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TGF-β affects development and differentiation of human natural killer cell subsets

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Summary

Human peripheral blood NK cells may be divided into two main subsets: $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$. Since TGF-β is known to influence the development of many leukocyte lineages, its effects on NK cell differentiation either from human $CD34^{+}Lin^{-}$ hematopoietic progenitor/stem cells *in vitro* or from peripheral blood NK cells were investigated. TGF-β represses development of NK cells from $CD34^{+}$ progenitors and inhibits differentiation of $CD16^{+}$ NK cells. Moreover, TGF-β also results in conversion of a minor fraction of $CD56^{bright}CD16^{+}$ cells found in peripheral blood into $CD56^{bright}CD16^{-}$ cells, highlighting a possible role of the former as a developmental intermediate and of TGF-β in influencing the genesis of NK subsets found in blood.

Keywords

NK cells; Cell differentiation; Innate immunity

Introduction

Two main subsets of NK cells are present in human peripheral blood, termed $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ [1,2]. $CD56^{dim}CD16^{+}$ NK cells make up the vast majority of peripheral blood NK cells (>90%). They are highly cytotoxic towards tumors and some virus infected cells, but produce only low levels of cytokines [2,3]. In contrast, $CD56^{bright}CD16^{-}$ NK cells, representing <10% of blood NK cells, display less cytotoxic activity *ex vivo*, but efficiently produce IFN-γ, TNF-α, LTα, IL-10, and GM-CSF in response to non-specific stimulation or monokines [2,3]. These two subsets also differ in the expression of many other cell surface receptors, including killer-cell immunoglobulin-like receptors (KIR) and CD94/NKG2A heterodimers, which are preferentially found on $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ NK cells, respectively. Microarray comparisons of the two subsets have identified numerous additional genes that are differentially expressed [4,5]. By contrast, most NK cells in lymph nodes have a $CD56^{bright}CD16^{-}$ phenotype [6,7]. Recently, several groups have identified an additional subset of NK-like cells in mucosa-

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associated lymphoid tissue that secrete IL-22 [8,9]. In mice, these NK-22 cells are non-cytotoxic and do not produce IFNγ, but are characterized by expression of the RORγt transcription factor and IL-7Rα (CD127) [8,9]. Decidual NK cells with a distinct phenotype are also found in abundance at the maternal-fetal interface during human pregnancy [4,10].

TGF-β1,2,3 are multi-functional pleiotropic cytokines, playing significant roles in embryogenesis, development, tissue renewal, and regulation of the immune system. TGF-β1 is the predominant form expressed by immune cells [11]. TGF-β is known to regulate many branches of hematopoiesis, affecting differentiation and proliferation of hematopoietic stem cells as well as progenitor cells of erythrocytes, granulocytes, monocytes/macrophages, dendritic cells, megakaryocytes, and other lineages [12]. The effects of TGF-β are often very dependent on the developmental stage and *in vivo* environmental context, with different (and often opposing) effects at different stages [12]. TGF-β also influences T cells at several different phases of development/differentiation, including controlling formation of both inflammatory Th17 cells and $Foxp3^+$ regulatory T cells [11].

To date, TGF-β has been shown to exert many effects on NK cells, including inhibition of proliferation, cytotoxicity, and IFNγ production, and down-regulation of activating receptors such as NKG2D and NKp30 [13–16]. In the present paper, its effect on NK cell development and differentiation has been explored, from both immature progenitors and from mature peripheral blood NK cells.

Results and Discussion

TGF-β affects the numbers and phenotype of NK cells developing from human $CD34^+$ progenitor cells

To investigate the effects of TGF-β on NK cell development, $CD34^+CD38^{low/-}$ hematopoietic progenitor/stem cells (HPC) from human bone marrow were cultured in the presence of OP9 stromal cells with IL-15, SCF, and Flt3L, cytokines known to facilitate NK cell development. Supplementation of the cultures with TGF-β1 (2ng/ml) resulted in lower percentages and dramatically reduced numbers of $CD56^+$ NK cells (Figure 1a). In similar cultures using $CD34^+CD38^{low/-}$ HPC isolated from human umbilical cord blood, TGF-β again repressed the numbers of NK cells that developed (Figure 1b). TGF-β also appeared to inhibit or delay the acquisition of markers of NK cell maturation or subset formation such as CD94, CD16, and KIR (Supporting Information Figure 1). TGF-β similarly inhibited or delayed the ability of developing NK cells to lyse the OP9 stromal cell monolayer.

Parallel results were observed in another experimental system. Bone marrow $CD34^+Lin^-$ HPC were grown without stromal cells in a different culture medium with IL-15, SCF, and IL-7. Under these conditions, when the activity of TGF-β was blocked with neutralizing anti-TGF-β1,2,3 monoclonal antibody (mAb), increased numbers of NK cells were observed at approximately 30 days of culture (Figure 1c). Larger numbers of other cell types were also observed, including adherent $CD56^-$ MHC class II^+ $CD11c^+$ cells. Blockade of TGF-β activity also affected the phenotype of NK cells that developed from HPC in these cultures. In the presence of anti-TGF-β mAb, greater percentages of NK cells expressed CD16 (Figure 1c; Supporting Information Figure 2). An increase in the number of KIR^+ NK cells was also observed upon neutralization of TGF-β (Supporting Information Figure 2). Both CD16 and KIR are characteristic of the $CD56^{dim}CD16^+$ peripheral blood NK cell subset. The TGF-β that mediated these effects may have been derived from the NK cells themselves or from other cell types that also developed in these cultures. However, it is likely that the TGF-β was derived from the serum in the culture system, since MyeloCult medium contains horse serum and FBS, and culture medium was additionally supplemented with FBS and human serum (HS). This medium showed similar levels of total TGF-β1 before and after

culture of developing NK cells (~ 50pg/ml) (measured by Biosource multispecies ELISA). It should be noted that addition of anti-TGF-β mAb did not augment NK cell numbers in OP9 stromal cell co-cultures (such as those depicted in Figure 1a–b). This discrepancy in the activity of anti-TGF-β mAb may have been due to: (i) differences in the quantity of endogenous serum TGF-β between the two varieties of culture medium, (ii) differences in cell number in culture that might overwhelm the 10μg/ml concentration of mAb or (iii) production or depletion of TGF-β by OP9 stromal cells. However, in clinical protocols for amplification of NK cells, it may be important to control the levels of TGF-β in culture to achieve maximal yields of NK cells. Effects of TGF-β on numbers of NK cells have also been observed in murine transgenic models [17].

TGF-β directly inhibits development/differentiation of NK cell subsets

In the preceding experiments, other cell types also developed in these cultures *in vitro*. Thus, the observed effects may have been indirect, with TGF-β influencing other cell lineages or stromal cells which, in turn, affected NK cell development. In addition, TGF-β has previously been shown to affect proliferation and differentiation of HPC [12]. Therefore, the effect of TGF-β on developing NK cells was investigated directly. CD161 (NKR-P1A) is one of the earliest markers to be expressed during human NK cell development [18]. Therefore, $CD161^+$ cells (that had not yet acquired CD94 or CD16 expression) were sorted from cultures undergoing NK development from umbilical cord blood HPC (as Figure 1b above). When placed back into medium with cytokines but without stromal cells, a proportion of these cells acquired CD94 and CD16 expression (Figure 2). However, addition of 2ng/ml TGF-β1 completely blocked this developmental progression/differentiation and greatly reduced proliferation of the cells (Figure 2). Analogous results were observed with sorted $CD56^+CD161^+CD94^-CD16^-$ cells (not shown).

TGF-β inhibits and down-regulates CD16 expression on $CD56^{bright}$ NK cells from peripheral blood

Several recent studies have provided insights into the developmental relationship between human NK cell subsets. Culture of $CD56^{bright}CD16^-$ NK cells with synovial or dermal fibroblasts was reported to cause differentiation into $CD56^{dim}CD16^+KIR^+$ cells exhibiting many characteristics of *ex-vivo* $CD56^{dim}CD16^+$ NK cells [19]. Related results were obtained upon culture with IL-2 [20,21], or after transfer of human NK cells into mouse models [19,22]. Similarly, when $CD56^{bright}CD16^-$ NK cells were sorted from human peripheral blood and cultured with IL-15 for 15 days, appearance of some $CD16^+$ cells was observed (Figure 3c column 1, Supporting Information Figure 3). However, percentages of KIR (or other features of $CD56^{dim}$ NK cells) did not increase substantially (not shown and Supporting Information Figure 4), suggesting that stromal cell factors or IL-2 (as opposed to IL-15) are required for complete transition to a $CD56^{dim}CD16^+$ phenotype [19,20]. Neutralization of TGF-β activity with mAb did not affect, or increased, CD16 expression (Figure 3c column 1, Supporting Information Figure 3). On the other hand, addition of 2ng/ml TGF-β1 largely blocked the appearance of $CD16^+$ cells (Figure 3c column 1, Supporting Information Figure 3). Here, sorted NK cells were highly pure, precluding indirect effects of other cell types. Thus, TGF-β inhibited the acquisition of CD16 on NK cells from peripheral blood, as well as on NK cells derived from bone marrow progenitors.

Many individuals possess a substantial population of $CD56^{bright}$ NK cells that also express CD16, appearing between $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ cells in two-color flow cytometric analysis (Figure 3a) [1]. These cells were hypothesized to be either a developmental intermediate between the other two subsets, or a distinct functional subset [1]. Thus, the effects of TGF-β were examined on each. Four populations of cells were sorted from peripheral blood NK cells ($CD56^{bright}CD16^-$, $CD56^{bright}CD16^{low}$,

$CD56^{bright}CD16^{high}$, and $CD56^{dim}CD16^{+}$ (Figure 3a,b)), then cultured for approximately two weeks with IL-15. Upon culture with anti-TGF-β neutralizing mAb, the $CD16^{+}$ subsets largely retained their CD16 expression (Figure 3c, Supporting Information Figure 3). In contrast, upon addition of TGF-β1, both $CD56^{bright}CD16^{low}$ and $CD56^{bright}CD16^{high}$ cells robustly lost CD16 expression to become $CD56^{bright}CD16^{-}$ (Figure 3c, Supporting Information Figure 3). $CD56^{dim}CD16^{+}$ NK cells showed a reduced tendency to down-regulate CD16 expression upon culture with TGF-β (Figure 3c, Supporting Information Figure 3). These results are consistent with a previously published observation, in which total $CD16^{+}$ blood NK cells sorted and cultured with TGF-β1 gave rise to a subset that lost CD16 expression, and another subset that remained $CD16^{+}$ [23]. Here, the $CD56^{bright}CD16^{+}$ NK cell subset was identified to be most responsive to TGF-β in terms of CD16 down-regulation. This loss of CD16 expression was also accompanied by proliferation [23] (Figure 3d). $CD56^{bright}CD16^{+}$ and $CD56^{bright}CD16^{-}$ NK cells showed similar average levels of proliferation in culture (Figure 3d), implying that these results were not due to outgrowth of rare sort contaminants. TGF-β inhibited proliferation of all subsets (Figure 3d). In these experiments with peripheral blood NK cells, effects of anti-TGF-β mAb were more limited, perhaps for the reasons mentioned above, or perhaps due to differential sensitivities or different responses of progenitors to TGF-β versus mature NK cells.

Therefore, for $CD56^{bright}$ NK cells, 2-week exposure to TGF-β inhibited acquisition of CD16 and down-regulated pre-existing CD16 expression. The majority of $CD56^{dim}CD16^{+}$ cells showed different responses to TGF-β (such as down-regulation of CD56 [Supporting Information Figure 3] and CX3CR1 chemokine receptors [Supporting Information Figure 4]). It is possible that divergent effects of TGF-β may be related to differences in TGF-β receptor expression (Supporting Information Figure 5). Although these may simply reflect subset-specific effects of TGF-β (e.g. on CD16 and ADCC function), these results may have relevance to NK cell differentiation pathways:

In accordance with recent reports [19,20,22], human NK cells may differentiate along a pathway from $CD56^{bright}CD16^{-}$ cells to $CD56^{bright}CD16^{+}$ intermediate cells, to fully cytotoxic $CD56^{dim}CD16^{+}$ NK cells. TGF-β may be able to block progression along this pathway, and is able to convert $CD56^{bright}CD16^{+}$ intermediate cells back to a $CD16^{-}$ phenotype. However, $CD56^{dim}CD16^{+}$ NK cells may be fully differentiated to a committed mature effector phenotype and thus refractory to these effects of TGF-β.

On the other hand, TGF-β may primarily generate decidual-like NK cells resembling those at the maternal-fetal interface [23]. TGF-β has been shown to up-regulate CD9 and CD103 (αE integrin) molecules found on decidual NK cells [23], and expression of CD9 is eliminated with addition of anti-TGF-β mAb on NK cells cultured in IL-15 (not shown).

For each of the three sorted $CD56^{bright}$ NK populations from peripheral blood, culture with TGF-β1 or anti-TGF-β did not markedly change percentages of KIR^{+} cells (not shown and Supporting Information Figure 4). Statistically significant changes in KIR percentage have previously been seen with TGF-β treatment of enriched total NK populations, likely attributed to survival/expansion of mature NK cells that had already acquired KIR [23] (Supporting Information Figure 4). TGF-β may differentially effect *de novo* acquisition of KIR on immature NK cells developing from HPC versus more mature circulating peripheral blood NK cells. Alternatively, in HPC cultures, adherent MHC class II^{+} $CD11c^{+}$ cells, or other cells (that also developed), or OP9 stromal cells, may have provided additional signals necessary for KIR expression.

CD56^{bright}CD16^{+} NK cells most closely resemble CD56^{bright}CD16^{-} NK cells

These experiments highlighted the CD56^{bright}CD16^{+} NK cell subset as a potential intermediate in NK cell subset differentiation. Although the phenotype of CD56^{bright}CD16^{+} NK cells from blood has been previously examined [1], many additional molecules have subsequently been shown to be expressed at higher levels and/or in greater percentages on either CD56^{bright}CD16^{-} NK cells or CD56^{dim}CD16^{+} NK cells (listed in Figure 3e and references [2,4,24–26]. Importantly, expression of each of these molecules on the intermediate CD56^{bright}CD16^{+} populations (both CD16^{low} and CD16^{high}) was similar to CD56^{bright}CD16^{-} NK cells, rather than to CD56^{dim} NK cells (Figure 3e). Similarly, intracellular staining of perforin was lower in CD56^{bright}CD16^{-}, CD56^{bright}CD16^{low}, and CD56^{bright}CD16^{high} NK cells, while CD56^{dim}CD16^{+} cells stained to a higher level (not shown). Thus, with the exception of CD16, for each of the markers examined, CD56^{bright}CD16^{+} NK cells more closely resembled CD56^{bright}CD16^{-} NK cells than CD56^{dim} NK cells. Effects of TGF-β on expression of these markers are depicted in Supporting Information Figure 4. Several of these molecules have been used to describe potentially analogous mouse NK cell subsets [27–29]. Another subset from human blood with intermediate phenotype, CD56^{dim}CD94^{high}, has recently been described [30], which could potentially be downstream of CD56^{bright}CD16^{+} cells in NK differentiation. Interestingly, CD56^{bright}CD16^{+} and CD56^{bright}CD16^{-} NK cells are more prevalent in the early period following bone marrow transplantation [31].

Concluding remarks

During the development of NK cells from bone marrow or umbilical cord blood CD34^{+} HPC *in vitro*, TGF-β was shown to influence the number of resulting NK cells (Figure 1). TGF-β also inhibited formation of CD16^{+} NK cells in sorted cultures of bone marrow HPC, immature CD161^{+}CD94^{-}CD16^{-} NK cells, or peripheral blood CD56^{bright}CD16^{-} NK cells (Figures 1,2,3). Furthermore, addition of TGF-β to CD56^{bright}CD16^{+} NK cells resulted in their conversion to CD56^{bright}CD16^{-} cells (Figure 3). Thus, levels of TGF-β at sites of NK development *in vivo* may influence the developmental progression and subset formation of NK cells.

Materials and methods

Cells

Bone marrow HPC were isolated from fresh normal whole human bone marrow (All Cells LLC., Emeryville, CA, USA) using RosetteSep human progenitor reagent (Stem Cell Technologies, Vancouver, BC, Canada), followed by flow cytometry sorting for CD34^{+}Lin^{-} (CD3^{-}CD14^{-}CD16^{-}CD19^{-}CD20^{-}CD56^{-}) cells (Figure 1c). Alternatively, CD34^{+}CD38$^{low/-}$CD56^{-} bone marrow HPC were sorted from frozen enriched human bone marrow CD34^{+} progenitors (Lonza, Basel, Switzerland) (Figure 1a). In accordance with approved guidelines established by the Research Ethics Board of Sunnybrook Health Sciences Center, umbilical cord blood HPC were isolated as previously described [32] by negative selection using the StemSep Human Progenitor Enrichment Kit (StemCell Technologies), and flow cytometry sorting for CD34^{+}CD38$^{low/-}$CD56^{-} cells. Peripheral blood NK cells were isolated from LeukoPaks (Massachusetts General Hospital Blood Apheresis Center, Boston, MA, USA) using RosetteSep human NK cell reagent (Stem Cell Technologies), then immediately analyzed or sorted by flow cytometry.

Cell culture conditions

Sorted cells were cultured *in vitro* in 24, 12, or 6 well plates with human cytokines (Peprotech, Rocky Hill, NJ, USA) in several culture media as indicated. MyeloCult medium

was from Stem Cell Technologies. Anti-TGFβ (clone 1D11) mAb was obtained from R&D Systems (Minneapolis, MN, USA). Medium, cytokines and neutralizing mAb were refreshed every 3–5 days by changing all or half the culture volume. Where indicated, cells were co-cultured on confluent monolayers of non-irradiated OP9 cells. Every 3–11 days, co-cultured cells were disaggregated as described [32] and placed onto new monolayers of OP9 (more frequently after stromal cell lysis was observed). Numbers of NK cells that developed were calculated by multiplying the percentage of $CD56^+$ cells (from flow cytometry) X the cell count X the factor by which the cells had been diluted during culture. Measurements taken at approximately the same duration from different experiments were averaged to single data points. OP9 cells support NK cell but not T cell development from HPC [32].

Flow cytometry

Samples were analyzed using FACSCalibur instruments (BD, Franklin Lakes, NJ, USA) and the following fluorescently conjugated mAb clones. From BD Pharmingen: CD56 (NCAM16.2, B159), CD16 (3G8), KIR (HP-3E4 ,CH-L, DX9), CD127 (hIL-7R-M21), CD27 (M-T271), CD94 (HP-3D9), CD2 (RPA-2.10), CD161 (DX12), HLA-DR (L243), CD62L (Dreg-56), CD25 (M-A251), CD122 (Mik-β2), CD34 (563, 581), CD38 (HIT2), and Lineage cocktail (340546). From R&D Systems: CXCR1 (42705), and CXCR3 (49801). From Biolegend (San Diego, CA, USA): CXCR2 (5E8), CD6 (BL-CD6), and CD44 (IM7). From MBL International (Woburn, MA, USA): CX3CR1 (2A9-1). From eBioscience (San Diego, CA, USA): CD16 (CB16).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

KIR	killer-cell immunoglobulin-like receptors
HPC	hematopoietic progenitor/stem cells
HS	human serum

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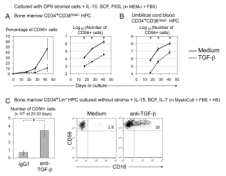

Figure 1. TGF-β affects the number of NK cells and the percentage of CD16^{+} NK cells developing from CD34^{+} HPC *in vitro*

(A) Approximately 5000 human CD34^{+}CD38$^{low/-}$CD56^{-} HPC were sorted from enriched bone marrow CD34^{+} cells, and cultured on OP9 stromal cells with IL-15 (20ng/ml), SCF (20ng/ml), and Flt3L (5ng/ml) in MEMα medium plus 20% heat-inactivated FBS. TGF-β1 (2ng/ml) was added to some cultures. Numbers of NK cells that developed were calculated as described in the Materials and methods. Plots represent the means of three experiments from two donors. (B) Similar cultures were initiated using approximately 5000 CD34^{+}CD38$^{low/-}$CD56^{-} HPC sorted from umbilical cord blood (Mean of three experiments from three donors). (C) 5000 to 12000 human CD34^{+}Lin^{-} HPC were sorted from bone marrow aspirates, then cultured with IL-15 (20ng/ml), SCF (20ng/ml), and IL-7 (20ng/ml) in MyeloCult medium plus 10% heat-inactivated human serum and 5% heat-inactivated FBS. Cultures were additionally supplemented with IgG1 control or anti-TGF-β neutralizing mAb (clone 1D11) (10μg/ml). Mean numbers of developing NK cells were calculated for 8 experiments from 4 donors. Flow cytometry dot-plots were gated on resulting CD56^{+} cells. No CD3^{+} cells developed in these cultures. In (A), (B), and (C), data show mean ± SEM. *$p<0.05$, two-tailed Student's t test comparing LOG_{10}-transformed values.

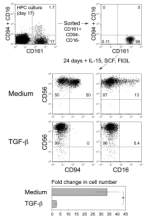

Figure 2. TGF-β inhibits acquisition of CD94 and CD16 on developing NK cells

Co-cultures of OP9 stromal cells and HPC isolated from human umbilical cord blood were initiated as described in Figure 1a–b. At day 17, $CD161^{+}CD94^{-}CD16^{-}$ cells were sorted to high purity and placed back into culture with IL-15 (20ng/ml), SCF (20ng/ml), and Flt3L (5ng/ml) without stromal cells in MEMα medium plus 20% FBS. TGF-β1 (2ng/ml) was added to some cultures. After 24 additional days, resulting cells were counted and stained for flow cytometry. Plots were gated on live cells by FSC/SSC/PI. Numbers indicate the percentage of cells in each quadrant. Mean fold change in cell number ± SEM from three similar independent experiments is also shown. *$p<0.05$ in Student's t test.

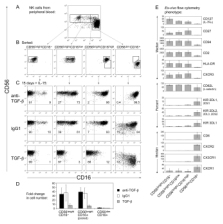

Figure 3. TGF-β inhibits and down-regulates CD16 expression on CD56^{bright} NK cells from blood

(A) NK cells were isolated from human peripheral blood by depletion of other cell lineages (CD3$^+$, CD4$^+$, CD19$^+$, CD36$^+$ or CD66b$^+$), then stained with anti-CD56 and anti-CD16 mAb, and sorted into sub-populations as indicated. (B) Purity of cells immediately after sorting. (C) Phenotype of sorted cells after culture for 15 days with IL-15 (20ng/ml) plus IgG1, or anti-TGF-β 1D11 mAb (10μg/ml), or added TGF-β1 (2ng/ml). MyeloCult medium was used (plus 10% HS and 5% FBS). Numbers indicate the percentage of cells in each quadrant. Only CD56$^+$ cells are shown in A; all live cells are shown in B and C (gated by FSC/SSC). Data in A/B versus C were collected with different staining and instrument protocols, so staining intensities are not directly comparable. (D) Changes in cell number in these experiments; data show mean ± SEM from three independent experiments. Results from CD56^{bright}CD16^{low} and CD56^{bright}CD16^{high} populations were pooled for analysis and labeled CD56^{bright}CD16$^+$. (E) *Ex-vivo* flow cytometry staining of human peripheral blood NK cell populations isolated, stained, and gated as depicted in (A). Data show median fluorescence intensity (minus median fluorescence of IgG control) or percentage of cells staining positively for each indicated mAb ± SD from 3–6 donors.