured with IL-7 in the presence of OP9 or OP9-DL1 cells with the addition of DMSO or DAPT for 14 days prior to analysis by flow cytometry. ** $p<0.01$, n=19.

Figure 4

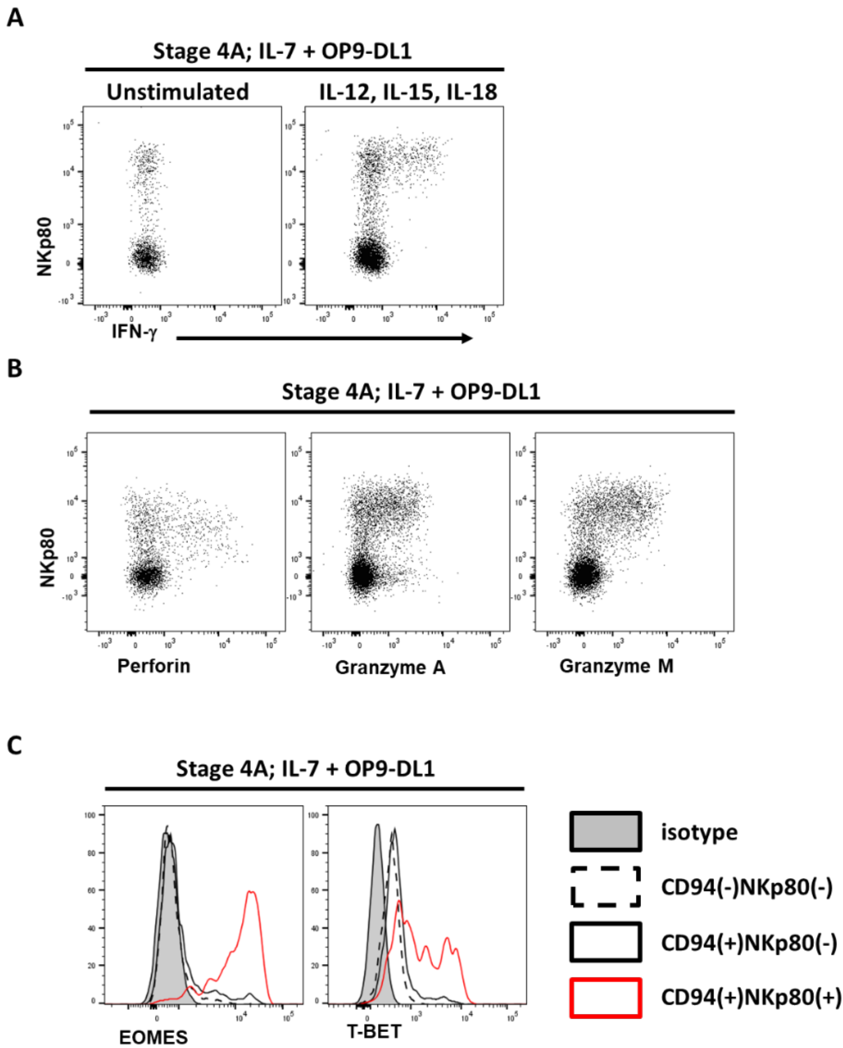

Figure 4. Culture-derived NKp80+ cells display a mature NK cell phenotype. (A) FACS-purified tonsillar stage 4A NK cells were cultured with IL-7 (10 ng/ml) in the presence of OP9-DL1 stromal cells for 14 days, then stimulated for 4 hr with IL-12, IL-15, and IL-18 (10 ng/ml each) and brefeldin A. Cells were harvested, followed by fixation and permeabilization for analysis by intracellular flow cytometry. (B, C) FACS-purified tonsillar stage 4A NK cells were cultured with IL-7 (10 ng/ml) in the presence of OP9-DL1 stromal cells for 14 days prior to analysis by intracellular flow cytometry.

References

1. Caligiuri, M.A. Human natural killer cells. *Blood* **112**, 461-469 (2008).

2. Freud, A.G. *et al.* A Human CD34(+) Subset Resides in Lymph Nodes and Differentiates in CD56^{bright} Natural Killer Cells. *Immunity* **22**, 295-304 (2005).

3. Freud, A.G. *et al.* Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med* **203**, 1033-1043 (2006).

4. Freud, A.G. & Caligiuri, M.A. Human natural killer cell development. *Immunol Rev* **214**, 56-72 (2006).

5. Freud, A.G. *et al.* NKp80 Defines a Critical Step during Human Natural Killer Cell Development. *Cell Reports* **16**, 379-391 (2016).

6. Kling, J.C. & Blumenthal, A. Roles of WNT, NOTCH, and Hedgehog signaling in the differentiation and function of innate and innate-like lymphocytes. *J Leukoc Biol* **101**, 827-840 (2017).

7. Andersson, E.R., Sandberg, R. & Lendahl, U. Notch signaling: simplicity in design, versatility in function. *Development* **138**, 3593-3612 (2011).

8. Artavanis-Tsakonas, S., Rand, M.D. & Lake, R.J. Notch Signaling: Cell Fate Control and Signal Integration in Development. *Science* **284**, 770-776 (1999).

9. Baron, M. An overview of the Notch signaling pathway. *Semin Cell Dev Biol* **14**, 113-119 (2003).

10. Scoville, S.D. *et al.* Rapid Column-Free Enrichment of Mononuclear Cells from Solid Tissues. *Scientific Reports* **5**, 12490 (2015).

11. Scoville, S.D. *et al.* A Progenitor Cell Expressing Transcription Factor RORgammat Generates All Human Innate Lymphoid Cell Subsets. *Immunity* **44**, 1140-1150 (2016).

12. Li, Y., Wu, S., Pu, J., Huang, X. & Zhang, P. Dengue virus upregulates expression of notch ligands Dll1 and Dll4 through interferon-β signaling pathway. *Immunology* **144**, 127-138 (2015).

13. Schmitt, T.M. & Zuniga-Pflucker, J.C. Induction of T Cell Development from Hematopoietic Progenitor Cells by Delta-like-1 In Vitro. *Immunity* **17**, 749-756 (2002).

14. Chen, L. *et al.* CD56 Expression Marks Human Group 2 Innate Lymphoid Cell Divergence from a Shared NK Cell and Group 3 Innate Lymphoid Cell Development Pathway. *Immunity* **49**, 464-476 (2018).

15. Baumgart, A. *et al.* Opposing role of Notch1 and Notch2 in a KrasG12D-driven murine non-small cell lung cancer model. *Oncogene* **34**, 578 (2014).

16. Gordon, S.M. *et al.* The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. *Immunity* **36**, 55-67 (2012).

17. Townsend, M.J. *et al.* T-bet Regulates the Terminal Maturation and Homeostasis of NK and Vα14i NKT Cells. *Immunity* **20**, 477-494 (2004).

18. Mundy-Bosse, B.L. *et al.* MicroRNA-29b mediates altered innate immune development in acute leukemia. *J Clin Invest* **126**, 4404-4416 (2016).