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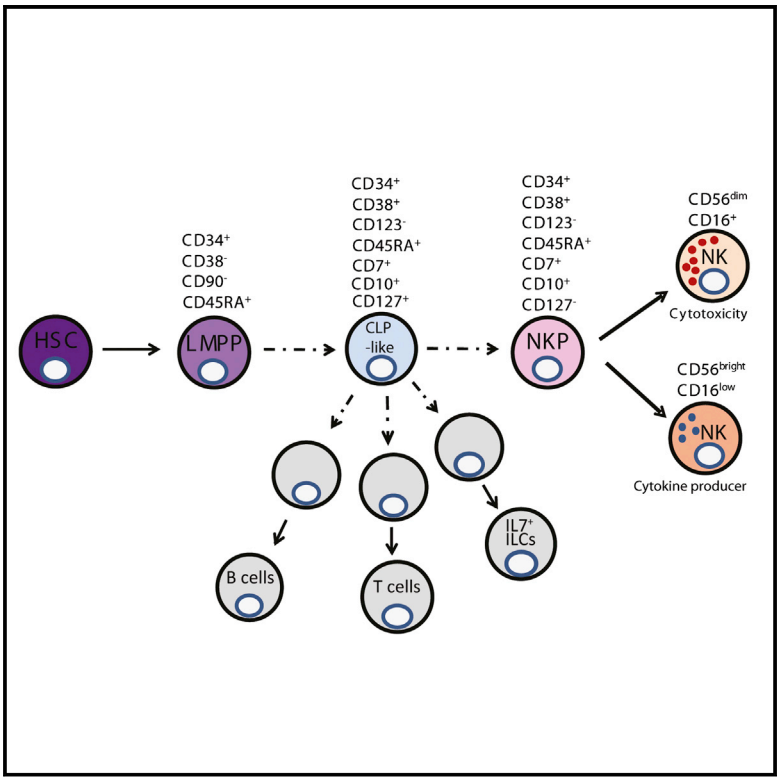
Immunity

Identification of a Human Natural Killer Cell Lineage-Restricted Progenitor in Fetal and Adult Tissues

Graphical Abstract



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In Brief

Natural killer (NK) cells are innate lymphocytes specialized in cytotoxicity against tumor and virus-infected cells and cytokine production, but their development in humans is not well understood. Sitnicka and colleagues identify a novel hematopoietic progenitor conserved throughout ontogeny that has robust NK cell potential, restricted to NK lineage.

Highlights

- $CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ cells represent NK lineage progenitor (NKP)
- NKP is located downstream of LMPP and CLP within the human hematopoietic hierarchy
- NKP generates mature functional NK cells in vitro and in vivo but no other lineages
- NKP is conserved throughout ontogeny and is found in human adult and fetal tissues

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Identification of a Human Natural Killer Cell Lineage-Restricted Progenitor in Fetal and Adult Tissues

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SUMMARY

Natural killer (NK) cells are cytotoxic lymphocytes and play a vital role in controlling viral infections and cancer. In contrast to B and T lymphopoiesis where cellular and regulatory pathways have been extensively characterized, the cellular stages of early human NK cell commitment remain poorly understood. Here we demonstrate that a Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ population represents a NK lineage-restricted progenitor (NKP) in fetal development, umbilical cord blood, and adult tissues. The newly identified NKP has robust NK cell potential both in vitro and in vivo, generates functionally cytotoxic NK cells, and lacks the ability to produce T cells, B cells, myeloid cells, and innate lymphoid-like cells (ILCs). Our findings identify an early step to human NK cell commitment and provide new insights into the human hematopoietic hierarchy.

INTRODUCTION

Developmental pathways from hematopoietic stem cells (HSCs) to mature functional blood cells involve multiple stages where progenitor cells, generated in the process, become increasingly restricted in their lineage choices, ultimately developing into fully committed unilineage cells (Orkin and Zon, 2008). In contrast to the mouse, the human hematopoietic hierarchy is less well characterized, and the coordination between lymphoid and myeloid programs is still under scrutiny (Laurenti et al., 2013). Despite the fact that several lymphoid-committed or primed progenitors have been defined downstream of the Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$CD49f$^+$ HSCs in human bone marrow (BM) and umbilical cord blood (CB) (Doulatov et al., 2010; Galy et al., 1995; Hoebeke et al., 2007), the existence and identity of a human common lymphoid progenitor (CLP) is currently less clear (Doulatov et al., 2010; Hoebeke et al., 2007). Moreover, although the early human B and T cell developmental stages have been characterized (Doulatov et al., 2010; Haddad et al., 2004; Hao et al., 2001; Hoebeke et al., 2007), the cellular and regulatory pathways of natural killer (NK) cell development are less understood.

NK cells are classically defined as CD3$^-$CD56$^+$CD16$^+$ cells in human. Several recent studies have identified new populations of innate-like lymphocytes (ILCs) and placed NK cells in group 1 of the expanding family of ILCs (Diefenbach et al., 2014; Hazenberg and Spits, 2014; Narni-Mancinelli et al., 2011; Walzer et al., 2007).

NK cells are found in the peripheral blood, lymph nodes, spleen, liver, peritoneal cavity, placenta, and uterine mucosa (Yu et al., 2013) and are important for controlling viral infections and cancer. NK cells can kill target cells that lack expression of major histocompatibility complex (MHC) class I molecules or that express ligands for activating receptors, e.g., NKG2D, two properties commonly associated with transformed cells (Raulet and Guerra, 2009). Furthermore, by producing cytokines (such as tumor necrosis factor alpha [TNF-α] and interferon gamma [IFN-γ]), NK cells can regulate subsequent adaptive immune responses (Colonna et al., 2011).

In patients with acute myeloid leukemia, transplanted donor NK cells expressing mismatched inhibitory receptors provided graft versus leukemia effect in the absence of graft versus host disease (Ruggeri et al., 2002). These and many other findings are the basis of a large number of ongoing studies and clinical trials for NK cell immunotherapy against cancer (Miller, 2013).

The current prevailing model suggests that adult BM is the primary site of NK cell development (Di Santo, 2006), although early lymphoid progenitors with NK lineage potential have been also identified in human tonsil (Freud et al., 2005), thymus (McClory et al., 2012; Res et al., 1996), and liver (Moroso et al., 2011), suggesting that some subsets of human NK cells develop extra medullary. Maturation of human NK cells is characterized by the loss of CD34 and C-KIT (CD117) expression (Freud et al., 2006), followed by sequential upregulation of CD94, CD16, and

killer cell Ig-like receptors (KIR) (Freud and Caligiuri, 2006), where the expression of the latter three receptors distinguishes NK cells from other members of ILC family. Two functionally distinct mature human NK cell populations exist in the peripheral blood: the $CD56^{dim}CD16^{+}$ cytotoxic NK cells and the $CD56^{bright}CD16^{-}$ NK cells that are mainly producing cytokines (Cooper et al., 2009). The origin of these two NK cell subsets, their developmental relationship, and their regulatory cues are not fully understood (Cooper et al., 2009; Di Santo, 2006). In addition, the identity of human progenitors uniquely restricted to the NK lineage (NKPs) remains unknown (Doulatov et al., 2010, 2012).

Using advanced multicolor flow cytometry with a new combination of markers important for human lymphoid commitment (Haddad et al., 2004; Hao et al., 2001; Kohn et al., 2012), we identified a $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ NK cell-restricted progenitor (NKP), downstream of LMPP ($Lin^{-}CD34^{+}CD38^{-}CD90^{-}CD45RA^{+}$) (Roy et al., 2012) and CLP-like cells ($Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{+}$), in human bone marrow, umbilical cord blood, tonsils, and fetal tissues. NKPs generated fully mature and functional NK cells both in vitro and in vivo and lacked the potential to produce T cells, B cells, ILCs, or myeloid cells at the single-cell level in vitro and after transplantation into newborn $NOD/SCID\gamma c^{null}$ mice. Our identification of the earliest steps of human NK cell commitment, conserved throughout ontogeny, provides new insights into human hematopoiesis with implications for the development of NK cell-based therapies.

RESULTS

$Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ Are Candidate NK Cell Progenitors

Given published reports linking the expression of early lymphoid markers (CD45RA, CD10, CD7) (Doulatov et al., 2010; Galy et al., 1995; Haddad et al., 2004; Hao et al., 2001; Hoebeke et al., 2007; Kohn et al., 2012) and IL-7 receptor (IL-7R, CD127) (Blom and Spits, 2006) to lymphoid development, we applied these markers to identify NKP candidates in human hematopoiesis. When 500, 10, or 1 $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ cells purified from CB (Figure 1A) or BM (Figure 1B) were co-cultured on OP9 or OP9DL1 stroma, all the wells contained viable $CD16^{+}$ and $CD16^{-}$ $CD19^{-}CD3^{-}CD56^{+}NKp46^{+}$ NK cells, but no other blood cell lineages (Figures 1C–1E, S1A–S1G, and S2A–S2C). Further phenotypic analysis demonstrated that NK cells generated in vitro from $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ candidate NKPs expressed multiple NK cell receptors, including NKp46, CD94, KIR, CD57, CD27, CD161, CD244, and NKG2D (Figure S1E). Purified multipotent CB or BM $Lin^{-}CD34^{+}$ cells were used as positive controls, and in addition to NK cells, they also produced $CD19^{+}$ B cells, $CD5^{+}CD4^{+/-}CD8^{+/-}CD3^{+/-}$ T cells, and $CD33^{+}CD14^{+}$ myeloid cells (Figures 1C–1E, S1A–S1E, and S3A), and multiple lineage output was observed at the single-cell level. Similar results were observed for purified $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{-}CD127^{-}$ cells (Figure S3B), whereas only lymphoid lineages (T, B, ILCs, NK cells) were generated from 10 or 1 purified $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{+}$ cells (CLP-like cells) (Figures 1C and S3A).

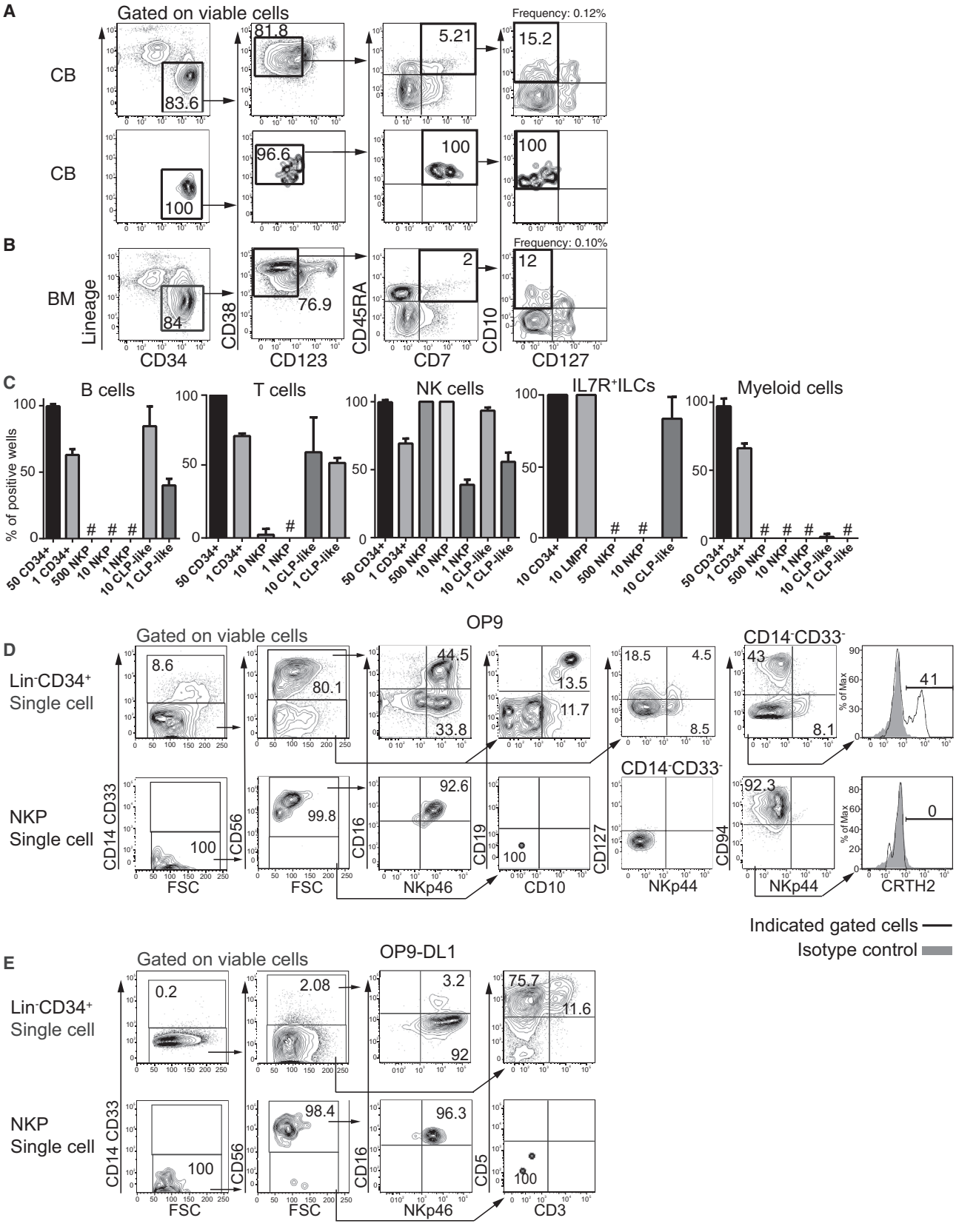
The $Lin^{-}CD34^{+}$ progenitors in addition to cytotoxic $CD56^{+}CD16^{+}NKp46^{+}$ NK cells (Figures 1C, S1A–S1G, S2A–S2C, and S3A) also generated IL-7R-expressing innate lymphoid-like cells (IL-$7R^{+}$ ILCs) that could be subdivided into ILC1 ($C\text{-}KIT^{-}NKp44^{-}IL\text{-}7R^{+}$), ILC2 ($C\text{-}KIT^{+/-}IL\text{-}7R^{+}CRTH2^{+}$), and ILC3 ($C\text{-}KIT^{+}NKp44^{+/-}IL\text{-}7R^{+}$) (Figures 1C, S1G, and S2A–S2C; Hazenberg and Spits, 2014). IL-$7R^{+}$ ILCs generated from $Lin^{-}CD34^{+}$ expressed RORγt and GATA-3, whereas the NKP-derived progeny expressed EOMES and T-BET, but not RORγt or GATA-3 (Figures S2A–S2C). IL-$7R^{+}$ ILCs were also clearly present in cultures generated from ten LMPPs and ten CLP-like cells (Figures 1C and S3A). In contrast, the progeny produced from the candidate NKP had a uniform $IL\text{-}7R^{-}CD56^{+}CD94^{+}CD16^{+}C\text{-}KIT^{-}$ phenotype compatible with conventional cytotoxic NK cells (Figures 1C, S1E, and S1G; Hazenberg and Spits, 2014), demonstrating that the NKP candidate had no ability to generate IL-$7R^{+}$ ILCs in vitro.

Next, we evaluated the generation of macrophages and granulocytes from NKPs and $Lin^{-}CD34^{+}$ cells using a single-cell myeloid culture assay (Terasaki culture). Whereas approximately 70% of single $Lin^{-}CD34^{+}$ cells produced medium or large clones, very few (1%–2%) single NKPs generated small clones (Figures S1B–S1D). The clones generated from $Lin^{-}CD34^{+}$ cells contained macrophages, granulocytes, and erythroid cells (Figure S1C), whereas the clones generated from NKPs contained cells with lymphoid morphology (Figure S1D). These findings established, at the single-cell level, that the $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ cell population has a robust NK lineage potential in vitro and lacks the ability to generate T, B, non-NK cell ILCs, and myeloid cells.

NKP Lacks Expression of Mature NK Cell Markers and Is Present in Fetal Tissues and Adult Tonsil

To further characterize the NKP candidate population, we investigated the expression of additional cell surface markers known to be important for the lymphoid commitment and NK cell development. CD62L and CD244 molecules were highly expressed on the NKP candidate both in CB and BM (Figures 2A and 2B). In contrast, cell surface receptors expressed by mature NK cells, such as NKp46 and NKG2D, were not detectable on NKPs. Approximately 70% of CLP-like cells (Figure 2A) and 50% of NKPs expressed C-KIT and FLT3 receptors, and only a few NKPs were positive for CSF-1R, the third member of the class III cytokine tyrosine kinase family. NKPs did not express detectable levels of the IL-2/IL-15 receptor β (CD122), but 30% to 40% expressed the common γ chain, CD132 (Figures 2A and 2B). Finally, NKPs did not express detectable levels of CD27 (TNF- receptor) or CD161 (Figures 2A and 2B).

The $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ NK cell-restricted progenitor was also detected in human fetal liver (FL) at gestational weeks 6, 10, 12, and 16, and in fetal bone marrow (FBM) at gestational weeks 12 and 16 (Figure 2C). Finally, NKPs were also present in adult tonsils (Figure 2D) and, after in vitro co-culture on OP9 stroma, these tonsil NKPs gave rise only to $CD56^{+}NKp46^{+}CD16^{+}NKG2D^{+}CRTH2^{-}$ NK cells, compatible with the phenotype of cells generated from CB and BM NKPs (Figure S3C).



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NK Cells Generated In Vitro from NKPs Are Functional

To determine whether in vitro produced NK cells were functionally competent, we performed a CD107a degranulation assay (Bryceson et al., 2005). NK cells generated from NKPs cultured with OP9 stromal cells upregulated CD107a after activation with PMA/ionomycin, anti-CD16 antibody, or the K562 leukemic cell line (Figures 3A and 3B). Similar results were obtained with NK cells generated from NKPs cultured with OP9-DL1 stromal cells (data not shown). In addition, the supernatants of the activated NK cells generated from NKPs contained higher amounts of IFN-γ and TNF-α, compared to unstimulated controls (Figures 3C and 3D and data not shown). Finally, NK cells generated from NKPs did not produce detectable amounts of IL-13, IL-5, and IL-22 cytokines (Hazenberg and Spits, 2014), in contrast to IL-7R$^+$ ILCs generated from Lin$^-$CD34$^+$ progenitors (Figures 3E and 3F).

Taken together, these data demonstrated that NK cells generated in vitro from the Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ NKPs are functionally mature.

The NKP Candidate Generates Only NK Cells after Transplantation into Newborn Immune-Deficient Mice

Next, we examined the ability of the NKP candidate to engraft and generate hematopoietic cells after transplantation into sublethally irradiated newborn NOD/SCIDγc^{null} mice (Ito et al., 2002). At 11 weeks after transplantation, 15 out of 17 mice transplanted with 600 Lin$^-$CD34$^+$ multipotent cells had detectable numbers of human cells in the peripheral blood, bone marrow, and spleen, and 9 out of 17 transplanted mice had detectable levels of human cells in the thymus (Figures 4A–4C and S4A–S4C). Furthermore, mice had a multilineage reconstitution where human B (CD56$^-$CD3$^-$CD19$^+$), T (CD19$^-$CD56$^-$CD5$^+$CD4$^{+/-}$CD8$^{+/-}$CD3$^{+/-}$), NK (CD19$^-$CD3$^-$CD56$^+$NKp46$^+$CD16$^{+/-}$), and myeloid (CD14$^+$CD33$^+$) cells were present in all analyzed tissues (Figures 4A–4C and S4A–S4C). In contrast, mice transplanted with 600 Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ NKPs had clearly detectable human CD19$^-$CD3$^-$CD56$^+$NKp46$^+$CD16$^{+/-}$ NK cells in the peripheral blood, bone marrow, spleen, and thymus, and no other lineages were detected (Figures 4A–4C and S4A–S4C). Essentially all NK cells produced in NOD/SCIDγc^{null} mice after transplantation with NKPs were CD56$^+$NKp46$^+$ (Figures 4A–4C and S4A–S4C). In contrast to the cohort transplanted with Lin$^-$CD34$^+$ population (Figure 4D), CD127$^+$ RORγt$^+$ or CRTH2$^+$ cells were undetectable in mice transplanted with NKPs, demonstrating that the NKP candidate did not have the ability to generate IL-7R-expressing ILCs in vivo (Figure 4D).

Collectively these results demonstrated that NKPs engraft in newborn NOD/SCIDγc^{null} mice and lead to exclusive NK cell reconstitution after transplantation.

NK Cells Generated In Vivo from NKP Candidate Are Cytotoxic and Secrete Cytokines

Next, we investigated whether in vivo generated NK cells were mature and cytotoxic. Human NK cells purified from spleens of reconstituted NOD/SCIDγc^{null} mice after activation with PMA/ionomycin or with K562 tumor cells had significantly higher expression of CD107a compared to unstimulated control, regardless of whether they were isolated from mice transplanted with NKPs or Lin$^-$CD34$^+$ cells (Figure 5A). When human NK cells produced in NOD/SCIDγc^{null} mice transplanted with NKPs were divided into CD16$^+$ and CD16$^-$ fractions, the CD16$^-$ cells showed significantly lower cytotoxic activity compared to the CD16$^+$ fraction (Figure 5B). Furthermore, in vivo generated NK cells produced detectable amounts of IFN-γ and TNF-α (Figure 5C) and expressed multiple NK cell receptors, including NKp46, KIR, CD94, CD57, CD27, NKG2D, CD161, and CD244 (Figure 5D). In summary, our data established that NK cells derived from NKPs after transplantation into NOD/SCIDγc^{null} mice were mature and were functionally and phenotypically similar to mature NK cells isolated from peripheral blood.

NK Cell Lineage-Associated Genes Are Specifically Upregulated in NKPs

To define the transcriptional signature of the NKPs, we performed a genome-wide microarray gene expression analysis on purified NKPs (Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$) and compared them to upstream multipotent progenitors LMPPs (Roy et al., 2012) (Lin$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^+$) and mature NK cells (CD56$^+$NKp46$^+$CD3$^-$CD19$^-$) purified from CB. The differentially expressed

Figure 1. Identification and Purification of the Human NK Cell Progenitor Candidate in Bone Marrow and Umbilical Cord Blood

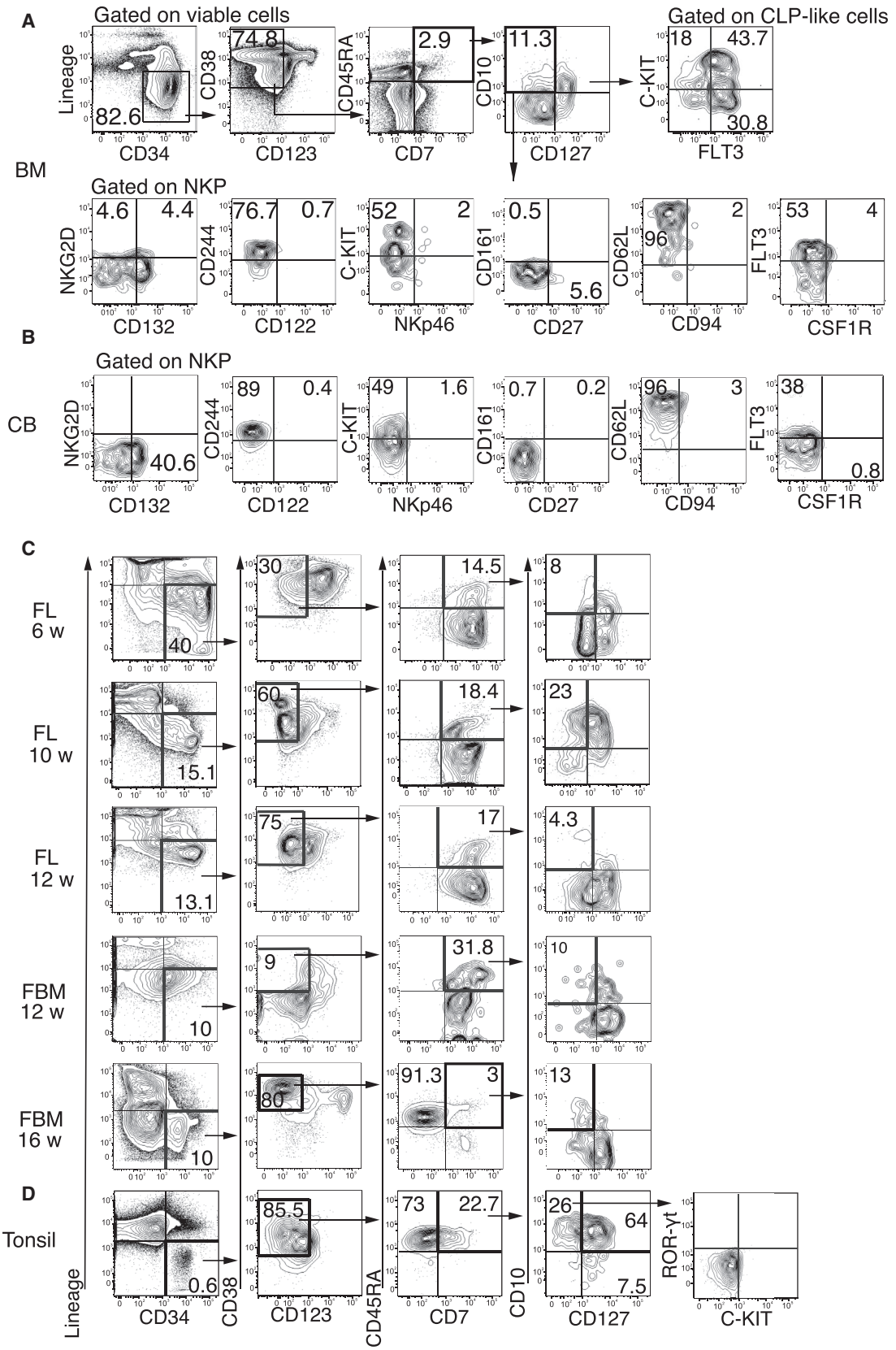
(A and B) Human CD34$^+$ cells (A) from umbilical cord blood (CB) and (B) bone marrow (BM) were analyzed for the expression of CD38, CD123, CD45RA, CD7, CD10, and CD127 markers and the NKP candidate (Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$) was sorted as indicated. Numbers represent percentages in indicated gates or boxes and arrows show the gating strategy.

(A) Top and bottom panels show the sort gates and the reanalysis after sort of the Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ population from CB. Data are representative of 13 different analyses of CB batches (2–6 donors for each batch).

(B) Panels show the sorting gates of NKPs from BM. Data are representative of 8 different BM donors.

(C) Percentage of wells containing NK cells (CD19$^-$CD3$^-$CD56$^+$NKp46$^+$CD16$^{+/-}$), B cells (CD56$^-$CD3$^-$CD19$^+$), T cells (CD19$^-$CD56$^-$CD5$^+$CD4$^{+/-}$CD8$^{+/-}$CD3$^{+/-}$), IL-7R$^+$ ILCs (CD19$^-$CD14$^-$CD94$^-$CD16$^-$CD56$^{+/-}$NKp44$^{+/-}$CD127$^{+/-}$C-KIT$^{hi/lo}$), and myeloid cells (CD14$^+$CD33$^+$) generated from purified CB Lin$^-$CD34$^+$, Lin$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^+$ LMPPs, Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^+$ CLP-like cells, and Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ NKPs after 4 weeks of culture on OP9 (B cells, NK cells, ILCs, and myeloid cells) and after 5 weeks of culture on OP9DL1 (T cells). Wells were considered positive when at least 30 viable human cells were detected and were scored positive for mature blood lineages when at least 20 cells with the required phenotype were detected. Data represent mean (SD) values from: 4 wells (4 independent experiments) with 500 NKPs, 61 wells (3 independent experiments) with 10 NKPs, 529 wells (5 independent experiments) with 1 NKP, 100 wells (5 independent experiments) with 50 Lin$^-$CD34$^+$ cells, 336 wells (3 independent experiments) with 1 Lin$^-$CD34$^+$ cell, 100 wells with 10 CLP-like cells (5 independent experiments), 120 wells with 1 CLP-like cell (3 independent experiments), and 12 wells with 10 CD34$^+$ cells, 10 LMPPs, 10 CLP-like cells, and 10 NKPs (in 3 independent experiments) for ILCs read-out. Hatch mark (#) indicates no potential detected. Similar results were obtained from BM cells (Figure S1).

(D and E) Representative plots showing the phenotype of progeny generated from single Lin$^-$CD34$^+$ cells and single NKPs after in vitro co-culture on (D) OP9 stroma for 4 weeks and (E) OP9DL1 stroma for 5 weeks. Numbers represent frequencies in indicated gates or boxes and arrows show the gating strategy. See also Figures S1, S2, and S3.



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probes (1,530) were clustered into 6 different groups via k-means, where a NKP-specific signature was identified (Figure 6A). Some key lymphoid-specific genes such as *EBF-1*, *RAG-1*, and *PAX5* were expressed at higher levels in NKPs as compared to LMPPs (Figure 6B). To determine whether this increase was specific to NKPs, we compared the expression of these genes to the CLP-like cells ($Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^+$) that, in the hematopoietic hierarchy, are placed upstream of NKPs and downstream to LMPPs. No significant differences were detected for *EBF-1*, *RAG-1*, and *PAX5* expression between the CLP-like cells and NKP populations (Figure 6B), which suggested that the expression of lymphoid genes was maintained but not upregulated in NKPs. To confirm these findings, we performed an expression analysis on 25 purified NKPs, CLP-like cells, LMPPs, mature NK cells, myeloid ($Lin^-CD34^+CD38^+CD123^+$), and B cell ($Lin^-CD34^+CD38^+CD123^-CD45RA^+CD19^+$) progenitors using quantitative gene expression analysis. The expression of erythroid-specific gene (*GATA1*), myeloid genes (*GCSF2R*, *PU.1*), and B-cell-specific genes (*CD79A*, *PAX5*, *EBF-1*) was lower in NKPs compared to upstream $CD34^+$ progenitors, LMPPs, CLP-like cells, as well as B cell and myeloid progenitors (Figure 6C and data not shown). Consistent with their robust NK cell potential, the NKPs expressed higher levels of several genes important for regulation of early lymphoid and NK cell development (*GATA3*, *EOMES*, *TBX21*, *CD122*, *CD247*) and several NK-cell-related genes (*NCAM1*, *NCR1*, *KLRB1*, *KIR2DL2*, *PERFORIN*, and *GRANZYME B*) (Figure 6C). Moreover, ILC-associated genes (*RORA*, *RORC*, *IL-17*, *IL-22*, *IL-5*, and *IL-13*) were not upregulated in NKP compared to LMPP or CLP-like cells (Figure S5A). Similar results were also obtained with the same cell populations isolated from human BM (Figure S5B).

These results demonstrate that the identified NKP exhibits strong NK lineage priming, whereas expression of genes associated with several other blood lineages is downregulated.

Human NKP Is Downstream of LMPP and CLP-like Cells in the Hematopoietic Hierarchy

To place the newly identified NKP within the human hematopoietic hierarchy, we analyzed the progenies generated from Lin^-CD34^+ multipotent progenitors, purified LMPPs, CLP-like cells, and NKPs in vitro and in vivo.

First, we analyzed the phenotype of the progeny of purified $Lin^-CD34^+CD38^-CD90^-CD45RA^+$ LMPPs, $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^+$ CLP-like cells, and $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ NKPs (Figure 7A) after co-culture on OP9 stroma at different time points. After 2 days of culture, LMPPs gave rise predominantly to CLP-like cells, whereas very few NKPs were detected at this time (data not shown). After 4 days, there were no detectable LMPPs left in the culture, although the generated progeny contained CLP-like cells, NKPs, and lineage-positive cells (Figure 7B). Purified CLP-like cells could be maintained in culture for 4 days while producing NKPs as well as lineage-positive cells (Figure 7B). In contrast, NKPs did not give rise to $CD127^+$ cells, and only lineage-positive cells were detected in the wells (Figure 7B). Although NKPs did not express detectable levels of CD122 ex vivo, more than 70% of the NKPs were CD122 positive after 4 days in culture, and all NKPs generated from CLP-like cells and LMPPs showed high cell surface expression of CD122 at day 6 (Figure 7B).

Next, we investigated the $CD34^+$ hematopoietic progenitor compartment in the BM of NOD/SCIDγc^{null} mice transplanted with human multipotent CB Lin^-CD34^+ cells, NKPs, and LMPPs. At 3 weeks after transplantation with Lin^-CD34^+ cells (Figure S6A), the BM of reconstituted mice predominantly contained a population of myeloid progenitors expressing IL-3 receptor ($CD123^+$) and a $CD45RA^+CD7^+CD127^+$ population that started to co-express CD10; however, the NKP population was not detectable at this time. Later, 4 weeks after transplantation (Figure S6A), the frequency of $CD123^+$ cells was reduced, and the lymphoid progenitors were increased: NKPs were clearly detectable in the BM, together with the CLP-like cells and other early hematopoietic progenitors expressing CD127, CD10, or CD7 lymphoid markers (Figure S6A). Importantly, NKPs generated in NOD/SCIDγc^{null} mice transplanted with human Lin^-CD34^+ cells produced only $CD56^+NKp46^+CD16^+$ NK cells in culture on OP9 stroma (Figure S6B). At 4 weeks after transplantation of NKPs into NOD/SCIDγc^{null} mice, NKPs were detected in the BM of 3 out of 14 mice (Figure S6C), and at this time, mature $CD56^+NKp46^+CD16^-$ NK cells were present mainly in the BM (data not shown).

Finally, the analysis of BM from NOD/SCIDγc^{null} mice transplanted with purified LMPPs supports that CLP-like cells were generated first followed by the generation of NKP (Figure S6D).

Collectively these data suggest that the candidate NKP represents a progenitor downstream of LMPP and CLP-like cells.

DISCUSSION

With the identification of a restricted NK cell progenitor in adult BM, tonsil, umbilical CB, and fetal tissues, we here report several findings that contribute to a greater understanding of human lymphoid and NK cell development.

Despite several recent advances in knowledge of NK cell biology, including their functional regulation and differentiation, the developmental stages from $CD34^+$ precursors to mature NK cells remain relatively less known. In particular, the identity of a NK-cell-restricted progenitor has remained elusive, despite

Figure 2. The NKP Lacks the Expression of Mature NK Cell Markers and Is Present in Fetal Tissues and Adult Tonsils

(A and B) Human $CD34^+$ cells from BM (A) and from CB (B) were analyzed for the expression of CD38, CD123, CD45RA, CD7, CD10, CD127, CD132, CD122, CD27, FLT3, C-KIT, CSF1R, CD62L, NKp46, NKG2D, CD244, and CD161. Data are representative of four independent experiments for each tissue.

(C) Representative FACS profile of liver (FL) from 6-, 10-, and 12-week-old fetuses and bone marrow (FBM) of 12- and 16-week-old fetuses. Three different livers and two different BM were analyzed for each time point.

(D) Representative FACS profile of adult tonsil, results are representative of three independent experiments (three different donors were analyzed). Numbers represents frequencies in indicated gates or boxes and arrows show the gating strategy.

See also Figure S3.

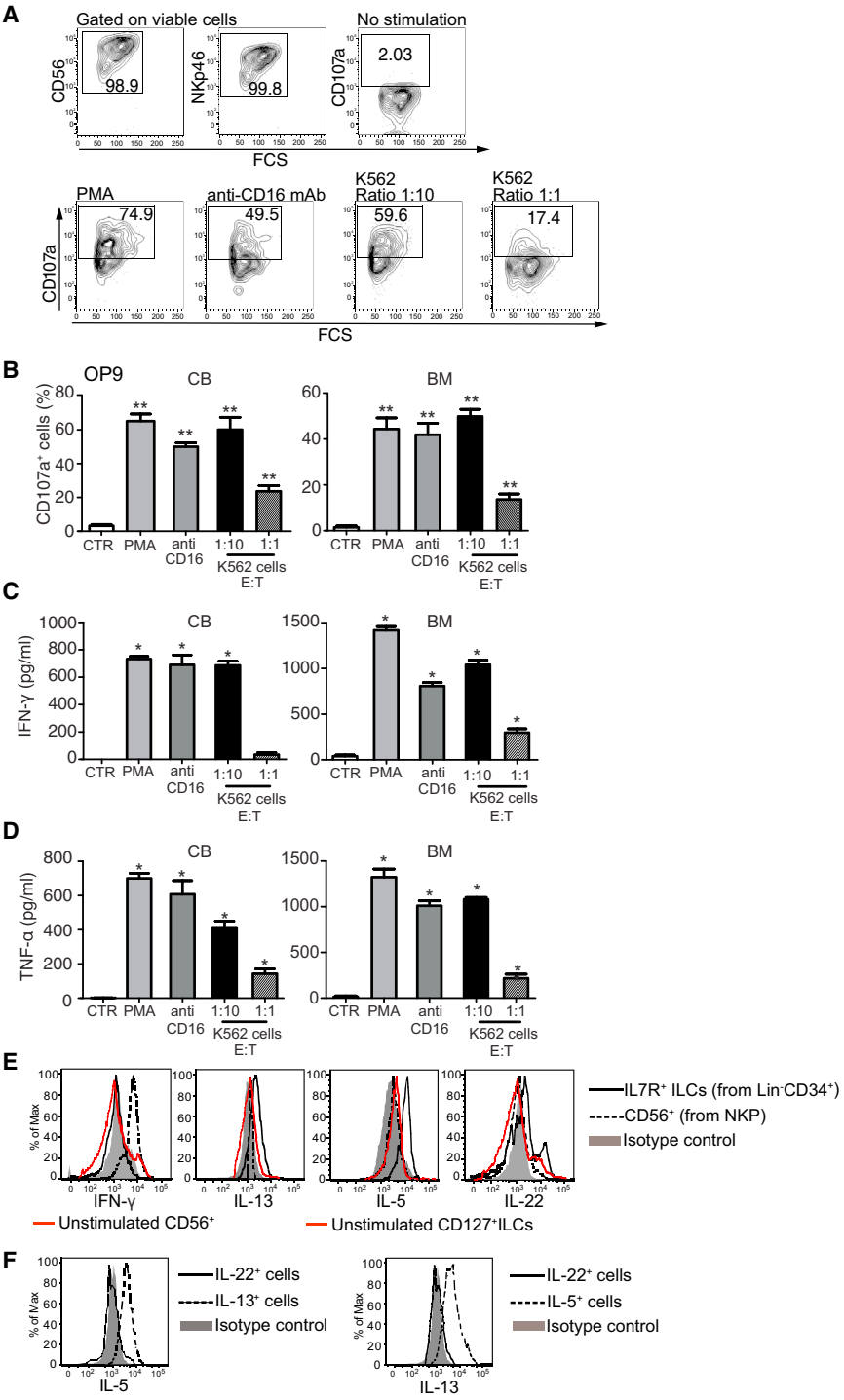


Figure 3. Human NK Cells Generated In Vitro from NKP Are Functionally Mature

(A) Representative FACS profiles of CD107a expression on NK cells generated in vitro from CB NKPs, either non-stimulated, or after stimulation with PMA/ionomycin, anti-CD16 antibody, or K562 tumor target cells using 1:1 and 1:10 effector to target (E:T) ratio. Data are representative of five independent experiments.

(B) Percentage of CD107a$^+$ NK cells generated in vitro from Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ NKPs purified from CB (left) and BM (right) cultured on OP9 stroma for 4 weeks. Data represent mean values and SD from five independent experiments for NK cells generated from CB NKP (ten wells seeded with ten cells were pooled for each experiment, on each stroma OP9 and OP9-DL1; data not shown) and four independent experiments for BM NKP-generated NK cells (ten wells seeded with ten cells were pooled for each experiment, on each stroma OP9 and OP9-DL1; data not shown). Mann-Whitney test was used for statistical analysis. **p value < 0.005.

(C and D) Levels of IFN-γ (C) and of TNF-α (D) produced by NK cells generated in vitro from CB (left) and BM (right) Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ NKPs cultured on OP9 stroma, non-stimulated (CTR) and after activation with PMA, anti-CD16 antibody, or K562 tumor target cells using 1:1 and 1:10 effector to target (E:T) ratio. Data represent mean values and SD from four independent experiments for CB- and BM-generated NK cells from NKPs. Similar results were obtained for NK cells generated on OP9DL1 (data not shown). Supernatants were generated from cells used in (A) and (B). Mann-Whitney test was used for statistical analysis. *p value < 0.05.

(E) Representative profiles showing secretion of IL-5, IL-13, IL-22, and IFN-γ by IL-7R$^+$ ILCs generated from Lin$^-$CD34$^+$ cells or progeny derived from NKPs after 3-week culture on OP9 stroma. Data are representative of two independent experiments with three wells analyzed for each cytokine.

(F) Representative profiles showing secretion of IL-5 and IL-13 by CD127$^+$ ILCs positive for IL-13 and IL-22 or for IL-5 and IL-22, respectively, generated from Lin$^-$CD34$^+$ cells after 3-week culture on OP9 stroma. Data are representative of two independent experiments with three wells analyzed.

several reports on hematopoietic precursor cells with the capacity to generate NK cells.

Previous studies have characterized the early lymphoid committed progenitors in human BM and CB as being Lin$^-$CD34$^+$CD38$^+$CD10$^+$ (Galy et al., 1995) and Lin$^-$CD34$^+$CD38$^-$CD7$^+$ (Hoebeke et al., 2007) cells, respectively, with the ability to generate T, B, and NK cells, but not myeloid cells. More recently a study has described a Lin$^-$CD34$^+$CD38$^-$CD45RA$^+$CD10$^+$CD7$^-$ immature lymphoid progenitor in human BM and CB, called MLP, that gives rise to T, B, and NK cells, but also maintains the ability to produce dendritic cells, monocytes, and macrophages (Doulatov et al., 2010). Here, we demonstrate that the Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^+$ cells represent a human CLP-like cell, with a lineage potential restricted to the lymphoid cells and a strong lymphoid gene priming.

More importantly, we demonstrate that a human NK-cell-restricted progenitor can be identified in adult bone marrow, tonsil, cord blood, and fetal tissues as a distinct population of $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ cells. This progenitor has a robust NK cell lineage potential and lacks the ability to generate other blood lineages at the single-cell level in vitro. The phenotype of this newly identified NKP is consistent with previous phenotypes of early lymphoid progenitors and represents a novel intermediate stage within the human hematopoietic hierarchy.

Recently, it has become evident that the family of ILCs is very heterogeneous, and in addition to cytotoxic NK cells, contains $CD127^+$ ILC1, ILC2, and ILC3 cells (Diefenbach et al., 2014; Hazenberg and Spits, 2014). Previously, a progenitor with NK cell potential was identified in human lymph nodes (Freud et al., 2005), which originates from BM $CD34^+$ progenitor cells that express CD45RA, CD10, CD7, C-KIT, and L-selectin (CD62L) (Freud et al., 2005, 2006). However, this population in addition to NK cells can generate T cells (Freud et al., 2006) and it remains to be determined whether they can also produce $CD127^+$ ILCs. Indeed, an ILC3-restricted $CD34^+CD45RA^+CD161^+CD117^+$ precursor (Montaldo et al., 2014), partially overlapping with the phenotype of the precursor described by Freud et al. (2005), was very recently identified in human tonsils. In contrast, the newly identified NKP had no ability to produce $IL\text{-}7R^+ROR\gamma t^+$ or $IL\text{-}7R^+CRTH2^+$ ILCs in vitro or in vivo, in agreement with a recently proposed model for the development of ILC lineages where NKP represents a separate branch downstream of the CLP, which generates only cytotoxic NK cells (Diefenbach et al., 2014). Our data are compatible and in agreement with this model, but most importantly provides the identity of a cytotoxic NK cell-restricted progenitor.

The $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ NKP identified here can also be detected in human tonsils, and also probably overlaps partially with the population described by Freud et al. (2005) based on the common co-expression of CD7, CD10, CD62L, and C-KIT. Importantly, however, the NKP identified here is clearly distinct from the recently identified ILC3 precursor, because it did not express RORγt or CD161, uniformly expressed CD7, and did not give rise to non-NK cell ILCs in vitro or in vivo.

The development of human NK cells in the fetus is not well characterized and it has been shown that populations of human T, B, and NK cells generated from fetal stem and progenitor cells have different functional properties than those generated postnatally in adult (Ivarsson et al., 2013; Mold et al., 2010; Punnonen et al., 1992). Although NK cells can be detected in human fetal liver already at gestational week 6 (Phillips et al., 1992), it is not known when and where NK cell progenitors arise during fetal development. Here we show that $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ NKPs were present in fetal liver between gestational weeks 6 and 16. In addition, we also detected NKPs in fetal bone marrow at gestational weeks 12, 15, and 16, which represent a time when the bone marrow becomes an active site for hematopoiesis.

Human NK cell maturation involves the sequential loss of CD34 and C-KIT expression (Freud et al., 2006), followed by upregulation of CD56, CD94, CD16, and KIRs (Freud and Caligiuri, 2006). The NKP candidate progenitor did not express detectable cell surface levels of proteins associated with mature NK cells ex vivo, whereas the NK cells generated in vitro and in vivo proceeded through the expected phenotypic and functional maturation stages.

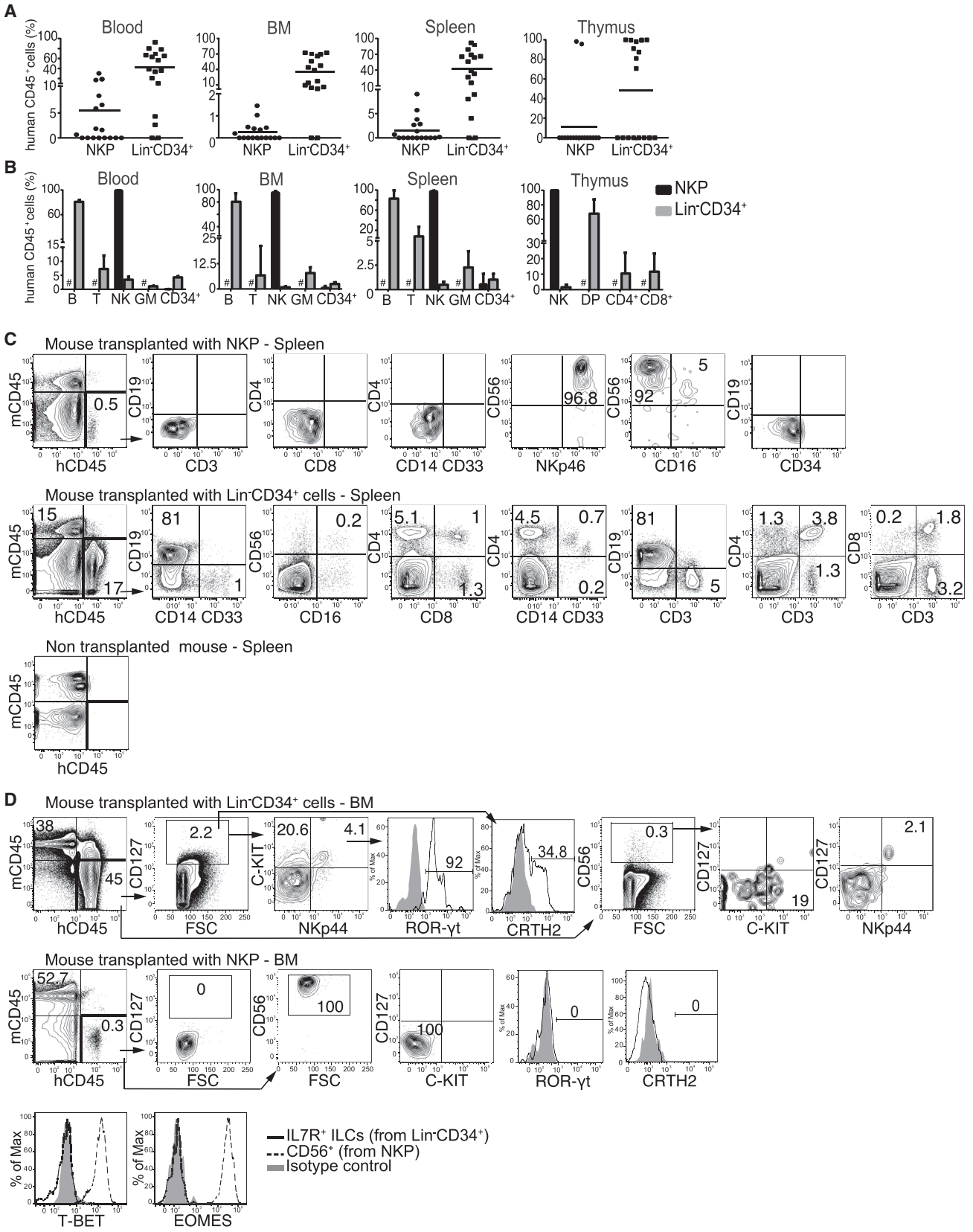
Interleukin-15 (IL-15) has been identified as a key cytokine important for in vitro and in vivo differentiation of mouse and human NK cells, and human $CD34^+$ cells respond to IL-15 in vitro (Cooper et al., 2002; Freud and Caligiuri, 2006; Freud et al., 2006; Ranson et al., 2003). The cell surface expression of the β subunit of the IL-15 receptor (CD122) on human lymphoid progenitors is, however, undetectable by flow cytometry (Freud and Caligiuri, 2006; Freud et al., 2006). In agreement with this, NKPs did not express detectable levels of CD122 ex vivo, but it was clearly detectable after 4 days in culture in the presence of cytokines, demonstrating that IL-15 is important for NK cell differentiation from NKPs.

A NOD/SCIDγc^{null} mouse xeno-transplantation model was previously shown to support engraftment of human hematopoietic progenitors and generation of T, B, myeloid, and $CD56^{dim}$ cytotoxic NK cells (Brehm et al., 2010; Chan et al., 2007; Ito et al., 2002).

In contrast to previous studies where the lineage potential of identified lymph node NK progenitor was not investigated in vivo (Freud et al., 2005, 2006), we show that 41%–53% of the mice transplanted with 600 purified NKPs had clearly detectable levels of human NK cell in the peripheral blood, spleen, and bone marrow. Importantly, no other blood lineages were produced, supporting our assertion that NKPs represent a novel, tightly restricted NK cell progenitor. Furthermore, human NK cells generated from transplanted NKPs expressed typical NK cell surface molecules and, according to the NK maturation stages, were phenotypically and functionally defined as $CD56^{dim}CD16^+$ cytotoxic NK cells and $CD56^{bright}CD16^-$ cytokine-producing NK cells (Chan et al., 2007; Cooper et al., 2009). Moreover, NKPs showed a significant upregulation of genes important for NK cell development (*EOMES* and *TBX21*), as well as genes involved in cytotoxic killing (*PERFORIN* and *GRANZYME B*), whereas the B cell and myeloid cell genes were downregulated.

In agreement with the recent studies on human hematopoiesis (Doulatov et al., 2010, 2012; Laurenti et al., 2013; Roy et al., 2012), the expression of *EBF-1* and other early lymphoid genes was upregulated already in early lymphoid progenitors MLP and LMPP. Interestingly, we found that *EBF-1* expression remained at comparable levels in CLP-like cells and NKPs, despite the fact that NKPs had no ability to generate B cells. NKPs also expressed high levels of CD62L on the cell surface, which coincides with lymphoid priming (Kohn et al., 2012). Genes related to NK cell effector functions, including NK-cell-mediated cytotoxicity, secretory pathways, and CD16 (FcγRIIIa), were upregulated in the NKP candidate, further supporting its NK lineage restriction and function. In contrast, and consistent with the finding that NKPs failed to generate $CD16^-IL\text{-}7R^+$ ILCs, there was no upregulation of non-NK ILC-associated genes, e.g., *RORA*, *RORC*, *IL-17*, *IL-22*, *IL-5*, and *IL-13* (Figures 6 and S5A).

Previous studies reported a high expression of GATA3 and identified GATA3 as an important regulator of mouse thymic NK cells (Vosshenrich and Di Santo, 2013). We found that the expression of *GATA-3* was upregulated in NKPs compared to other early lymphoid progenitors. This suggests that the newly



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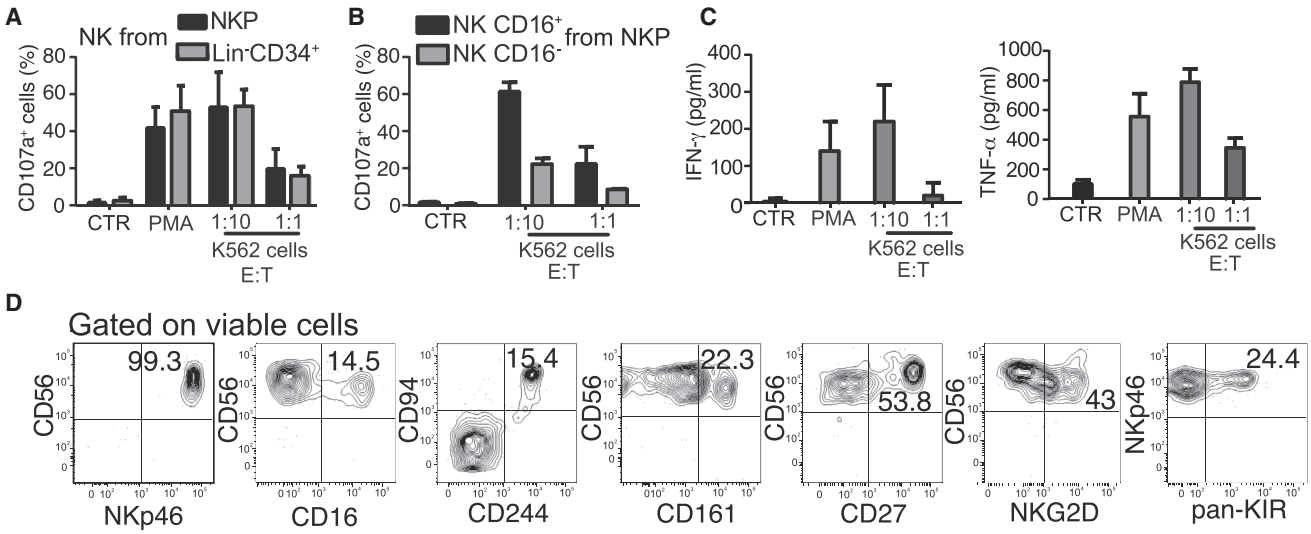

Figure 5. Human NK Cells Generated from NKP after Transplantation into Immune-Deficient Mice Are Functionally Mature

(A) Percentage of CD107a$^+$ CD45$^+$CD3$^-$CD56$^+$NKp46$^+$ human NK cells generated from 600 CB Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ NKPs (black bars) or 600 Lin$^-$CD34$^+$ cells (gray bars) sorted from NOD/SCIDγc^{null} mice spleen at 11 weeks after transplantation. Data represent mean values and SD from three independent experiments with six mice analyzed in total.

(B) Percentage of CD107a$^+$ NK cells within CD16$^+$ (black bars) and CD16$^-$ (gray bars) NK cell fractions generated in vivo from 600 CB Lin$^-$CD34$^+$CD38$^+$ CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ NKPs and 600 Lin$^-$CD34$^+$ control cells, non-stimulated (CTR) and after activation with PMA or K562 tumor target cells using 1:1 and 1:10 effector to target (E:T) ratio. Data represent mean values and SD from two independent experiments with three mice analyzed in total.

(C) Levels of TNF-α and IFN-γ produced in vitro by human CD45$^+$CD3$^-$CD56$^+$ NK cells generated in vivo from 600 Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$ CD10$^+$CD127$^-$ NKPs at 11 weeks after transplantation into NOD/SCIDγc^{null} mice, non-stimulated (CTR) and after activation with PMA and K562 target tumor cells using 1:1 and 1:10 E:T ratio. Data represent mean (SD) values from three independent experiments with six mice analyzed in total.

(D) Representative FACS profile of CD56, CD16, NKp46, CD94, CD244, CD161, CD27, KIR receptors, and NKG2D expression on sorted NK cells generated after transplantation of NKP into NOD/SCIDγc^{null} mice. Data are representative of two independent experiments with four mice analyzed in total.

identified NKP could also represent a progenitor for thymic NK cells. In support of that, human NK cells were present in the thymus of NOD/SCIDγc^{null} mice reconstituted after transplantation with human NKPs.

To investigate the developmental relationship between LMPP, CLP, and NKP, we performed in vitro and in vivo studies and established that LMPP can give rise to both CLP-like and NKP cells, whereas CLP-like cells can generate only NK progenitors, and NKPs cannot produce CLP-like cells. The placing of NKPs in the hierarchy downstream of CLP and LMPP was also supported by C-KIT and FLT3 expression, which were high in LMPP, decreased in CLP-like cells, and more reduced in NKPs, both at the protein and mRNA level.

The proposed model of human hematopoietic hierarchy suggests that HSCs differentiate into multilymphoid progenitors that give rise to the bi-potent B-NK cell precursors in the bone marrow (Doulatov et al., 2010). Recent studies on hematopoiesis in rhesus macaque by Wu et al. (2014) have challenged the current hierarchy and suggested that NK cells originate from a distinct progenitor with no clonal overlap with B, T, and myeloid cell lineages (Wu et al., 2014). Our functional in vitro and in vivo studies and the analysis of expression patterns of lineage-specific genes in NKPs, CLP-like cells, and LMPPs support that NKPs are placed in the hematopoietic hierarchy downstream of CLPs and LMPPs, with no ability to generate T, B, and myeloid cells. Supporting this model, the previous work by Doulatov et al. (2010, 2012) have demonstrated that generation of mature NK cells from hematopoietic stem and progenitor cells occurs through the intermediate stages including lympho-myeloid and B-NK cell progenitors. The conclusions from the studies by Wu

Figure 4. The Human NKP Candidate Generates NK Cells but No Other Lineages after Transplantation into Immune-Deficient Mice

(A) Proportion of human CD45$^+$ cells in PB, BM, spleen, and thymus in recipient mice transplanted with human cells. Data represent mean values and SD from 3 independent experiments with 17 recipient mice transplanted with NKPs (circles) and 17 mice transplanted with Lin$^-$CD34$^+$ cells (squares).

(B) Proportion of human NK (CD19$^-$CD3$^-$CD56$^+$NKp46$^+$CD16$^{+/-}$), B (CD56$^-$CD3$^-$CD19$^+$), T (CD19$^-$CD56$^-$CD5$^+$CD4$^{+/-}$CD8$^{+/-}$CD3$^{+/-}$), and myeloid (CD14$^+$CD33$^+$) cells within human CD45$^+$ cells in different tissues of engrafted mice. Data represent mean (SD) values of hCD45$^+$ mice from 3 independent experiments with 17 recipient mice transplanted with NKPs (black bars) and 17 mice transplanted with Lin$^-$CD34$^+$ cells (gray bars). Hatch sign (#) indicates no potential detected.

(C) Representative FACS profiles of spleen from NOD/SCIDγc^{null} mice transplanted with 600 Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$ NKPs (top), 600 Lin$^-$CD34$^+$ cells (middle), or non-transplanted control (bottom), 11 weeks after transplantation.

(D) Representative FACS profiles of BM from NOD/SCIDγc^{null} mice transplanted with 600 Lin$^-$CD34$^+$ cells (top), 600 Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$ CD7$^+$CD10$^+$CD127$^-$ NKPs (bottom) showing the generation of IL-7R$^+$ ILCs (CD127$^+$CD56$^{+/-}$NKp44$^{+/-}$C-Kit$^{hi/lo}$), 11 weeks after transplantation.

See also Figure S4.

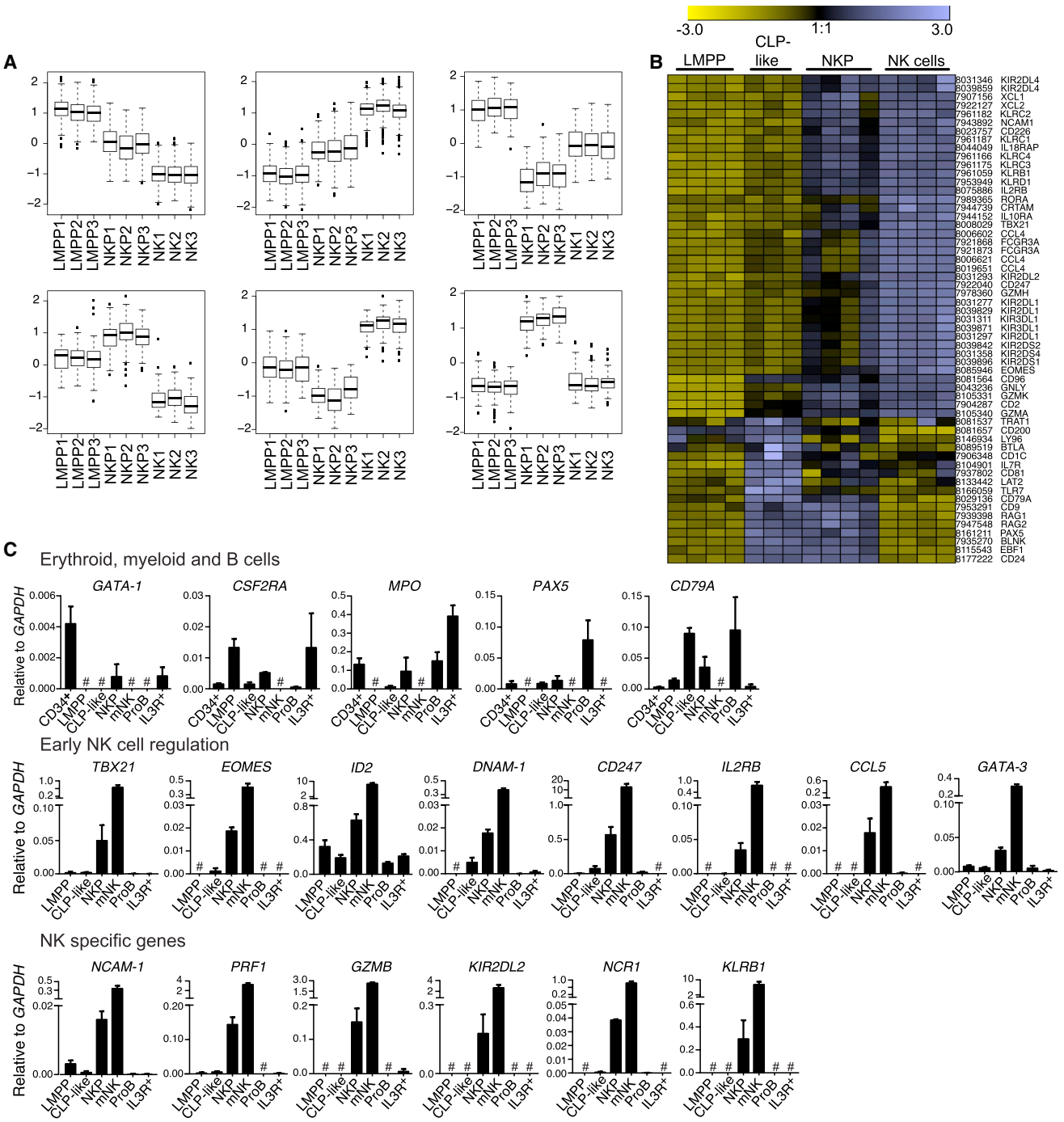

Figure 6. The NKP Candidate Has a Distinct Genetic Signature from LMPP, CLP, and Mature NK Cells

(A) K-means clusters of differentially expressed genes between LMPP, NKP candidates, and mature NK cell populations.

(B) Gene expression of selected lymphoid genes in LMPP (Lin$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^+$), CLP-like (Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^+$), NKP (Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$CD7$^+$CD10$^+$CD127$^-$), and mature NK (NKp46$^+$CD56$^+$CD3$^-$CD19$^-$CD33$^-$) cells. Data are from two different Affymetrix analyses with three replicates for each population.

(C) Quantitative gene expression analysis of purified cell populations. Data show average expression levels of three experiments (three replicates per population per experiment using 25 cells). #p $\leq$ 0.001, below detection.

See also Figure S5.

et al. (2014) might differ due to the fact that the CD16$^+$CD56$^-$ phenotype used to identify NK cells is not entirely NK cell specific.

Here, we show that the Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^+$ CD7$^+$CD10$^+$CD127$^-$ population represents a NK cell lineage-restricted progenitor in human umbilical cord blood, adult bone

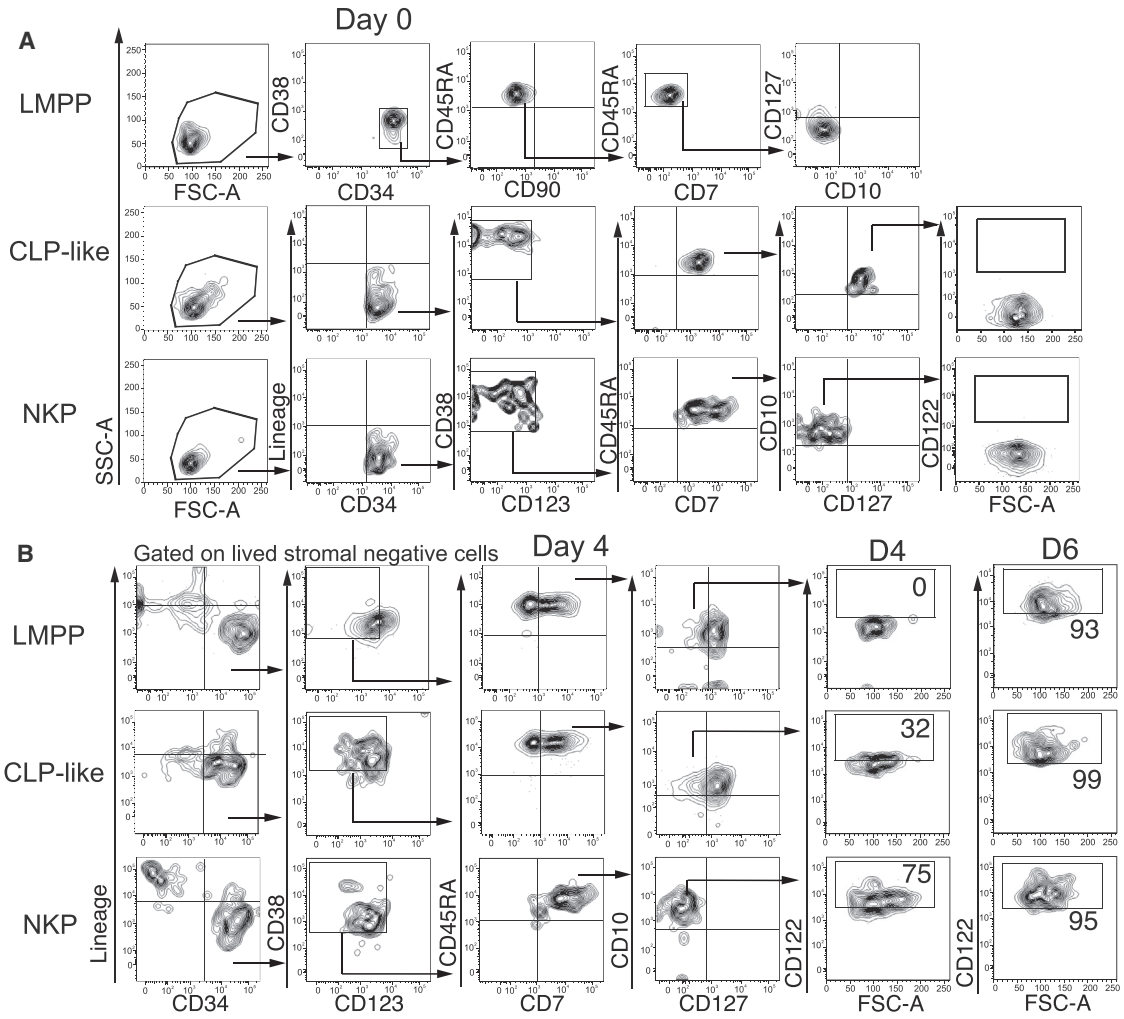

Figure 7. The Human NKP Is Downstream of LMPPs and CLP-like Cells in the Hematopoietic Hierarchy

500 CB purified LMPP ($Lin^-CD34^+CD38^-CD90^-CD45RA^+$), CLP-like ($Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^+$), and NKP ($Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$) cells were cultured for 4 and 6 days on OP9 stroma and the phenotype of the maintained/generated cells was analyzed by flow cytometry at day 0 (A) and at days 4 (D4) and 6 (D6) (B). The expression of CD122 was also evaluated on generated NKPs from the different progenitors. Data are representative of three independent experiments using different batches of CB cells with six wells plated for each population for each experiment. See also Figure S6.

marrow, tonsil, and fetal tissues. Given the fact that NK cells have a central role in eliminating virus-infected and transformed cancer cells, the identification of NKP will generate a greater depth of understanding of the origin of human NK cells and contribute to the development of new protocols for NK cell-based therapy.

EXPERIMENTAL PROCEDURES

Tissues

Human tissues were collected after informed consent and with the approval from the ethical review boards at Lund University and Karolinska Institutet. Donated human cord blood (CB) samples from Lund, Malmö, and Helsingborg hospitals were diluted in heparin immediately after delivery and processed within 24 hr. Human bone marrow (BM) was obtained from healthy adult volunteers via aspiration of 60 ml from the hip bone and processed directly after. Mononuclear cells from CB and BM were isolated on Lymphoprep (Axis-Shield). Human tonsils were obtained from adults undergoing surgery at the Karolinska University Hospital, Stockholm. Fetal tissues were donated from elective abortions as well as purchased from Novogenix Laboratories. Human fetal tissues were isolated as previously described (Medvinsky et al., 2008) and procedures for preparation of single-cell suspensions are provided in the Supplemental Experimental Procedures.

Antibodies, Flow Cytometry, and Cell Sorting

The single-cell suspensions were blocked by incubation with human Fc Receptor binding inhibitor (eBioscience) and then stained with specific human monoclonal antibodies (mAb) listed in Table S1. Fluorescence minus one (FMO) controls, with proper isotype controls, were used to determine the positive signals. 7-aminoactinomycin D (7-AAD, Sigma-Aldrich), TO-PRO-1 iodide (1 mM, Invitrogen), and DAPI (Invitrogen, 0.5 μg/ml) were used to exclude dead cells from the analysis. Intracellular stainings were performed using Foxp3 transcription factor staining buffer kit (eBiosciences) as recommended by the manufacturer and antibodies are listed in Table S2.

To identify the NKP candidates, the following Abs were mixed to define the lineage-negative (Lin^-) population: anti-CD3, CD4, CD11b, CD11c, CD14, CD15, CD19, CD33, CD56, CD66b, and CD235a. For the fetal liver (FL) enriched $CD34^+$ population, only anti-CD3, CD14, CD15, CD19, CD33, CD56, CD66b,

and CD235a antibodies were used. Samples were analyzed on an LSR II (BD Biosciences) and analysis was performed using FlowJo software (v.9.3; TreeStar).

The $CD34^+$ fractions were obtain from human CB, BM, and FL (from 6- to 16-week-old fetuses staged based on the developmental age) using CD34 positive selection (Miltenyi Biotec). After the staining with directly conjugated antibodies, the $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ cells were sorted. All sorts were performed on a BD FACSAria III (BD Biosciences) and the average purity of sorted cells in all experiments was 93%.

OP9 and OP9-DL1 Cultures

OP9 and OP9-DL1 stroma cells were kindly provided by Dr. J.C. Zúñiga-Pflücker, and cultures were carried out as previously described (Nozad Charoudeh et al., 2010; Schmitt and Zúñiga-Pflücker, 2002). Purified $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ NKPs and Lin^-CD34^+ cells (used as positive control) were co-cultured at 500, 10, or 1 cell per well on OP9 and OP9DL1 stroma and generated progeny was analyzed by flow cytometry. Details are provided in the Supplemental Experimental Procedures.

Terasaki Single-Cell Culture Assay

Single sorted $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ or Lin^-CD34^+ cells from CB or BM were cultured for 14 days in the presence of myeloid cytokines and scored for the size and morphology of generated clones. See the Supplemental Experimental Procedures for details.

Transplantation Assay

Newborn NOD/SCIDγc^{null} (NSG) mice (Ito et al., 2002) were used as recipients for human cell transplantation. The estimated delivery dates were determined 21 days after the vaginal plug was observed, as previously described (Tang et al., 2012). Newborn recipient mice were irradiated with a 100 Gy 12 hr after the delivery and were injected into the facial vein with 600 CB Lin^-CD34^+ or CB $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ cells in 25 μl, between 2 and 6 hr after irradiation, as previously described (Park et al., 2008). Mice were analyzed for the human cell engraftment at 4, 5, and 11 weeks after transplantation. Details are provided in the Supplemental Experimental Procedures.

Evaluation of NK Cell Activity

Functional activity of NK cells generated in vitro and after transplantation into NOD/SCIDγc^{null} mice was evaluated using expression of CD107a (lysosomal associated membrane protein 1; LAMP-1) (Bryceson et al., 2005) and secretion of IFN-γ and TNF-α. See the Supplemental Experimental Procedures for details.

Microarray Analysis

Global gene expression analysis was performed on the $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ (NKP), $Lin^-CD34^+CD38^-CD90^-CD45RA^+$ (multipotent progenitors LMPP), $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^+$ (common lymphoid progenitor [CLP]-like cell), and $CD3^-CD56^+NKp46^+$ (mature NK cells) purified from CB (two experiments, three replicates for each population). See the Supplemental Experimental Procedures for details.

Quantitative Gene Expression Analysis by Fluidigm

Multiplex real-time PCR analysis was performed using BioMark 48.48 Dynamic Array platform (Fluidigm) and TaqMan Gene Expression Assays (Life Technologies). For cDNA synthesis and preamplification of target Genes, CellsDirect One-Step qRT-PCR kit (Invitrogen) was used. Twenty-five cells were sorted directly into 96-well PCR plate containing 2.5 μl gene specific 0.2× TaqMan gene expression assays (Applied Biosystems), 5 μl of CellsDirect 2× Reaction mix (Invitrogen), 1.2 μl CellsDirect RT/Taq mix, 1.2 μl TE buffer, and 0.1 μl SUPERase-In RNase Inhibitor (Ambion). Conditions for reverse transcription and target gene amplification were: 15 min at 50°C; 2 min at 95°C; and 22 cycles of 95°C for 15 s and 60°C for 4 min. Preamplified products were diluted 1:5 in TE buffer and analyzed on Dynamic Array with the following PCR cycling condition: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 60 s. Data were analyzed via the ΔCt method: results were normalized to *GAPDH* expression and expressed as mean expression level relative to *GAPDH* (±SEM). TaqMan primers used for the analysis are listed in Table S3.

Statistics

All results are expressed as mean (SD) or frequency of viable single cell (determined with Flow Jo software). The statistical significances between groups were determined by the Mann-Whitney test.

ACCESSION NUMBERS

Data have been deposited into NCBI Gene Expression Omnibus portal under the accession number GEO: GSE60448.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.07.011.

AUTHOR CONTRIBUTIONS

V.M.R. and E.S. designed and conceptualized the overall research, analyzed the data, and wrote the manuscript. V.M.R., A.Z., C.P., J.M., and S.S. performed experiments and analyzed the data. D.F. collected human tonsil tissues.

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