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A Human CD34(+) Subset Resides in Lymph Nodes and Differentiates into CD56^{bright} Natural Killer Cells

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Summary

In humans, T cells differentiate in thymus and B cells develop in bone marrow (BM), but the natural killer (NK) precursor cell(s) and site(s) of NK development are unclear. The CD56^{bright} NK subset predominates in lymph nodes (LN) and produces abundant cytokines compared to the cytolytic CD56^{dim} NK cell that predominates in blood. Here, we identify a novel CD34^{dim} CD45RA(+) hematopoietic precursor cell (HPC) that is integrin $\alpha_4\beta_7^{bright}$. CD34^{dim}CD45RA(+)β_7^{bright} HPCs constitute <1% of BM CD34(+) HPCs and 6% of blood CD34(+) HPCs, but >95% of LN CD34(+) HPCs. They reside in the parafollicular T cell regions of LN with CD56^{bright} NK cells, and when stimulated by IL-15, IL-2, or activated LN T cells, they become CD56^{bright} NK cells. The data identify a new NK precursor and support a model of human NK development in which BM-derived CD34^{dim}CD45RA(+)β_7^{bright} HPCs reside in LN where endogenous cytokines drive their differentiation to CD56^{bright} NK cells in vivo.

Introduction

Human natural killer (NK) cells are CD3(–)CD14(–) CD56(+) large granular lymphocytes that can kill infected or transformed cells that fail to express normal MHC class I molecules, thereby complementing protection provided by T cells (Colucci et al., 2003). Similar to other lymphocytes, the total human NK cell population is heterogeneous, with the CD56^{bright} and CD56^{dim} subsets representing two phenotypically and functionally distinct subsets (Cooper et al., 2001). CD56^{bright} NK cells have few cytotoxic granules, and low expression of the low-affinity Fc receptor CD16 and killer immunoglobulin-like receptors (KIR), all consistent with poor cytolytic properties, but are capable of potent activation-induced cytokine and chemokine production (Cooper et al., 2001; Fehniger et al., 2003). By comparison, CD56^{dim} NK cells have abundant cytolytic granules and high surface density expression of CD16 and KIR for potent antibody-dependent and natural cytolytic function, with little ability to produce immunomodulatory cytokines. Currently, the developmental relationship between the CD56^{bright} and CD56^{dim} human NK subsets is unclear, as is their site(s) of differentiation. While $\geq$90% of NK cells in peripheral blood (PB) are CD56^{dim}, >90% of NK cells in lymph nodes (LN) are CD56^{bright} (Cooper et al., 2001; Fehniger et al., 2003). A recent study by Munz and colleagues showed that the resident CD56^{bright} NK cells in LN and tonsils could be induced with interleukin (IL)-2 to adopt functional and phenotypic qualities of PB CD56^{dim} NK cells (Ferlazzo et al., 2004), suggesting that CD56^{bright} NK cells may be less mature than CD56^{dim} NK cells in a sequential scheme of human NK development. Considering these findings, we hypothesized that LN might be a site of early human NK development in vivo.

A correlate to this hypothesis is that the human NK cell precursor would need to reside in LN. Similar to other leukocyte populations, human NK cells are ultimately derived from CD34(+) hematopoietic precursor cells (HPCs), yet the precise phenotype of the human NK precursor cell is unknown (Colucci et al., 2003; Yokoyama et al., 2004). Culture of purified human bone marrow (BM) CD34(+) HPCs in either IL-2 or IL-15 primarily results in the generation of CD56^{bright} NK cells (Mrozek et al., 1996). Similarly, mouse NK cells can be generated by in vitro culture of immature BM progenitors in IL-2 or IL-15 (Williams et al., 1997). Both IL-2 and IL-15 signal in part via a common IL-2/IL-15 receptor (R) β chain (Waldmann et al., 1998), and IL-2/IL-15Rβ-deficient mice are severely deficient in mature NK cells (Suzuki et al., 1997). Indeed, the lineage (Lin)(–)IL-2Rβ(+) population in mouse BM has clearly been identified as the committed mouse NK precursor cell (Rosmaraki et al., 2001); however, IL-2/IL-15Rβ expression on freshly isolated human CD34(+) HPCs is below the limits of detection by flow cytometry (Shibuya et al., 1993; Yu et al., 1998). Therefore, while the human NK precursor can be defined by its functional ability to differentiate into a CD56^{bright} NK cell in response to IL-2 or IL-15, the precise phenotype of this CD34(+) HPC remains elusive.

Previous work by other laboratories has provided invaluable insight into the phenotype of the CD34(+) human NK precursor by associating surface antigen expression with NK precursor function. For example, Miller and colleagues provided early evidence that coexpression of CD7 on CD34(+) HPCs selectively enriches for NK precursors (Miller et al., 1994). In addition, work by the Chen laboratory demonstrated that the coexpression of CD10 on BM CD34(+) HPCs identified the human common lymphoid progenitor (CLP) that included the NK precursor (Galy et al., 1995). Despite these advances, both CD34(+)CD7(–) and CD34(+) CD10(–) HPC populations also contain some NK pre-

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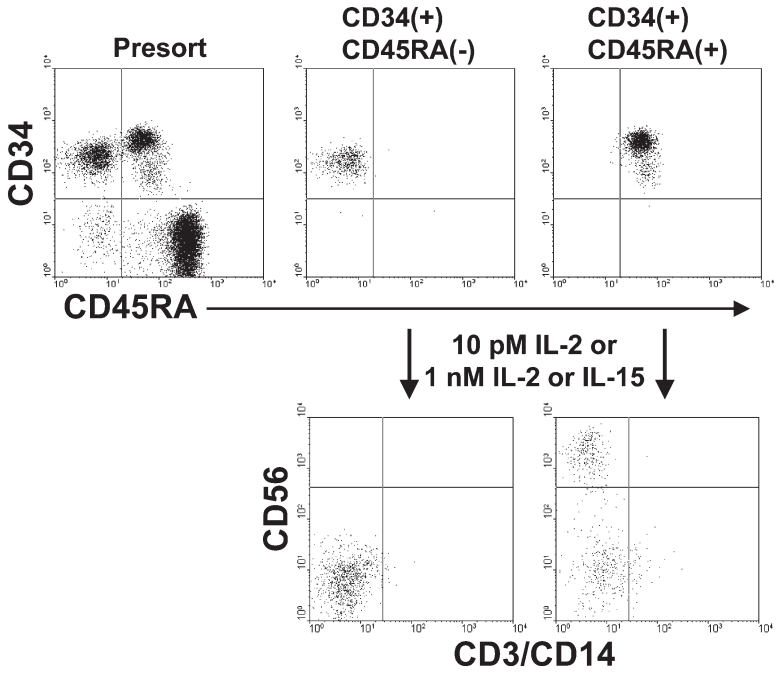

Figure 1. Coexpression of CD45RA on CD34(+) HPCs Identifies the Human NK Precursor

CD34(+)CD45RA(–) and CD34(+)CD45RA(+) populations from BM (n = 3) and PB (n = 7) were sorted and cultured for 2 weeks in 10 pM IL-2, 1 nM IL-2, or 1 nM IL-15. Results shown are representative. Only CD34(+) CD45RA(+) cells differentiate into CD56^{bright} NK cells in IL-2 or IL-15.

cursors, as determined by differentiation into CD56^{bright} NK cells following incubation in IL-2 or IL-15 (Canque et al., 2000; Galy et al., 1995; Miller et al., 1994). Thus, the "all-inclusive" human CD34(+) NK precursor cell remains to be identified.

Here, we identify a novel subset of CD34^{dim} HPCs that constitutively expresses CD45RA and high-surface density integrin $\alpha_4\beta_7$, with functional evidence for expression of the heterotrimeric high-affinity (HA) IL-2R$\alpha\beta\gamma$. The CD34^{dim}CD45RA(+)β_7^{bright} subset of CD34(+) HPCs is selectively and highly enriched for within human LN and resides in the T cell-rich regions along with CD56^{bright} NK cells. The CD34^{dim}CD45RA(+)β_7^{bright} subset is unique in that differentiation into functional CD56^{bright} NK cells appears to occur exclusively within this CD34(+) HPC subset and can occur in the presence of IL-15 or at concentrations of IL-2 that only saturate its HA IL-2R. Further, we show that activated LN T cells can also promote the differentiation of the CD34^{dim}CD45RA(+)β_7^{bright} NK precursor into a CD56^{bright} NK cell in vitro. The data support a model of human NK development in which a BM-derived CD34^{dim}CD45RA(+)β_7^{bright} NK cell precursor population selectively resides in LN where endogenous cytokines can drive its differentiation into CD56^{bright} NK cells in vivo.

Results

Human NK Precursors Are Enriched within Peripheral Blood

The CD56^{bright} NK cell is the only resting lymphocyte population in blood to constitutively express a heterotrimeric HA IL-2R$\alpha\beta\gamma$ (Caligiuri et al., 1990; Nagler et al., 1990). To identify its precursor, we first searched for a subset of CD34(+) HPCs that might also express the HA IL-2R$\alpha\beta\gamma$ and differentiate into a CD56^{bright} NK cell via signaling through this receptor. The HA IL-2R$\alpha\beta\gamma$ is unique in that it can signal following the binding of very low concentrations (10 pM or 2.3 U/ml) of IL-2, and this binding can be completely abrogated with the anti-IL-2Rα monoclonal antibody (mAb) (Queen et al., 1989). This population was found entirely within the CD45RA(+) subset of CD34(+) HPCs of both BM and PB (Figure 1). We observed identical results in nanomolar concentrations of either IL-15 or IL-2 that utilize the shared IL-2/IL-15R$\beta\gamma$, but not the IL-2Rα (Waldmann et al., 1998) (not shown). Importantly, there was a significant difference in the absolute numbers of CD56^{bright} NK cells derived from 2 × 10^4 CD34(+) BM HPCs (537 ± 340, n = 9) versus an equal number of PB CD34(+) HPCs (6831 ± 4329, n = 5) (p = 0.0013), suggesting that PB contains a much higher percentage of CD34(+) NK precursors than BM (see below).

Functional and Phenotypic Characterization of the CD34(+)-Derived CD56^{bright} NK Cells

The CD56^{bright} NK cells derived under these conditions from CD34(+) HPCs were cytotoxic against the NK-sensitive K562 cell line (Figure 2A) and capable of cytokine production when costimulated in recombinant monokines (Figure 2B and Table S1; see the Supplemental Data available with this article online). Similar to PB CD56^{bright} NK cells, these cells could not produce cytokines when only cocultured with K562 targets (not shown). Figures 2C and 2D provide a representative phenotype of CD34(+)-derived CD56^{bright} NK cells. We did not observe any consistent phenotypic differences between BM or PB CD34(+)-derived CD56^{bright} NK cells, but a few distinct differences in phenotype were noted between these cells (Figure 2C) and mature PB CD56^{bright} NK cells (Figure S1). For example, fresh or in vitro-cultured mature PB CD56^{bright} NK cells display uniform expression of CD94 (bright) and leukocyte function-associated antigen-1 (LFA-1), whereas most of

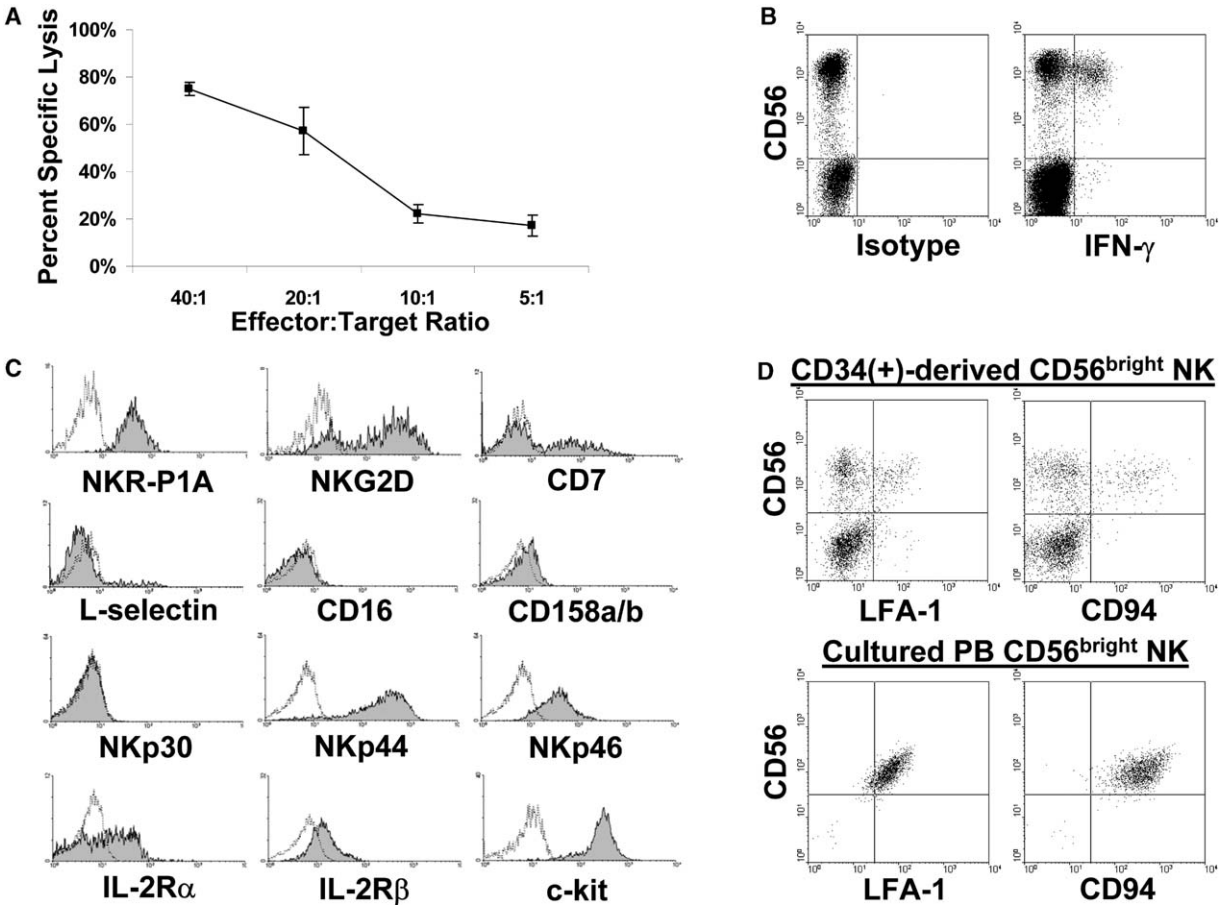

Figure 2. Functional and Phenotypic Attributes of CD34(+)-Derived CD56^{bright} NK Cells

(A) CD34(+)-derived CD56^{bright} NK cells are cytotoxic against K562 target cells at the indicated effector:target cell ratios. Results are representative of six experiments.

(B) IFN-γ production by CD34(+)-derived CD56^{bright} NK cells. The dot plots were gated on CD3(–)CD14(–) events.

(C) Surface expression of CD34(+)-derived CD56^{bright} NK cells. The histograms were gated on CD3(–)CD14(–)CD56^{bright} events; shaded regions represent staining with the specific mAbs as indicated, whereas dotted lines (open regions) represent isotype controls. No consistent differences in function or phenotype were observed between CD56^{bright} NK cells derived from CD34(+) cells of BM or PB origin.

(D) Representative phenotypic comparison of CD56^{bright} NK cells derived from CD34(+) HPCs following a 2 week culture in IL-2 or IL-15 versus purified mature PB CD56^{bright} NK cells cultured for 2 weeks in IL-2 or IL-15. Comparing Figure 2C and Figure S1 also shows a difference in NKp44 expression.

the CD34(+)-derived CD56^{bright} NK cells lack these antigens (Figure 2D). Given these qualitative differences in phenotype, the consistent high purities of our CD34 preparations (Figure S2), and the very limited growth potential of mature PB CD56^{bright} NK cells cultured ex vivo (Fehniger et al., 2000), the detection of CD56^{bright} NK cells following prolonged cultures of CD34(+) HPCs with IL-2 or IL-15 cannot result from contamination by mature PB CD56^{bright} NK cells. Rather, these data collectively show that the human NK precursor cell that differentiates to a CD56^{bright} NK cell in the presence of IL-15 or IL-2 is found exclusively within CD34(+)CD45RA(+) HPCs and is more abundant in PB than in BM.

Refinement of the Phenotype of the CD34(+)CD45RA(+) NK Cell Precursor

Our statistical analyses of NK precursor frequency in BM and PB noted above suggested that these CD34(+)CD45(+) NK precursor cells may be trafficking out of the BM to the periphery. We therefore analyzed surface expression of homing and chemokine receptors and cell adhesion molecules (CAMs) on PB CD34(+) HPCs. We observed that all PB CD34(+) cells express similar levels of $\alpha_4\beta_1$ integrin and PEN5 (not shown), whereas CD34(+)CD45RA(+) cells display relatively higher levels of LFA-1 compared to CD34(+) CD45RA(–) HPCs (Figure 3). While PB CD56^{bright} NK cells express CCR7 and CXCR3 (Cooper et al., 2001; Vitale et al., 2004), we could not detect the expression of either of these chemokine receptors on any PB CD34(+) subset (not shown). Interestingly, we found that among total PB CD34(+) cells, a unique CD34^{dim}CD45RA(+) subset expresses very high levels of L-selectin and integrin $\alpha_4\beta_7$ (represented by integrin β_7, Figure 3). Of note, these cells are distinct from CD34^{dim}CD45RA(+)CD4(+) IL-3Rα^{bright} pro-DC2 cells (Blom et al., 2000), which are IL-2Rα(–)c-kit(–)$\alpha_4\beta_7^{dim}$ (unpublished data) and were depleted from our PB CD34 preparations with an anti-

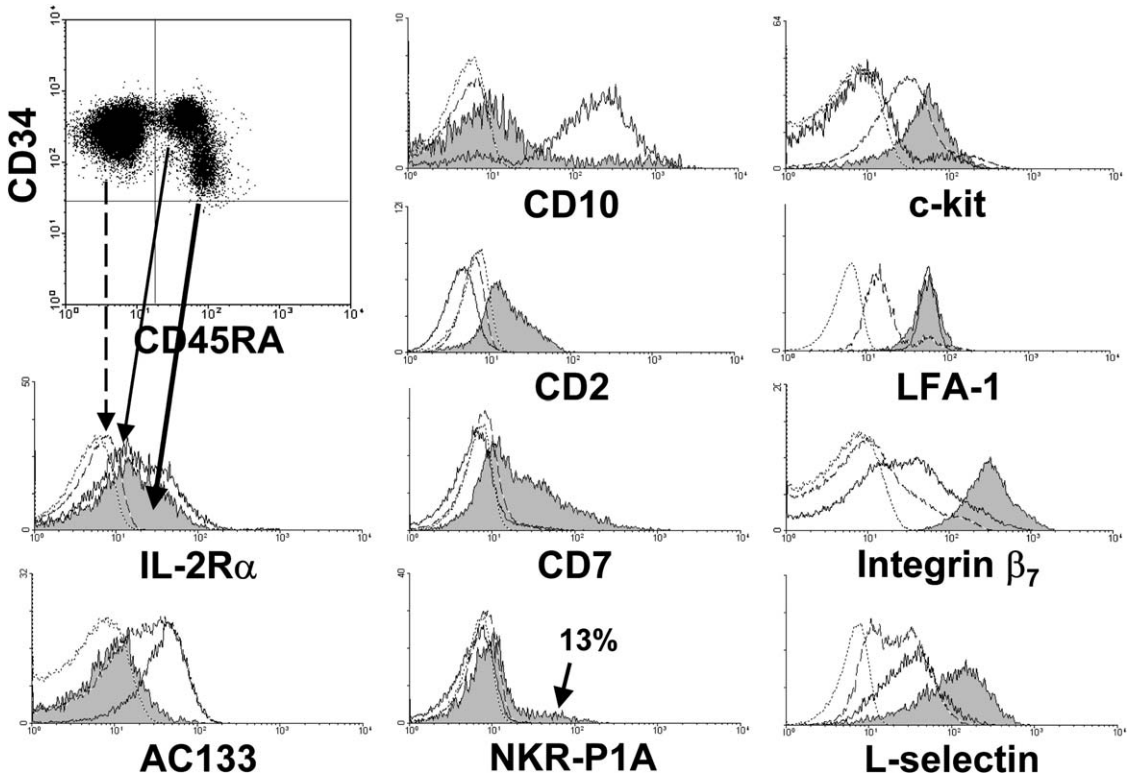

Figure 3. Phenotypic Analysis of PB CD34(+) HPC Subsets

Dotted lines (open regions) in the histograms represent isotype control staining of total PB CD34(+) cells, shaded regions represent surface expression on CD34^{dim}CD45RA(+) cells, solid lines (open regions) represent expression on CD34^{bright}CD45RA(+) cells, and dashed lines (open regions) represent expression on CD34(+)CD45RA(–) cells. Prior to staining for flow cytometry, CD19(+) and CD4(+) cells, including pro-B (Lassoued et al., 1993) and pro-DC2 (Blom et al., 2000) HPCs, were removed (see the Experimental Procedures). The data shown are representative of ten donors.

CD4 mAb (see the Experimental Procedures). The PB CD34^{dim}CD45RA(+) subset we describe here is strikingly reminiscent of mature PB CD56^{bright} NK cells based on its expression of CD2, CD7, NKR-P1A, and c-kit (shaded histograms in Figure 3 and Figure S1) (Cooper et al., 2001). In contrast, the majority of PB CD34^{bright}CD45RA(+) cells (open regions with solid lines in Figure 3) lack these markers yet express surface CD10, suggesting that this population may be functionally similar to the CD34(+)CD10(+) CLP previously described in adult BM (Galy et al., 1995). In addition, the CD34^{bright}CD45RA(+) subset expresses the early stem cell marker, AC133, potentially indicating that this subset is relatively immature compared to the CD34^{dim} CD45RA(+) subset that displays no AC133 (Figure 3) (Yin et al., 1997).

Subtle overlaps in phenotype between the two PB CD34(+)CD45RA(+) populations presented an initial challenge to sort these subsets to high purity. For example, not all the CD34^{dim}CD45RA(+) cells are CD10(–), and not all the CD34^{bright}CD45RA(+) cells are CD7(–) (Figure 3). Among the surface markers we analyzed, relative bright expression of integrin β_7 best differentiated the CD34^{dim}CD45RA(+) subset not only from other CD34(+) subsets, but also from mature CD56^{bright} and CD56^{dim} NK cells, which have absent or low expression of integrin β_7, respectively (Figure S1 and data not shown). Therefore, we used a combination of mAbs against CD34, CD45RA, and integrin β_7 to purify the two PB CD34(+)CD45RA(+) subsets and to reduce the potential for mature NK cell contamination in our sorts, a representative of which is shown in Figure 4. After 2 weeks of culture in 10 pM IL-2 or 1 nM IL-15 or IL-2, we observed that the CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ subset repeatedly gave rise to CD3(–)CD14(–)CD56^{bright} NK cells (n = 11) by FACS with excellent purity (Figure 4). Generally, we observed only ~1- to 3-fold expansion when the cells were cultured in 10 pM IL-2, and ~15-fold expansion when cultured in 1 nM IL-2 or IL-15 (Figure S3). However, there was much greater expansion of these cells when cultured on the AFT024 fetal liver stromal cell line (Miller et al., 1999) in the presence of 1 nM IL-2 and 100 ng/ml flt3 ligand (FL), 100 ng/ml c-kit ligand (KL), and 10 ng/ml IL-7 (Figure S3), indicating that these cells are highly proliferative when cultured in the presence of excess cytokines and cellular support. The CD56^{bright} NK cells derived on the AFT024 line displayed the same phenotype as that shown in Figures 2C and 2D (not shown). In stark contrast to the results obtained from the CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ subset, most of the CD34^{bright}CD45RA(+)$\beta_7{}^{dim/neg}$ cells died in culture with only IL-2 or IL-15 (not shown), and none of the

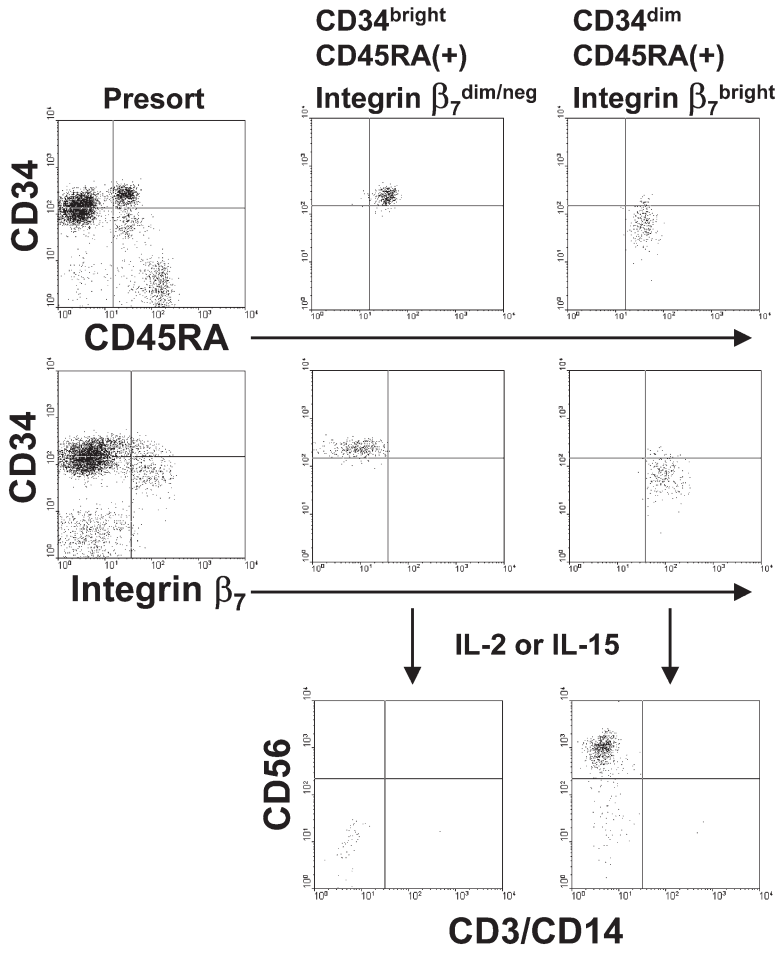

Figure 4. The PB CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ Subset Uniquely Differentiates into CD56^{bright} NK Cells in IL-2 or IL-15

PB CD34^{bright}CD45RA(+)$\beta_7{}^{dim/neg}$ and CD34^{dim} CD45RA(+)$\beta_7{}^{bright}$ cells were sorted and subsequently cultured for 2 weeks in 10 pM IL-2 or 1 nM IL-2 or IL-15, followed by FACS analysis to assess for CD3(–)CD14(–)CD56^{bright} NK cell development. The results are representative of 11 donors.

live cells had the CD3(–)CD14(–)CD56^{bright} phenotype (Figure 4). Based on these results and those from Figure 1, we conclude that the CD56^{bright} NK precursor population is contained exclusively within the CD34^{dim} CD45RA(+)$\beta_7{}^{bright}$ PB subset.

CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ Cells Reside in Human LN

In contrast to PB, where $\geq$90% of NK cells are CD56^{dim}, >90% of NK cells in human LN are CD56^{bright} and are located in the parafollicular T cell-rich regions (Fehniger et al., 2003). One potential model predicts that CD56^{bright} NK cells develop in the BM and traffic to LN, where they participate in the immune response through release of IFN-γ and other cytokines (Colucci et al., 2003; Cooper et al., 2004; Fehniger et al., 2003; Ferlazzo et al., 2004; Vitale et al., 2004). An alternative model is that BM-derived NK precursors first traffic through PB to LN, where they can differentiate into CD56^{bright} NK cells in response to endogenous cytokines. Indeed, consistent with the significantly greater number of CD56^{bright} NK cells derived from PB versus BM CD34(+) HPCs noted above, <1% of BM CD34(+) HPCs display the phenotype of the CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ population shown in Figure 3 (n = 5), compared to ~6% of PB CD34(+) HPCs (n = 20). These data support the possibility that at least a subset of NK precursors is destined for the periphery. Further, in addition to integrin $\alpha_4\beta_7$, our data also show that the PB CD34^{dim} CD45RA(+)$\beta_7{}^{bright}$ subset displays very high surface density expression of LFA-1 and L-selectin (Figure 3), all three of which can facilitate the extravasation of leukocytes across LN high endothelial venules (von Andrian and Mempel, 2003).

To test the hypothesis that CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ cells reside in LN, phenotypic analyses of CD34(+)-enriched single-cell LN suspensions from eight individual donors were performed. In striking contrast to PB, nearly the entire CD34(+) population discovered within human LN was CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ (Figures 5A and 5B). Thus, while the CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ subset represents only ~6% of all PB CD34(+) HPCs, this subset represents >95% of all LN CD34(+) HPCs (n = 8). This natural and nearly exclusive enrichment for the CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ subset of CD34(+) HPCs in LN eliminates PB contamination as the source of these cells. Further, PB CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ cells express high levels of surface L-selectin, while the LN CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ cells had lower or absent expression (compare Figure 3, bottom right panel to Figure 5B). This suggests that L-selectin may be involved in the extravasation of PB CD34^{dim}CD45RA(+)$\beta_7{}^{bright}$ cells into LN and may subsequently downregulate upon

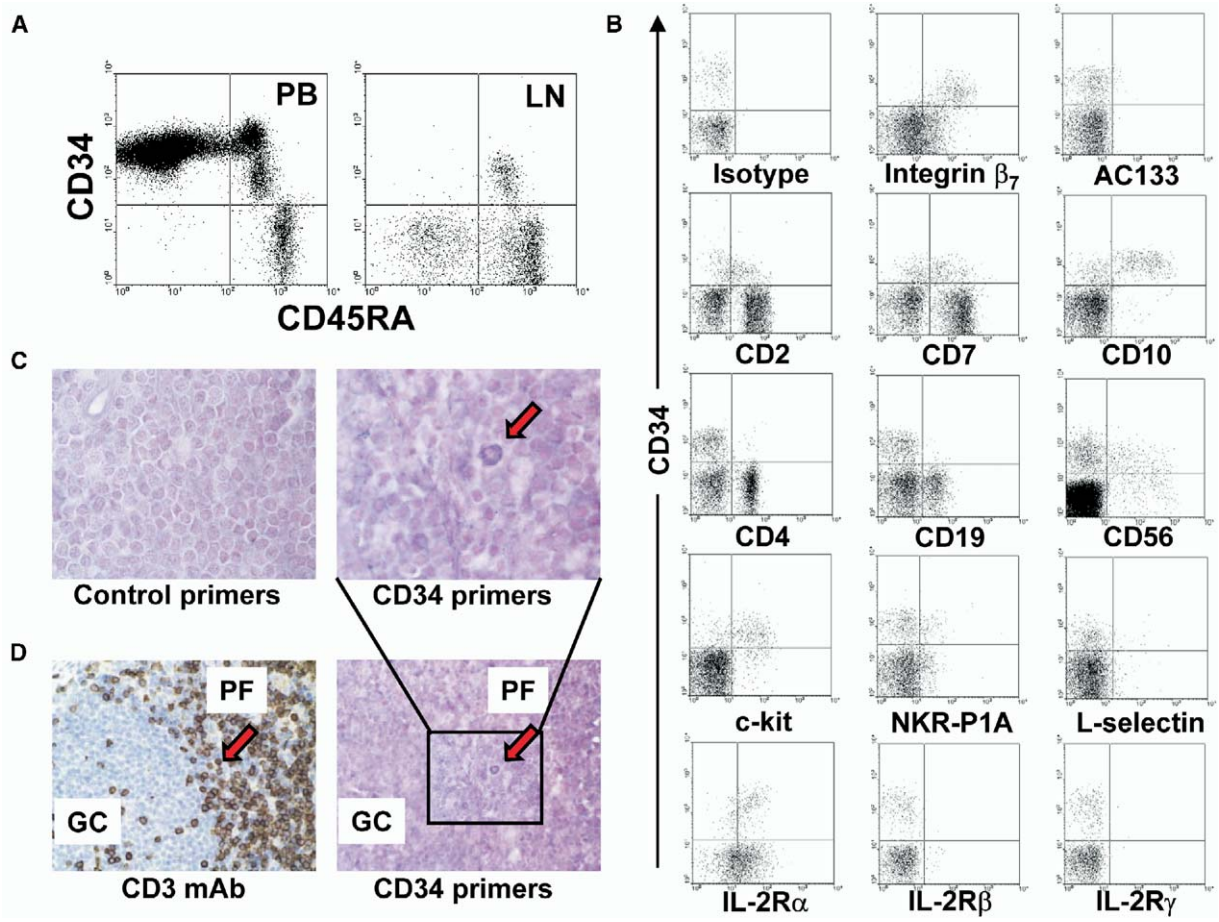

Figure 5. Discovery of a Unique Population of CD34(+) HPCs in Human LN

(A) Comparative flow cytometric analysis of CD34(+) HPC subsets in PB and LN following enrichment for CD34(+) cells in each tissue. In contrast to PB, LN CD34(+) cells are almost exclusively $CD34^{dim}CD45RA(+)\beta_7^{bright}$.

(B) Surface antigen expression of enriched LN CD34(+) cells as determined by flow cytometric analysis. The $CD34^{dim}CD45RA(+)\beta_7^{bright}$ subset in LN is strikingly similar to the subset in PB, except for the former's low or absent L-selectin expression (compare with bottom right panel in Figure 3).

(C) In situ RT-PCR for CD34 mRNA on human LN sections. The power of these images is 1000×. Control primers used in the left panel were specific for the human papilloma virus (HPV) *p16* gene (Nuovo et al., 1999). The red arrow in the right panel indicates a representative LN CD34(+) cell detected by this method. Note the uniform cytoplasmic signal coming from this cell, which is indicative of cytoplasmic CD34 mRNA.

(D) CD34(+) cells reside within the parafollicular T cell-rich regions of LN. Shown in the right panel is the same LN section depicted on the right in (C), but at a lower power (200×). The red arrow in both images indicates the location of the CD34(+) cell detected by in situ RT-PCR. On the left is a serial section from the same region of the LN stained with an anti-CD3 mAb. Cells with brown staining are CD3(+). GC, germinal center; PF, parafollicular region.

entrance (von Andrian and Mempel, 2003). As shown in Figure 5C, we were able to detect CD34(+) cells within LN tissue sections by using in situ RT-PCR (note the uniform cytoplasmic staining within the indicated cell). The frequency of CD34(+) cells detected by this method corresponds to an estimate of ~1 CD34(+) cells per 35,000–50,000 total LN cells. This is in agreement with the predicted frequency from our flow cytometry data that shows the cell to represent <0.05% of all events without CD34(+) enrichment when using a live forward scatter/side scatter gate (not shown). To our knowledge, this represents the first identification of CD34(+) HPCs in human LN. Collectively, these results support the notion that among all PB CD34(+) HPCs, it is the $CD34^{dim}CD45RA(+)\beta_7^{bright}$ subset that exclusively contains the $CD56^{bright}$ NK precursor and selectively resides in LN. Formal proof that this cell actually traffics from PB to LN will await further study.

T Cell Activation Promotes $CD56^{bright}$ NK Differentiation In Vitro

Immunohistochemistry (IHC) staining on serial LN sections with an anti-CD3 mAb revealed that the CD34(+) HPCs we observed by in situ RT-PCR (Figure 5C) were located within T cell-rich regions of LN sections (Figure 5D), where we previously reported that $CD56^{bright}$ NK cells also reside (Fehniger et al., 2003). To recapitulate what might occur in vivo during T cell activation, LN

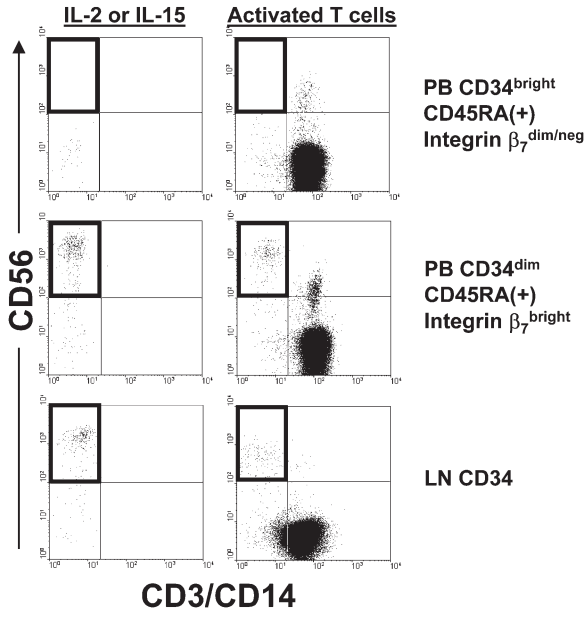

Figure 6. T Cell Activation Supports CD56^{bright} NK Cell Differentiation from CD34(+) HPCs In Vitro

PB CD34^{bright}CD45RA(+)$\beta_7^{dim/neg}$ and CD34^{dim}CD45RA(+)β_7^{bright} subsets (n = 6) or total LN CD34(+) HPCs (>95% CD34^{dim} CD45RA(+)β_7^{bright}) (n = 3) were cultured for 7 days in exogenous IL-2 or IL-15 (left column) or cocultured for 7 days with autologous PB or LN CD3(+) T cells activated via CD3/CD28 stimulation (right column). The CD3(–)CD14(–)CD56^{bright} NK cells derived from these cultures are seen in the upper left quadrant of each histogram. In data not shown, a culture of sorted LN CD3(+) T cells stimulated with anti-CD3/CD28 beads did not produce any CD56^{bright} NK cells without coculture of the CD34(+) HPCs.

CD34(+) HPCs were cocultured with autologous LN CD3(+) T cells in the presence of CD3/CD28 stimulation. As shown in Figure 6, we observed CD3(–)CD14(–) CD56^{bright} NK cell development after only 7 days under these conditions, along with a >10-fold increase in activated T cell numbers (not shown). We similarly cultured purified PB CD34^{bright}CD45RA(+)$\beta_7^{dim/neg}$ and CD34^{dim} CD45RA(+)β_7^{bright} subsets with autologous PB CD3(+) T cells and observed that, similar to the results obtained by a 7-day culture in exogenous IL-2 or IL-15, only CD34^{dim}CD45RA(+)β_7^{bright} cells gave rise to NK cells in the presence of CD3/CD28-stimulated T cells (Figure 6). The addition of an anti-IL-2 mAb to these cocultures resulted in variable (average 25%, n = 9) reduction in CD56^{bright} NK cell development, likely due to the contribution of other endogenous factors (e.g., IL-7, IL-15, IL-21, KL) that might directly or indirectly contribute to this process. Thus, activated LN T cells, in close proximity to LN CD34^{dim}CD45RA(+)β_7^{bright} HPCs, can induce human CD56^{bright} NK cell differentiation without the addition of exogenous cytokines.

Discussion

NK cells are innate immune effectors that serve a number of important functions in the body's defense against infection and malignant transformation. In one instance, the NK cell's provision of IFN-γ to monocytes/macrophages recently infected by obligate intracellular pathogens results in a critical short-term containment of infection while the more sustaining T cell response can be mounted (Bendelac and Fearon, 1997). In other instances, donor NK cell KIR mismatch with host MHC class I expression on acute myeloid leukemia blasts appears to be critical for predicting a favorable response following haplo-identical bone marrow transplantation (Ruggeri et al., 2002). Thus, both cytokine production and cytolytic activity are important NK cell properties in mediating effective host defense against disease. In humans, these two NK functions can be broadly assigned to two NK subsets. CD56^{bright} NK cells have few cytolytic granules and low-absent KIR expression but produce abundant cytokines and chemokines when activated by monokines. CD56^{dim} NK cells do not produce appreciable amounts of cytokines yet have abundant expression of KIR and perforin that readily promote their potent cytolytic functions (Cooper et al., 2001). Greater than 90% of NK cells in LN are CD56^{bright}, while ~90% of NK cells in PB are CD56^{dim} (Fehniger et al., 2003).

While human NK cells originate from CD34(+) HPCs, the site(s) of their differentiation and the developmental relationship between CD56^{bright} and CD56^{dim} NK subsets are currently unresolved (Colucci et al., 2003). To date, no one CD34(+) subset has been shown to contain all human NK precursors when differentiated to CD56(+) NK in the presence of IL-2 or IL-15. We previously noted that the CD56^{bright} NK cell has unique constitutive expression of the heterotrimeric HA IL-2R$\alpha\beta\gamma$ among lymphocytes in resting blood (Caligiuri et al., 1990) and hypothesized that its CD34(+) precursor might also express this receptor and differentiate into a CD56^{bright} NK cell when the HA IL-2R was saturated. In this report, we used this functional assay to initially identify a novel CD34(+) subset of HPCs, subsequently characterized as CD34^{dim}CD45RA(+)β_7^{bright}, which, when cultured in IL-15 or IL-2, appears to contain the NK precursor population in its entirety. The CD34^{dim}CD45RA(+)β_7^{bright} NK precursor cell coexpresses other surface receptors and CAMs that facilitate trafficking to LN (von Andrian and Mempel, 2003), and we discovered that, among CD34(+) HPCs, the CD34^{dim}CD45RA(+)β_7^{bright} population is uniquely enriched within human LN. In addition to its NK development in the presence of IL-2 or IL-15, we demonstrate that endogenous T cell-derived cytokines can also drive the CD34^{dim}CD45RA(+)β_7^{bright} HPCs toward NK cell differentiation. We therefore propose that this unique subset of CD34(+) HPCs is produced in the BM and traffics through the blood to finally reside in LN, where it differentiates into a CD56^{bright} NK cell under the influence of endogenous cytokines.

Despite the results presented here, such a model challenges the current conception of NK development in vivo. First of all, while it is well established that the human thymus contains NK precursors (Lanier et al., 1992; Spits et al., 1998), it is generally accepted that, in adults, NK development primarily occurs in the BM, as its ablation results in the loss of lytic NK cells that can be restored by BM transplantation (Colucci et al., 2003; Hackett et al., 1985). However, this does not preclude

the possibility that BM-derived NK precursors may traffic to the periphery for final maturation. For instance, adult human PB contains immature CD34(–)NKRP1A(+)CD16(–)CD56(–) NK cells that mature in the presence of IL-2 and IL-12 (Bennett et al., 1996). Further, Ferlazzo et al. (2004) recently suggested that late events in human CD56^{bright} NK maturation likely occur in peripheral lymphoid tissue, as the resident CD56^{bright} NK cells in LN and tonsils can be induced with low-dose IL-2 to adopt functional and phenotypic qualities of PB NK. Our new finding that human CD34^{dim} CD45RA(+)β_7^{bright} HPCs reside within LN and our previous finding that their CD56^{bright} NK progeny are highly enriched in LN (Fehniger et al., 2003) strongly implicate the LN as a site of early human NK cell differentiation.

In adult mice, NK development is similarly thought to occur in the BM, yet it is noteworthy that selective NK deficiencies are found in mice lacking LN, while T or B cells are not deficient (Colucci et al., 2003). During mouse embryonic development, integrin $\alpha_4\beta_7$(+) fetal lymphoid tissue-inducer (LTi) cells traffic to developing LN and Peyer's patches (PP) and induce formation of these tissues (Mebius, 2003). Id2$^{-/-}$ mice lack fetal LTi cells and therefore fail to develop peripheral LN and PP (Yokota et al., 1999). Similarly, the absence of membrane lymphotoxin (LT)β expression on LTi cells also results in failure of these tissues to develop (Mebius, 2003). As fetal LTi cells have the potential to differentiate into mature NK cells (Mebius et al., 1997), it is noteworthy that both Id2$^{-/-}$ and LT$\beta^{-/-}$ mice lack mature NK cells (Alimzhanov et al., 1997; Yokota et al., 1999). In light of these reports, it is interesting to speculate that human CD34^{dim}CD45RA(+)β_7^{bright} HPCs may represent a similar cell population in man, and, if so, potentially regulate or maintain the generation of human secondary lymphoid organs in vivo.

Our ex vivo studies show that soluble IL-15, like IL-2, specifically acts on the CD34^{dim}CD45RA(+)β_7^{bright} HPCs to drive CD56^{bright} NK cell differentiation. Further, cultures with other cytokines, such as KL, FL, IL-7, and the AFT024 fetal liver stromal cell line also indicate that either IL-2 or IL-15 are required for NK cell differentiation. Thus, in vivo, these two cytokines can likely both induce NK cell differentiation from the LN CD34^{dim} CD45RA(+)β_7^{bright} precursor, but their expression on different immune cells and their differential expression during immune quiescence and activation may dictate how and when each contributes to this process.

While the LN CD34^{dim}CD45RA(+)β_7^{bright} HPCs constitutively express the HA IL-2R$\alpha\beta\gamma$ that selectively binds IL-2, the highly restricted availability of IL-2 strongly suggests that it alone cannot be responsible for CD56^{bright} NK cell development in the absence of T cell activation. We propose that in the absence of antigen-specific immune activation, CD56^{bright} NK cell differentiation is regulated by cytokines known to be constitutively expressed by stroma and antigen-presenting cells (e.g., FL, KL, and the requisite IL-15). Then, during processes that activate resident T cells in LN, endogenously produced IL-2, along with other cytokines, might contribute to drive CD56^{bright} NK cell differentiation from LN CD34^{dim}CD45RA(+)β_7^{bright} HPCs. These cytokines also promote autocrine LN T cell activation and paracrine LN CD56^{bright} NK cell activation (Fehniger et al., 2003). In this way, NK stores that were depleted during the early phase of infection could be replenished during the late, antigen-specific phase of infection. Future studies with in vivo models will be important to address the hypothesis that adaptive immune activation can drive NK development.

In any of these scenarios, we would propose that IL-2 is not required for the survival of either the CD34^{dim}CD45RA(+)β_7^{bright} HPC subset or the CD56^{bright} NK cell, because of IL-2's restricted expression (Waldmann et al., 1998). We believe that the tyrosine kinase receptors, flt3 and c-kit, which we observed are also expressed on CD34^{dim}CD45RA(+)β_7^{bright} precursors, likely mediate the survival signal(s) for this subset and may be involved in its development, as incubation of BM CD34(+) HPCs in either FL or KL increases the frequency of NK cell precursors (Yu et al., 1998). Indeed, FL is produced in the BM, while KL is normally abundant in both BM and human serum (Colucci et al., 2003; Mrozek et al., 1996; Wodnar-Filipowicz, 2003). Further, KL binding and signaling through c-kit expressed on CD56^{bright} NK cells mediates BCL-2-dependent survival in serum-free medium (Carson et al., 1994). In addition, other cytokines signaling through the common IL-2Rγ chain (Barao et al., 2003; Sivori et al., 2003) as well as membrane bound LTβ (Iizuka et al., 1999) also likely play important roles in the generation, survival, and/or maturation of CD34^{dim}CD45RA(+)β_7^{bright} HPCs.

The insights gained from our study do not appear to help resolve the issue of human CD56^{bright} and CD56^{dim} NK subset development. CD56^{bright} NK cells may have a pathway of development that is distinct from the CD56^{dim} NK cell that lacks a functional HA IL-2R (Caligiuri et al., 1990). In our short-term cultures of the CD34^{dim}CD45RA(+)β_7^{bright} precursor with IL-2 or IL-15, we never observed the development of bona fide [CD16(+)KIR(+)c-kit(–)] CD56^{dim} NK cells (Cooper et al., 2001), yet we currently have no data to suggest that the CD56^{dim} NK cell is derived from a separate HPC. Therefore, an alternative hypothesis is that a developing NK cell may first proceed from CD56^{bright} to the CD56^{dim} NK cell, but again, data supporting this pathway are lacking (Colucci et al., 2003).

In summary, we identify a novel CD34^{dim}CD45RA(+) β_7^{bright} NK precursor cell that is uniquely enriched to reside within the parafollicular T cell-rich region of human LN. We demonstrate that incubation of the CD34^{dim}CD45RA(+)β_7^{bright} NK precursor cell with activated LN T cells can induce differentiation into a CD56^{bright} NK cell. These data implicate the human LN as a site for CD56^{bright} NK cell development.

Experimental Procedures

Purification of CD34(+) HPCs from Human Tissue

All protocols were approved by The Ohio State University (OSU) Institutional Review Board. Fresh human BM was donated or purchased from AllCells, LLC (Berkeley, CA) and was received within 24 hr of harvest. PB leukopaks were obtained from the American Red Cross. BM or PB mononuclear cells were enriched by a ficoll centrifugation step and then total CD34(+) cells were either enriched over one magnetic column or purified (>97%, Figure S2) over two magnetic columns by using the Miltenyi CD34(+) enrichment kit (Miltenyi Biotec, Auburn, CA). For sorting experiments and phenotypic analyses, total PB was treated with a rosette cocktail

against CD3, CD4, CD19, CD36, and glycophorin A (StemCell Technologies, Vancouver, BC, Canada) to deplete cells expressing these markers during the ficoll centrifugation step. Human LN were retrieved fresh from surgically discarded tissue from non-cancer patients by the OSU Tissue Procurement Resource and from the National Disease Research Interchange. LN single-cell suspensions were prepared as described (Fehniger et al., 2003), and total CD34(+) cells were enriched as described above. LN CD34(+) HPCs were either stained for flow cytometric analyses or sorted to purity for cell culture (Figure S2).

Flow Cytometry and Cell Sorting

All conjugated and unconjugated experimental and isotype control mAbs used in this report were purchased from BD Biosciences, except CD16, CD56, CD122 (IL-2Rβ), CD158a, CD158b, NKp30, NKp44, NKp46 (Coulter, Miami FL), NKG2D (R&D Systems, Minneapolis, MN), and AC133 (Miltenyi Biotec, Auburn, CA). Nonspecific binding was minimized by preincubation with whole mouse IgG (direct primary staining) or whole goat IgG (indirect staining) (Sigma, St. Louis, MO). Cells were assessed on a FACSCalibur analyzer and analyzed with CellQuest (BD Biosciences) or WinMDI (J. Trotter, Scripts Institute, La Jolla, CA) software. For culture experiments of purified CD34(+) subsets, cells were sorted on a FACSVantage cell sorter (BD Biosciences), and then sort purities were verified on a FACSCalibur.

NK Cell Development Cultures

IL-2 was provided by Hoffman LaRoche (Nutley, NJ) and was used at the indicated concentrations. FL and IL-15 were provided by Amgen (Thousand Oaks, CA), and KL and IL-7 were from R&D Systems. Unless otherwise indicated, human NK development cultures were initiated with 2×10^4 CD34(+) HPCs in 200 μl complete medium consisting of 1640 RPMI with GlutaMAX, 10% heat-inactivated human AB serum (ICN Biomedicals, Irvine, CA), antibiotics, 10 mM HEPES, 100 μM nonessential amino acids, 1 mM Na pyruvate (all from Invitrogen), and 50 μM 2-βME (Sigma) plus exogenous cytokines. Half of the culture medium was replaced every 3–4 days. At the time of final harvest, total cells/well were determined on a hemacytometer by using trypan blue dye exclusion. The absolute numbers of mature CD56^{bright} NK cells/well were calculated by multiplying the average numbers of total viable cells from triplicate wells by the average percent of CD3(–)CD14(–)CD56^{bright} NK cells determined by FACS analyses. For CD34(+) HPC/T cell coculture experiments, $0.5–2 \times 10^3$ PB CD34(+) HPC subsets or total LN CD34(+) HPCs were incubated with 2.5×10^3 autologous PB or LN CD3(+) T cells (purified via FacsVantage sorting, Figure S2) stimulated with 5×10^3 anti-CD3/CD28 beads (Dynal Biotech, Brown Deer, WI) in the presence or absence of 10 μg/ml anti-IL-2 or control Ab (R&D Systems). After 7 days of culture, cells were harvested, counted for viability, and stained for flow cytometry.

Functional Analyses of In Vitro-Derived NK Cells

Purified CD34(+) cells were cultured as described above for 2–4 weeks. To assess IFN-γ production by intracellular flow cytometry, cultured cells were pooled and then resuspended at 10^6 cells/ml in complete medium plus 10 ng/ml IL-12 (Genetics Institute, Cambridge, MA) and 100 ng/ml IL-18 (BASF, Worcester, MA) for overnight culture at 37°C. Golgi-plug (BD Biosciences) was added for a 4 hr incubation, and cells were next stained for surface CD3, CD14, and CD56 and then permeabilized with the Cytofix/Cytoperm reagent (BD Biosciences). Cells were stained with either anti-IFN-γ-FITC mAb or isotype control-FITC mAb, washed, and then analyzed by FACS. Alternatively, cultured cells were stimulated with recombinant monokines or 5×10^3 K562 target cells for 72 hr, and then supernatants were analyzed by ELISA for cytokine production by using Quantikine kits from R&D Systems. CD34(+)-derived CD56^{bright} NK cells were tested for their ability to lyse K562 target cells in a standard chromium-release assay as described (Mrozek et al., 1996; Yu et al., 1998).

Immunohistochemistry and In Situ RT-PCR

The protocols we used have been previously described (Fehniger et al., 2003; Nuovo et al., 1999). Briefly, optimal protease digestions of LN sections were followed by overnight incubation in RNase-free DNase (10 U per sample, Boehringer Mannheim, Indianapolis, IN) and one-step RT-PCR by using the rTth system and digoxigenin dUTP (Nuovo et al., 1999). The primer sequences for CD34 mRNA detection were: forward, 5′-accctgtgtctcaacatggca-3′; reverse, 5′-tctctgatgcctgaacat-3′. Additional controls included pretreatment with RNase digestion as well as RT-PCR with irrelevant primers (HPV p16-specific primers) that have been described (Nuovo et al., 1999). IHC with an anti-human CD3 mAb (Zymed Laboratories, Inc., San Francisco, CA) was performed as described (Fehniger et al., 2003).

Statistical Analyses

The paired data were analyzed by using an exact Wilcoxon Signed Rank test, and the unpaired data were analyzed by using an exact Wilcoxon Rank Sum test. S-Plus version 6.0 and SAS version 8.02 were used for the analyses.

Supplemental Data

Supplemental Data including phenotypic analysis of PB CD56^{bright} NK cells, sample purities of sorting experiments, cell proliferation data of purified CD34^{dim}CD45RA(+)β_7^{bright} PB NK precursors, and cytokine production data from CD34(+)-derived NK cells are available at http://www.immunity.com/cgi/content/full/22/3/295/DC1/.

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