Stage three immature human natural killer cells found in secondary lymphoid tissue constitutively and selectively express the T_H17 cytokine interleukin-22

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ABSTRACT

Considerable functional heterogeneity within human natural killer (NK) cells has been revealed through the characterization of distinct NK cell subsets. Accordingly, a small subset of CD56(+)NKp44(+)NK cells termed “NK-22” cells, was recently described within secondary lymphoid tissue (SLT) as IL-22(-) when resting, with a minor fraction of this population becoming IL-22(+) when activated. Here we discover that the vast majority of stage three immature (i) NK cells in SLT constitutively and selectively express IL-22, a TH17 cytokine important for mucosal immunity, while earlier and later stages of NK developmental intermediates do not express IL-22. These iNK cells have a surface phenotype of CD34(-)CD117(+)CD161(+)CD94(-), largely lack expression of NKp44 and CD56, and do not produce IFN-γ nor possess cytolytic activity. In summary, stage three iNK cells are highly enriched for IL-22 and IL-26 messenger RNA, IL-22 protein production, but do not express IL-17A or IL-17F.
INTRODUCTION

It is established that T and B cell developmental intermediates reside in the thymus and bone marrow (BM), respectively. Recent evidence shows human natural killer (NK) developmental intermediates are within secondary lymphoid tissue (SLT) (1, 2). We characterized four stages of NK cell development in SLT by lineage commitment, response to cytokines, and NK cell effector function (2). CD34(+)CD117(-) stage one pro-NK cells and CD34(+)CD117(+) stage two pre-NK cells retain multipotency with T and DC cell potential, whereas CD34(-)CD117(+)CD94(-) stage three immature (i) NK cells and CD34(-)CD117(+/-)CD94(+) stage four NK cells are committed to the NK cell lineage. Stage three iNK cells lack certain NK cell surface receptors, capacity for IFN-γ production, and cytolytic activity, all characteristic of mature NK cells including the more differentiated stage four cells.

Colonna recently identified a population of SLT NK cells, termed “NK-22”, that is characterized by a CD3(-)NKp44(+)CD56(+)CCR6(+) phenotype (3). NK-22 express little or no IL-22 mRNA at rest, yet <15% become IL-22(+) upon activation. IL-22 is a TH17 cytokine that engages a receptor expressed by epithelial tissues resulting in production of antimicrobial peptides which promote the local innate immune response (4, 5). Here we show that the majority of stage three resting, human iNK cells found in SLT selectively express abundant transcripts for IL-22 and IL-26, and produce IL-22 protein without prior stimulation.
MATERIALS AND METHODS

Isolation of human NK precursors from SLT

All procedures were approved by The OSU Institutional Review Board. NK developmental intermediates were isolated from fresh normal tonsil and analyzed as described by Freud et al (2). All populations were sorted to purity >99%.

cDNA preparation

RNA was purified from < 5 x 10^5 NK cells using Absolutely RNA Nanoprep Kit (Stratagene) or using RNeasy (Qiagen) if > 5x10^5. cDNA was synthesized using MMLV reverse transcriptase kit (Invitrogen) and random hexamers.

Real-Time PCR

Taqman primer/probe sets for IL-22, IL-26, IL-17A, and IL-17F were purchased from ABI (Applied Biosystems). Real-Time PCR was performed on an ABI Prism 7900HT, analyzed by the ΔΔCt method, and normalized to an 18S internal control.

Flow cytometric analyses

Total CD3(-)CD19(-)CD34(-) tonsillar mononuclear cells were isolated as previously described (2). All mAbs were purchased from BD Biosciences, except NKp44 APC, BDCA-2 APC, CD161 APC (Miltenyi), CD94 FITC (clone 131412, R & D Systems), CD56 APC, NKp44 PE (Beckman Coulter). Intracellular staining was performed immediately after CD34 depletion, and without a protein transport inhibitor, using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit and anti-IL-22-PE mAB (clone 142928; R & D). Non-specific staining was detected through the use of an appropriately labeled isotype control mAB in all analyses. Cytometric and data analyses were performed as previously described (1).

Statistical analysis

Data were analyzed using a Student 2-tailed t-test. P < 0.05 was considered significant.
RESULTS AND DISCUSSION

We quantified transcripts of IL-17A and IL-17F, IL-22, and IL-26 in purified human NK developmental intermediates from SLT. IL-17 mRNA was not detected in any of the four stages (not shown). IL-22 mRNA was only detected in resting stage three iNK cells in amounts that were > 92-fold greater than trace quantities found in stages one, two, and four NK cells. In subsequent donors, IL-22 mRNA expression was 138 ± 1.6-fold higher in stage three iNK cells compared to stage four (P = 0.0001; n = 7) (Figure 1A).

We measured IL-17A, IL-17F and IL-26 mRNA in stage three and stage four NK cells from individual donor SLT. Again, no IL-17A or IL-17F was detected in > 10 donors (not shown). IL-26 mRNA was expressed in resting stage three iNK cells from five of seven donors, and absent or barely detectable in stage four NK cells from all seven donors. When present in both stages, IL-26 transcript was 34.8 ± 1.6-fold higher in stage three iNK cells when compared to stage four cells (P = 0.002, n = 5; data not shown).

We detected IL-22 protein in stage three resting iNK cells using intracellular staining with an anti-IL-22 antibody. The majority of CD117(+)CD94(-) stage three iNK cells, a population previously described in our lab as NKp46(-)2B4(+) (2), co-express intracellular IL-22, whereas staining is very low in the CD117(+/−)CD94(+) stage four population (Figure 1B). Indeed, 88.0 ± 5.3% of stage three iNK cells were IL-22(+) compared to 6.7 ± 3.5% of stage four NK cells (Figure 1C; P < 0.00002, n = 6). We utilized an ELISA to confirm that resting and IL-15 stimulated stage three iNK cells, but not stage four NK cells, secrete IL-22 protein (not shown). Supplemental Figure 1 documents the localization of these cells to the lamina propria and parafollicular region of the tonsil.

We found that most CD117(+)CD94(-)IL-22(+) cells express CD161, but do not express BDCA-2, ruling out inclusion of NK dendritic cells (6) (Figure 2A). Less than 3% of stage three iNK cells express CD56 and < 15% of stage three iNK cells express NKp44, two markers associated with the inducible NK-22
phenotype as previously described (3). The average proportion of IL-22(+) cells expressing CD56, NKp44, CD117, or CD161 from four donors is summarized in Figure 2B. Finally, in quantitative data not shown, we ascertained that stage two and stage three NK cell intermediates selectively expressed RORC mRNA, the gene required for lymph node formation and TH17 differentiation (7, 8).

Here we show that the vast majority of stage three iNK cells in SLT display robust constitutive IL-22 expression \textit{ex vivo}. These findings are distinct from the earlier identification of NKp44(+)CD56(+) cells in SLT that express little or no IL-22 at baseline yet < 10% of these cells express IL-22, IL-26, and leukemia inhibitory factor (LIF) upon \textit{in vitro} culture with various cytokines or activated monocytes (3). The surface phenotype of NK-22 cells in our study includes a small percentage of CD56(+)NKp44(+) cells, but these markers are neither necessary nor sufficient to unambiguously identify resting IL-22(+) cells within human SLT. Rather, the surface phenotype of stage three iNK cells, CD34(-)CD117(+)CD161(+)CD94(-), specifically identifies the NK-22 subset in SLT.

The finding that IL-22 transcript is absent from stages one, two and four during NK development raises two possibilities: (1) IL-22 expression must be lost prior to differentiation to stage four; or (2) the NK-22 subset represents a separate, terminally differentiated NK cell population. In support of the latter, < 10% of stage three iNK cells differentiate to the IFN-\(\gamma\)-producing stage four NK cell (2), which may reflect iNK cell heterogeneity.

The abundant intracellular IL-22(+) staining in stage three iNK cells isolated from human tonsil is consistent with \textit{in vivo} exposure to a cellular milieu within SLT that is permissive for IL-22 production. Factors previously implicated in TH17 production and IL-22 synthesis by T cells include RORC and inflammatory cytokines such as IL-6, IL-23, and IL-1\(\beta\), as well as Toll-like receptor (TLR) activation on DC and monocyte/macrophages. Whether this selective population of iNK cells that constitutively produce IL-22 in SLT has a role in NK development, lymph node development or mucosal immunity is
unknown. It will be insightful to determine the contribution of Th17 polarizing cytokines and TLR activated cells to the differentiation of IL-22(+) stage three iNK cells, as well as the effects of iNK cell IL-22 on surrounding cells within SLT.
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AUTHOR CONTRIBUTIONS: TH performed the majority of experiments and contributed to the written manuscript. BB contributed to conceptual design, oversaw experimental design and contributed to the written manuscript. SM, EB, HM, and GN performed some of the experiments. XZ contributed to the statistical analysis. AGF contributed to the ideas put forth in the manuscript and assisted with experiments. JY contributed to experimental design, experimental work, writing and editing of the manuscript. MAC contributed to conceptual idea for the manuscript, experimental design, writing and editing of the manuscript. The authors have no relevant financial conflict of interest to disclose.
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FIGURE LEGENDS

Figure 1: IL-22 mRNA and protein expression during NK development.  (A) (Left) Quantitative (Q) RT-PCR analysis of IL-22 expression was performed on FACS-sorted NK stages one through four from human tonsil after pooling mRNA of each purified stage from 6-7 donors to achieve sufficient quantities for cDNA synthesis. Relative quantification was performed using the ΔΔCt method, and gene expression levels were normalized to 18S RNA. Y axis indicates fold increase over level of IL-22 mRNA quantified in stage four NK cells, arbitrarily normalized to 1. IL-22 was virtually absent from stages one and two, so (Right) subsequent RT-PCR measurements were performed using stage three iNK and stage four NK cells using cDNA from seven individual donors. The average fold change in IL-22 mRNA present in stage three iNK cells compared to stage four NK cells is ~138. Error bars represent standard error of the mean from n = 7 donors. *P = 0.0001. (B) IL-22 intracellular protein expression during NK development. Total CD3(-)CD19(-)CD34(-) tonsillar mononuclear cells were stained for surface expression of CD117 and CD94, followed by assessment for intracellular expression of IL-22 protein. Lin(-)CD117(+)CD94(-) identify stage three iNK cells which are then stained for intracellular expression of IL-22 as shown in this representative donor, compared to isotype control. Lin(-)CD117(-)CD94(+) identify stage four NK cells which are then stained for intracellular expression of IL-22 as shown in this representative donor, compared to isotype control. (C) The average proportion of IL-22(+) cells in stage three iNK versus stage four NK in all donors examined (n = 6). Error bars represent standard error of the mean. *P = 0.00002

Figure 2. Surface phenotype of IL-22(+) stage three iNK cells. Total CD3(-)CD19(-)CD34(-) resting tonsillar mononuclear cells were stained for surface expression of lineage markers, CD117, CD94, followed by intracellular expression of IL-22, and events were gated on total Lin(-)CD117(+)CD94(-)IL-22(+) stage three iNK cells. (A) Representative histograms show expression for each indicated surface marker (shaded)
in a donor, compared to isotype control (clear). (B) Graphical summary of the mean proportion of IL-22(+) stage three iNK cells expressing various surface markers from all (n = 4) donors is summarized. Error bars represent standard error of the mean.
Figure 2

A

BDCA-2

Lin(-)
CD117(+)
CD94(-)
IL-22(+)

CD161

0%
90.35%
2.17%

CD56

11.78%

NKp44
Figure 2

B

% of IL-22(+)Lin(-) cells

CD56(+)  NKp44(+)  CD117(+)  CD161(+)

0  10  20  30  40  50  60  70  80  90  100
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