Human Natural Killer Cell Maturation Defect Supports In Vivo CD56\textsuperscript{bright} to CD56\textsuperscript{dim} Lineage Development

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Abstract

Two populations of human natural killer (NK) cells can be identified in peripheral blood. The majority are CD3\textsuperscript{−}CD56\textsuperscript{bright} cells while the minority exhibits a CD3\textsuperscript{−}CD56\textsuperscript{dim} phenotype. \textit{In vitro} evidence indicates that CD56\textsuperscript{bright} cells are precursors of CD56\textsuperscript{dim} cells, but \textit{in vivo} evidence is lacking. Here, we studied NK cells from a patient that suffered from a melanoma and opportunistic fungal infection during childhood. The patient exhibited a stable phenotype characterized by a reduction in the frequency of peripheral blood CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cells, accompanied by an overt increase in the frequency and absolute number of CD3\textsuperscript{−}CD56\textsuperscript{bright} cells. These NK cells exhibited similar expression of perforin, CD57 and CD158A, the major activating receptors CD16, NKp46, NKG2D, DNAM-1, and 2B4, as well as the inhibitory receptor CD94/NKG2A, on both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells as healthy controls. Also, both NK cell subpopulations produced IFN-γ upon stimulation with cytokines, and CD56\textsuperscript{dim} NK cells degranulated in response to cytokines or K562 cells. However, upon stimulation with cytokines, a substantial fraction of CD56\textsuperscript{dim} cells failed to up-regulate CD57 and CD158, showed a reduction in the percentage of CD16\textsuperscript{+} cells, and CD56\textsuperscript{dim} cells did not down-regulate CD62L, suggesting that CD56\textsuperscript{dim} cells could not acquire a terminally differentiated phenotype and that CD56\textsuperscript{bright} cells exhibit a maturation defect that might result in a potential altered migration pattern. These observations, support the notion that NK cells of this patient display a maturation/activation defect that precludes the generation of mature NK cells at a normal rate accompanied by CD56\textsuperscript{dim} NK cells that cannot completely acquire a terminally differentiated phenotype. Thus, our results provide evidence that support the concept that \textit{in vivo} CD56\textsuperscript{bright} NK cell differentiation is impaired in CD56\textsuperscript{dim} NK cells, and contribute to further understand human NK cell ontogeny.

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Introduction

Natural Killer (NK) cells exert cytotoxic functions and secrete IFN-γ and other pro-inflammatory cytokines against virus-infected and tumor cells. Also, NK cells are key regulators of the adaptive immune response through their cross talk with dendritic cells (DCs) that promotes DC maturation, and T helper (Th) 1- and cytotoxic T lymphocyte (CTL)-mediated immunity [1,2]. NK cell activity is regulated by cytokines such as interleukin (IL)-2, IL-12, IL-15, IL-18 and type I interferons (IFNs) [3]. NK cell effector function is also triggered upon recognition of target cells through activating receptors such as NKG2D, DNAM-1, 2B4, the Natural Cytotoxicity Receptors (NCRs) NKp46, NKp44 and NKp30, and members of the Killer Immunoglobulin-like Receptor (KIR) family that carry a short cytoplasmic tail (KIR2DS and KIR3DS) [4,5]. Conversely, normal cells are preserved from NK cell mediated functions because they promote engagement of inhibitory KIR receptors that carry a long cytoplasmic tail (KIR2DL and KIR3DL), CD94/NKG2A and members of the Immunoglobulin-Like Transcript (ILT) receptor family [4,5]. Human NK cells, defined as CD3\textsuperscript{−}CD56\textsuperscript{+} cells, can be subdivided in two main cell populations. About a 90% are cytotoxic CD56\textsuperscript{dim}CD16\textsuperscript{+} cells, while the remaining 10% are CD56\textsuperscript{bright}CD16\textsuperscript{dim}−/− non-cytotoxic cells that are abundant in secondary lymphoid organs [6,7]. This subpopulation expresses CD62L (L-selectin) and CCR7, which directs their homing to these niches but upon activation, CD62L down-regulation facilitates NK cell trafficking to inflamed tissues [8].}

\textit{In vitro} evidence indicates that CD56\textsuperscript{bright} NK cells are precursors of CD56\textsuperscript{dim} NK cells. However, \textit{in vivo} evidence about such lineage development is still lacking. As mouse NK cells are different from human NK cells in many aspects [1], results from knockout mice cannot be extrapolated to humans. Conversely, human NK cell deficiencies are helpful to unravel the immunobiology of NK cells but these conditions are very rare [9]. Absolute or functional NK cell deficiencies are
Figure 1. Lung infection and analysis of blood mononuclear cells. A) CT scan of the lungs of the patient, showing an area of consolidation of 43×60 mm in right lower lobe parenchyma (indicated by arrows). B) Lung biopsy (sliced in two pieces) with visible cavitated lesions with mucoid material. C) Hematoxylin/eosin staining of the lung biopsy showing the infiltration of inflammatory cells with predominance of histiocytes and multinucleated giant cells containing PAS-positive spherical bodies of 5 to 10 μm in diameter (indicated by arrows). The morphological characteristics of these PAS-positive spherical bodies are compatible with Cryptococcus neoformans infection. D) Absolute numbers of total NK cells, CD3+CD56\textsuperscript{dim} cells and CD3+CD56\textsuperscript{bright} cells in blood of 6 healthy normal donors (N) and in blood from two draws from different dates (indicated in parentheses) from the patient (P). E) PBMCs from 9 different normal donors (N) and from different blood draws from different dates (indicated in parentheses) from the patient (P) were stained with anti-CD3 and anti-CD56 mAbs, and the percentage of NK cells (CD3+CD56\textsuperscript{dim}), CD3+CD56\textsuperscript{dim} and CD3+CD56\textsuperscript{bright} cells within the whole mononuclear cell population (PBMCs) were calculated and depicted as dot plots. Also, the relative abundance of CD3+CD56\textsuperscript{dim} and CD3+CD56\textsuperscript{dull} cells was calculated and depicted (CD3+CD56\textsuperscript{dull} are shown in black bars; CD3+CD56\textsuperscript{dull} cells are shown in white bars). Each sample of the patient was tested at least twice, and individual values obtained are shown as a dot in the graphs. Intercuartile ranges (IQR) are indicated in the graphs of panels D and E. F) Representative dot plots of lymphoid cells gated according to their FSC and SSC parameters. The numbers in each region correspond to the percentage of CD3+CD56\textsuperscript{dim} and CD3+CD56\textsuperscript{bright} cells within the lymphoid population (gated according to their FSC and SSC parameters). G) Representative dot plots to show the percentage of CD3+CD56+CD16+ NK cells within the CD3+ lymphoid cell population in a healthy normal control (N) and in one sample of the patient (P). The numbers in each quadrant correspond to the percentage of CD16+CD56\textsuperscript{dim}+ CD16+CD56\textsuperscript{bright} NK cells. Right graph: data from 3 healthy normal controls and 3 blood samples from different dates from the patient (P). Percentages depicted correspond to the percentage of CD3+CD56+CD16+ NK cells within the total NK cell population (CD16+CD56\textsuperscript{dim}+ CD16+CD56\textsuperscript{bright} cells). H) PBMCs from normal donors (N) and from the patient (P) were stained with anti-CD3, anti-CD14 and anti-HLA class I mAbs to assess HLA class I expression (continuous line) on T cells (CD3 cells) and monocytes (CD14+ cells). Data presented correspond to representative histograms. Dashed lines: IC mAb. The numbers inserted in the graphs correspond to the MFI. I) PBMCs from normal donors (N) and from the patient (P) were stained with anti-CD4, anti-CD25 and anti-Foxp3 to assess the percentage of Tregs. The dot plots correspond to Foxp3 vs. CD25 in CD4+ cells gated from the FSC vs. SSC plots. The percentages in each quadrant are shown. The right graph shows the percentage of Tregs in PBMCs from normal healthy controls and in two blood samples from different dates (indicated in parentheses) from the patient (P). One representative experiment from 3 independent analyses is shown in C and D. PBMCs from blood samples obtained in February 2011 were used for panels F, H and I. doi:10.1371/journal.pone.0051677.g001

Methods

Studies have been approved by the institutional review board and written informed consent was obtained from the parents of the patient (as he was not major at the beginning of these studies). In addition, written consent was obtained from the parents on behalf of the minors involved in our study as well as from the major healthy volunteers that provided blood samples for this study. Also, all the participants (or the parents of the participants) of this study wrote written consent for the publication of the clinical data gathered along this investigation.

Antibodies, cytokines and reagents

Recombinant human IL-2, IL-12 and IL-15 were from PeproTech; recombinant human IL-18 was from MBL International. The following mAbs against human molecules were used: FITC-, PE- and SPRD-labeled anti-CD3 (UCHT-1, Southern Biotech); PE/Cy7-labeled anti-CD3 (UCHT-1, Biolegend); FITC-labeled anti-CD14 (HCD14 Biologend); PE-labeled anti-HLA class I (W6/32, Biolegend); PE/Cy5-labeled anti-CD56 (N901, Beckman Coulter); FITC-labeled anti-CD16 (eBioCB16, eBioscience); FITC-labeled anti-CD57 (HCD57, Biolegend); FITC- or PE-labeled anti-CD62L (DREG-36, Southern Biotech); FITC-labeled anti-perforin (pdp, dG9, Biolegend); PE-labeled anti-IFN-γ (AS.B3, Biolegend); FITC-labeled anti-CD107a (1D4B, BD Biosciences); PE-labeled anti-CD25 (BC96, Biolegend); FITC-labeled anti-CD69 (FN50, BD); PE-labeled anti-NKG2D mAb (clone 1D11, Biolegend); PE-labeled anti-NKp46 (clone 9E2, Biolegend); FITC-labeled anti-CD226 (DNAM-1, clone DX1, BD Pharmingen); PE-labeled anti-CD107a (1D4, clone C1.7, Biolegend); PE-labeled anti-CD94 (clone DX22, Biolegend); APC-labeled anti-NKG2A (clone 131411, R&D); mAb isotype-matched control mAb (IC, eBioscience). KIR analysis was performed with a mix of PE-labeled anti-CD158a, h (EB6.B) and the anti-CD158b1, b2 (GL183) mAbs (both from Beckman Coulter). For regulatory T cell staining, the Alexa Fluor 488, FoxP3, CD4, CD25 FoxP3 staining kit from BD was used. 7AAD Viability Staining Solution was from Biolegend.

PBMCs and NK cells

Human peripheral blood mononuclear cells (PBMCs) and NK cells were isolated from blood of healthy volunteers or from the patient described in this work (called “P”) using Ficoll-Paque™ Plus (Amersham Biosciences) centrifugation and the RosetteSep kit (StemCell), respectively. During the initial testing, we recruited age-matched healthy donors to compare the patient with these age-matched samples (hematological data, percentage of NK cells and CD56\textsuperscript{dim} and CD56\textsuperscript{bright} populations). However, as the values of this age-matched population was similar to the values for adult population, and considering that the patient reached the age of 17 at the end of this study, in subsequent (retroactive) experiments we used healthy donors that correspond to young adults (ages between 25 and 30). Purity of isolated NK cells (CD3+CD56\textsuperscript{+} cells) was above 90%, as assessed by flow cytometry (FC). In some experiments, PBMCs were cultured with IL-2 (0.8 ng/ml) or IL-15 (10 ng/ml) in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (NatoCor, Córdoba, Argentina), sodium pyruvate, glutamine and gentamicin (Sigma) for up to 5 days.
**Table 1.** Laboratory findings in the patient along time.

| Age (years) | 13       | 14       | 16       | 17       | Reference values |
|-------------|----------|----------|----------|----------|------------------|
| Serum IgG (mg/dl) | 1190     | 1270     | 1130     | 1040     | 984–1544         |
| Serum IgA (mg/dl)  | 290      | 247      | 112      | 144      | 112–252          |
| Serum IgM (mg/dl)  | 196      | 80       | 100      | 91       | 82–220           |
| Anti-TT Ab (UI/ml) * | 0.5      | 0.8      | ND †      | ND       | >0.1             |
| Anti- pneumococcus Ab (mg/dl) | 88       | 270      | 216      | 139      | >113 †           |
| Anti-measles virus Ab | +        | ND       | ND       | ND       |                  |
| Anti-HIV Ab        | –        | –        | ND       | ND       |                  |
| Granulocyte percentage in whole blood | 72       | 54       | 58       | 49       | 40–80%           |
| Monocyte percentage in PBMCs (cells/mm³) | 7 (168)  | 8 (156)  | 8 (208)  | 8 (557)  | 4–13%            |
| T cell percentage in PBMCs (cells/mm³) | 57 (1368)| 66 (1289)| 66 (1719)| 58 (4041)| 65–85%           |
| CD3⁺       | 32 (768) | 39 (729) | 36 (936) | 35 (2450)| 36–46%           |
| CD4⁺       | 19 (456) | 23 (430) | 25 (650) | 20 (1400)| 19–40%           |
| CD8⁺       | 28 (672) | 24 (467) | 20 (520) | 26 (1812)| 7–23%            |
| CD20⁺      | 40000    | 115000   | ND       | ND       | 74.000–135.000   |
| PHA        | 79000    | 101000   | ND       | ND       | 54.000–104.000   |
| Anti-CD3  | 46000    | 180000   | ND       | ND       | 55.000–91.000    |
| PMA + Ionomycin | 69000   | 275000   | ND       | ND       | 61.000–149.000   |
| DTH  | N ‡      | ND       | ND       | ND       |                  |

*TT: Tetanus toxoid; †ND: not determined; ‡As established by the Argentinean Society of Pediatrics; §N: normal.

**Flow cytometry and cell sorting**

Cells were stained with specific fluorochrome-labeled mAbs, analyzed in a FACS Aria flow cytometer (BD), and data were processed with the FlowJo software (Tree Star Inc., Ashland, OR). Numerical data presented are the percentage of cells in the quadrants in the dot plots or to the geometric mean fluorescence intensity (MFI) in the histograms. Also, CD3⁺CD56dim and CD3⁺CD56bright cells were sorted with the FACS Aria cell sorter up to 99% of purity.

**IFN-γ production by NK cells**

PBMCs from normal donors or from the patient were stimulated overnight at 37°C with IL-12 (10 ng/ml), IL-15 (1 ng/ml) and IL-18 (10 ng/ml). During the last 4 h of culture, GolgiStop and GolgiPlug reagents (BD) were added, following the instructions provided by the manufacturer. Thereafter, cells were stained with anti-CD3 and anti-CD56 mAbs, permeabilized with Cytofix/Cytoperm (BD) and stained with the anti-IFN-γ mAb to assess IFN-γ producing cells within the CD3⁺CD56dim and CD3⁺CD56bright NK cell subpopulations by FC.

**Degranulation of NK cells**

NK cells were cultured overnight at 37°C with IL-12, IL-15 and IL-18 or K562 target cells. During the last 4 h, the anti-CD107a or an IC mAbs were added together with Golgi-Plug and Golgi-Stop (BD). Thereafter, cells were stained with anti-CD3 and anti-CD56 mAbs, and the percentage of CD107a⁺ cells within the CD3⁺CD56⁺ or within the CD3⁺CD56dim cell populations was determined.

**Statistical analysis**

A paired t-Student test was used to compare expression of NK cell receptors between CD3⁺CD56dim and CD3⁺CD56bright cells.

**Results**

**NK cell number and phenotype in blood of a human patient with melanoma and opportunistic fungal infection**

We studied a case of a pediatric patient (P) that presented repeated upper airway infections and that at the age of 12 developed an ulcerative spitzoid melanoma in his right ear. Although the melanoma was successfully removed by surgery, one year later the patient presented pneumonia in the lower right lobe, accompanied by fever and bloody stools. A computed tomography (CT) of the chest revealed the presence of an opaque mass (Fig. 1A) for which a lung biopsy was taken (Fig. 1B). The analysis of this biopsy revealed the presence of *Cryptococcus neoformans* (Fig. 1C). The fungal infection was non-invasive, there was no evidence about other infectious agent in the lung to suspect that the cryptococcosis could have been a super-infection or a case of commensalism, and patient was successfully treated with fluconazole. Nonetheless, the fact that within one year the patient suffered from a melanoma and a deep fungal infection in the lung led to the suspicion that the patient might be experiencing some sort of immunodeficiency. Therefore, further immunological studies were performed. Standard laboratory tests (Table 1) ruled out classical primary or secondary immunodeficiencies (this patient was HIV-negative). Also, subclasses of IgG (IgG1, IgG2, IgG3 and IgG4),
accumulation of CD3\(^+\)CD56\(^{bright}\) NK cells in peripheral blood when compared to healthy normal controls (Fig. 1D, left graph and Fig. 1E). However, only the altered frequency of NK cells was stable and consistent along time. Therefore, we centered our efforts in a deeper analysis of this cell compartment. This reduced frequency of peripheral blood NK cells was mostly due to the presence of reduced percentages of CD56\(^{bright}\) NK cells (Fig. 1D and 1E) accompanied by increased percentages and absolute numbers (cells/mm\(^3\)) of CD56\(^{dim}\) NK cells in PBMCs (Fig. 1D–F). These results indicate that this patient exhibits a stable phenotype characterized by an accumulation of CD3\(^+\)CD56\(^{bright}\) cells accompanied by a reduction in CD3\(^+\)CD56\(^{dim}\) cells in peripheral blood and suggest that there could be a blockade in the transition of CD3\(^+\)CD56\(^{bright}\) cells to CD3\(^+\)CD56\(^{dim}\) cells in vivo. A more detailed analysis revealed that the patient also seems to exhibit an accumulation of CD3\(^+\)CD56\(^{dim}\) CD62\(^+\) NK cells (Fig. 1G).

Accumulation of CD56\(^{bright}\) NK cells has been observed in certain TAP deficiencies and after treatment with Daclizumab, a humanized anti-CD25 mAb [10]. TAP deficiencies cause reduced expression of HLA class I molecules, but unaltered amounts of HLA class I molecules were detected on T cells and monocytes of this patient (Fig. 1H). Daclizumab interferes with the IL-2 signaling, which is a pathway necessary to generate regulatory T cells (Treg, CD4\(^+\)CD25\(^+\)Foxp3\(^+\) cells). However, this patient exhibited percentages of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) cells in peripheral blood that were similar to healthy normal donors (Fig. 1I). Thus, this patient does not seem to exhibit a TAP deficiency and it is unlikely that he exhibits a blockade in the IL-2 signaling pathway.

To further characterize NK cells from this patient, we performed phenotypic and functional studies. A phenotypic analysis demonstrated percentages of perforin\(^+\), CD16\(^+\), CD57\(^+\), CD158\(^+\) and CD62L\(^+\) cells within the CD56\(^{dim}\) and CD56\(^{bright}\) cell populations similar to healthy donors, as well as slightly reduced percentages of CD57\(^+\)CD158\(^+\) cells within the total NK cell population and also within gated CD3\(^+\)CD56\(^{dim}\) NK cells, when compared to healthy controls (Fig. 2 and Table 2). In addition, we analyzed the expression of a large panel of NK cell receptors on CD56\(^{bright}\) and CD56\(^{dim}\) NK cells (Fig. 3). We observed higher amounts of CD16 on CD56\(^{dim}\) cells than on CD56\(^{bright}\) cells from both, healthy controls and the patient, and these differences were statistically significant (expression on CD56\(^{dim}\) vs. CD56\(^{bright}\) NK cells: p<0.01 for healthy donors and p<0.001 for the patient). Conversely, we observed higher amounts of Nkp46, NK2D, CD94 and NKG2A on CD56\(^{bright}\) cells than on CD56\(^{dim}\) cells from both, healthy controls and the patient. The higher expression of Nkp46, NK2D, CD94 and
Conversely, the expression of DNAM-1 and 2B4 were similar population.

CD56dim NK cells in the dot plots (compare Fig. 2 to Fig. 5B cells of the patient remained clearly distinguishable from healthy donors. In addition, functional analysis revealed statistically significant for each receptor and for NK cells from the patient up-regulated expression of perforin (Fig. 5), CD25 (the \(\alpha\) chain of the high affinity receptor of IL-2, not shown) and CD69 (not shown) in a similar manner as NK cells from healthy donors. However, upon stimulation with these cytokines, CD56\textsuperscript{bright} NK cells of the patient remained clearly distinguishable from CD56\textsuperscript{dim} NK cells in the dot plots (compare Fig. 2 to Fig. 5B and Fig. 5D). Conversely, it was hard to distinguish the CD56\textsuperscript{bright} NK cells from the CD56\textsuperscript{dim} NK cells in healthy donors, appearing both as only one CD56\textsuperscript{dim} homogeneous cell population.

Moreover, less CD56\textsuperscript{dim} NK cells from the patient, compared to healthy controls, acquired CD57 and CD158 upon stimulation with IL-2 or IL-15 (Fig. 5A and 5B, and Fig. 6A). A substantial fraction of CD56\textsuperscript{dim} cells showed a reduction in the percentage of CD16\textsuperscript{+} cells (Fig. 6A and Table 2), and less total NK cells and CD56\textsuperscript{dim} cells from the patient, compared to healthy controls, became CD57\textsuperscript{+}CD158\textsuperscript{+} upon stimulation with IL-2 or IL-15 (Fig. 5B, Fig. 5E and Fig. 6B). Overall, these results suggest that in the patient, less NK cells could progress to an activated phenotype and acquire a terminally differentiated phenotype in response to IL-2 or IL-15. In addition, we observed that CD3\textsuperscript{+}CD56\textsuperscript{bright} cells of the patient exhibited a defective down-regulation of CD62L upon stimulation with IL-2 (Fig. 5A, Fig. 6C and Table 2) that was not due to a slower kinetic of CD62L down-regulation as even at prolonged stimulation periods with IL-2 or IL-15, more than 60% of CD3\textsuperscript{+}CD56\textsuperscript{bright} NK cells of the patient while less than 20% of CD3\textsuperscript{+}CD56\textsuperscript{bright} cells from healthy controls remained CD62L\textsuperscript{+} (Fig. 6D). Of note, the inability to down-regulate CD62L and the sustained high expression of CD56 was not due to apoptosis induction as CD56\textsuperscript{bright} NK cells remained viable after 5 days of stimulation with IL-15 (not shown).

Experiments performed with sorted CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells stimulated with IL-2 revealed that both NK cell subpopulations (from the patient and from the healthy control that was simultaneously analyzed) up-regulated CD56 expression (Fig. 5C). However, sorted CD56\textsuperscript{dim} cells from healthy controls and from the patient did not up-regulate CD158 in response to IL-2, while sorted CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells did not down-regulate expression of CD62L. These results indicate that IL-2 alone cannot provide sufficient maturation signals to sorted NK cell subpopulations.

In summary, the results presented suggest that this patient exhibits a clinical picture that is compatible with a novel immunodeficiency-like condition characterized by a) low frequency of CD56\textsuperscript{dim} NK cells in peripheral blood that cannot properly acquire expression of CD57 and CD158, and maintain expression of CD16 upon stimulation with IL-2 or IL-15; and b) an increase in the frequency and absolute numbers of CD56\textsuperscript{bright} NK cells that do not down-regulate CD62L upon stimulation with these cytokines. Overall, these data might suggest the existence of a defect that compromises NK cell maturation and activation and support the idea that \textit{in vivo} CD3\textsuperscript{+}CD56\textsuperscript{bright} cells differentiate into CD3\textsuperscript{+}CD56\textsuperscript{dim} NK cells.

### Discussion

NK cells are pivotal players of the immune response against tumors and viruses in humans and mice. Although much information about NK cell lineage development has been obtained using genetically modified animals, information about human NK cell ontogeny is limited mostly due to the scarcity of human NK cell deficiencies. Some data have emerged from studies performed in blood of patients after bone marrow transplantation [11]. Also, four NK cell developmental intermediates have been identified in human lymph nodes and tonsils [12]. However, an open and important question is whether CD56\textsuperscript{(dim)} and CD56\textsuperscript{bright} NK cells belong to separate lineages of whether CD56\textsuperscript{bright} NK cells are immature precursors of CD56\textsuperscript{dim} NK cells. In \textit{in vivo} evidence favors the second possibility [10,13–18], but \textit{in vitro} evidence is still lacking. Here, we describe the first human immunodeficiency-like...
Figure 4. Functional response of NK cells. A) NK cells from a normal (healthy) donor (N) or from the patient (P) were stimulated overnight with IL-12, IL-15 and IL-18, and the percentage of IFN-γ-producing NK cells was assessed by FC. Upper panels: Dot plots presented correspond to CD56^dim NK cells from one healthy normal donor and from one sample of the patient. Lower graph: individual data from three healthy normal donors and from two different samples of the patient. B, C) NK cells from a healthy normal donor (N) or from the patient (P) were cultured overnight in the absence (Resting) or in the presence of IL-12, IL-15 and IL-18 (+ Cytokines) or K562 cells at an 1:1 ratio (+ K562), and cell surface CD107a (red histograms) was assessed on gated CD3^- NK cells (B) or on gated CD3^-CD56^dim cells (C) by FC and depicted as histograms. The numbers in the histograms indicate the percentage of CD107a^+ cells. Markers were set with cells stained with the IC mAb (black histograms) to leave less than 1% of positive cells. Experiments were repeated twice with NK cells from two different healthy donors and two different blood draws from the patient with similar results. PBMCs from blood samples obtained in May 2007 were used for panels B and C. doi:10.1371/journal.pone.0051677.g004

A condition characterized by low frequency of CD56^dim NK cells with an overt and persistent increased frequency and absolute numbers of CD56^bright NK cells in peripheral blood. This particular phenotype was accompanied by a slight increased frequency of CD3^-CD56^-CD16^- NK cells, which are expanded during chronic viral infections, are thought to be a source of chemokines relevant for anti-viral immunity, and tentatively derive from CD56^dim NK cells [19]. Increased percentage of CD56^bright NK cells with normal numbers of total NK cells have been observed in different physiological and pathological conditions [20–24], but none of them seem to be plausible in the patient herein studied. Recently, two reports described mutations in the minichromosome maintenance complex component 4 (MCM4) that caused NK cell deficiency and adrenal insufficiency [25,26]. In one report, reduced NK cell numbers were observed but their phenotype and function was not investigated [26]. In the other report it was observed that the patients that carry the mutation exhibited reduced amounts of CD56^dim NK cells and lower percentages of pfp^+ NK cells in blood, and it was concluded that CD56^bright NK cells are largely depend on MCM4 to proliferate and generate CD56^dim NK cells [25]. However, normal amounts of CD56^bright NK cells in blood of the patients were detected. Conversely, in the patient that we present in this work, a consistent increase in absolute counts of CD56^bright NK cells was detected and he did not exhibit a growth retardation or adrenal insufficiency.

A detailed phenotypic analysis of NK cells from our patient revealed percentages of perforin^+, CD16^+, CD57^+, CD158 within the CD56^dim subpopulation, and CD62L^+ cells within the CD56^bright subpopulation that were similar to healthy donors. However, the patient exhibited slightly decreased percentages of CD57^-CD158^- CD56^dim NK cells. Expression of CD57 and CD158 has been associated with the acquisition of a terminally differentiated phenotype by NK cells [27,28,29] for which it appears that the patient presents less terminally differentiated NK cells in peripheral blood. Also, CD56^dim and CD56^bright NK cell subpopulations expressed unaltered amounts of the main receptors involved in NK cell activation (CD16, Nkp46, NK2D and DNAM-1) and regulation (CD94/NKG2A), and did not exhibit a defect in IFN-γ production upon stimulation with IL-12, IL-15 and IL-18. Also, CD3^-CD56^dim NK cells did not present an altered degranulation in response to cytokines and K562 cells. Since we observed a decrease in CD56^dim NK cell frequency with a concomitant increase in the frequency and absolute numbers of CD56^bright NK cells in peripheral blood, we reasoned that the patient may course with a mild immunodeficiency that affects NK...
Table 2. Expression of NK cell markers in CD56dim and CD56bright cells from two healthy normal donors (N1, N2) and from the patient (P).

| Marker       | CD56dim (N1) (resting) | CD56dim (N2) (resting) | CD56dim (P) (resting) | CD56bright (N1) (resting) | CD56bright (N2) (resting) | CD56bright (P) (resting) | CD56bright (N1) (stimulated) | CD56bright (N2) (stimulated) | CD56bright (P) (stimulated) |
|--------------|-------------------------|-------------------------|------------------------|----------------------------|---------------------------|----------------------------|-------------------------------|-------------------------------|-------------------------------|
| CD16         | 98.2                    | 95.6                    | 91.7                   | 90.8                       | 93.0                       | 63.1                       | 65.6                          | 56.6                          | 50.1                          |
| CD57         | 52.4                    | 58.0                    | 56.4                   | 58.8                       | 56.6                       | 43.9                       | 73.3                          | 65.2                          | ND                            |
| CD158        | 34.9                    | 29.8                    | 29.8                   | 71.0                       | 63.6                       | 19.3                       | 12.4                          | ND                            | ND                            |
| CD62L        | 28.6                    | 24.7                    | 16.9                   | 16.9                       | 19.3                       | ND                         | ND                            | ND                            | ND                            |

Numbers correspond to the percentage of cells expressing the indicated marker.

Previous reports [31,32,33] described the existence of a small subpopulation of CD56bright NK cells characterized by the expression of CD16 (CD56brightCD16+) that would be a maturation intermediate. We may speculate that the blockade in the differentiation of CD56bright cells into CD56dim cells in the patient affects a fraction of the CD56brightCD16− cells during their transition to CD56brightCD16+ cells because the former cells remain detectable upon stimulation with IL-2 or IL-15. On the other hand, down-regulation of CD158 [28,29,30]. The unusual behavior of the patient's NK cells was observed when IL-2 stimulations were performed using whole PBMCs. When sorted CD3−CD56dim and CD3−CD56bright cells were stimulated, we were unable to observe up-regulation of CD158 on CD56dim cells and down-regulation of CD62L on CD56bright from the patient and from the healthy control, suggesting that NK cells require more complex signals than IL-2 alone to trigger differentiation and maturation.

Of note, as the altered NK cell phenotype and response to cytokines described is partial and a fraction of NK cells from the patient achieved terminal differentiation, we suggest that he carries some sort of mild immunodeficiency or immunodeficiency-like condition that is probably the result of a heterozygous genotype that generates a “bottleneck” (instead of a complete blockade) that precludes CD56dim NK cell development and activation at rates similar to healthy individuals. Such putative heterozygous genotype would preclude normal differentiation of CD56bright NK cells to CD56dim cells and in the acquisition of a CD62Llow/CD16−/CD57−CD158+ phenotype by CD56dim cells but would allow a sufficient number of NK cell maturation of CD56bright cells and, eventually, activation of CD56dim cells. Surprisingly, stimulation of PBMCs of this patient with IL-2 or IL-15 led to three major observations. First, the CD56bright NK cell subpopulation remained clearly visible and distinguishable from the CD56dim NK cell subpopulation. Second, these CD56bright NK cells could not down-regulate CD62L upon stimulation with IL-2 or IL-15 but remained viable. And third, only a small fraction of CD56dim NK cells acquired CD57 and CD158 upon stimulation with IL-2 or IL-15, while a reduction in the percentage of CD56dimCD16+ was observed. Although this difference indicates that some results obtained in vitro with cytokine-stimulated NK cells may not reflect an in vivo situation, our results indicate that CD56bright cells of this patient could not progress along the normal pathway of maturation and that part of the CD56dim NK cell pool of the patient could not acquire a terminally differentiated phenotype characterized by up-regulated expression of CD16, CD57 and CD158 [28,29,30]. The unusual behavior of the patient’s NK cells was observed when IL-2 stimulations were performed using whole PBMCs. When sorted CD3−CD56dim and CD3−CD56bright cells were stimulated, we were unable to observe up-regulation of CD158 on CD56dim cells and down-regulation of CD62L on CD56bright from the patient and from the healthy control, suggesting that NK cells require more complex signals than IL-2 alone to trigger differentiation and maturation.
cells to mature adequately and respond to activation stimuli, which in turn would provide a sufficient degree of NK cell activity to protect the patient from suffering major infections, in particular viral infections that are characteristic in patients with absolute or severe functional defects in NK cells [9]. Despite these speculations, our results provide evidence that indicates that in vivo, CD56bright cells differentiate into CD56dim NK cells, which constitute a contribution to a better understanding of NK cell ontogeny in humans.

![Figure 5. Phenotype of NK cells stimulated with IL-2 or IL-15.](image)

PBMCs from two healthy normal donors (N1 and N2) and from the patient (P) (A, B) or sorted CD56dim and CD56bright NK cells from one healthy normal donor and from the patient (C) were stimulated for 3 days with IL-2. Also, PBMCs from two healthy normal donors (N1 and N2) and from the patient (P) were stimulated for 3 days with IL-15 (D, E). Thereafter, cells were harvested and stained with anti-CD3 and anti-CD56, and a third fluorochrome-labeled mAb (indicated on the y-axis). Also, expression of CD57 and CD158 in NK cells (gated as CD3-CD56+ cells) from N1, N2 and the patient (P), and on CD3+CD56dim cells from P was plotted for cells stimulated with IL-2 or IL-15 (B, E). Expression of CD57 and CD158 on sorted and IL-2-stimulated CD56dim NK cells from one healthy normal donor and from the patient is also shown (C). Data are depicted as dot plots. The percentages in each quadrant are shown. Results correspond to one from 3 independent experiments.

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![Figure 6. Percentage of CD16+, CD57+, CD158+ and CD62L+ NK cells before and after stimulation with IL-2 or IL-15.](image)

A) Percentage of CD16+, CD57+, CD158+, and CD62L+ NK cells in total NK cells (CD3+CD56+ cells, upper line of graphs) and in CD56dim NK cells (lower lane of graphs) from healthy normal donors (blue) and from the patient (red). B) Percentage of CD57+CD158+ NK cells before (-) and after stimulation of PBMCs with IL-2 or IL-15 (+) in total NK cells (CD3+CD56+ cells) and in CD56dim NK cells from the same samples. Blue: NK cells from healthy donors; red: NK cells from the patient. C) Percentage of CD56dim NK cells before (-) and after stimulation of PBMCs with IL-2 or IL-15 (+) in CD3+CD56+ cells and in CD3+CD56dim cells from the same samples. Blue: CD3+CD56+ cells from healthy donors; red: CD3+CD56+ cells from the patient. D) Percentage of CD62L+ cells before (0) and after 3 to 5 days of stimulation of PBMCs with IL-2 (left graph) or IL-15 (right graph) in CD3+CD56dim and CD3+CD56bright NK cells from healthy donors and from the patient. Black: CD3+CD56dim cells from healthy donors; green: CD3+CD56dim cells from healthy donors; red: CD3+CD56bright cells from healthy donors; red: CD3+CD56bright cells from the patient. Six healthy donors samples were used for analysis of CD16 and CD57; two healthy donor samples were used for analysis of CD158, CD57 and CD62L expression. One representative experiment from 3 independent analyses is shown. PBMCs from blood samples obtained in December 2007 were used.

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**Author Contributions**

Conceived and designed the experiments: CID MFB MVG NWZ. Performed the experiments: CID MFB MVG NWZ. Analyzed the data: CID MFB NWZ. Contributed reagents/materials/analysis tools: MBG LB NWZ. Wrote the paper: NWZ. Performed laboratory studies and initial flow cytometry analysis in PBMC of the patient: IU JS DIC MIG. Performed the medical follow up of the patient: DDG LB.