Research Article

Cancer Immunology Research

IL15 Infusion of Cancer Patients Expands the Subpopulation of Cytotoxic CD56^{bright} NK Cells and Increases NK-Cell Cytokine Release Capabilities

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Abstract

The cytokine IL15 is required for survival and activation of natural killer (NK) cells as well as expansion of NK-cell populations. Here, we compare the effects of continuous IL15 infusions on NK-cell subpopulations in cancer patients. Infusions affected the CD56^{bright} NK-cell subpopulation in that the expansion rates exceeded those of CD56^{dim} NK-cell populations with a 350-fold increase in their total cell numbers compared with 20-fold expansion for the CD56^{dim} subset. CD56^{bright} NK cells responded with increased cytokine release to various stimuli, as expected given their immunoregulatory functions. Moreover, CD56^{bright} NK cells gained the ability to kill various target cells at levels that are typical for CD56^{dim} NK cells. Some increased cytotoxic activities were also observed for CD56^{dim} NK cells. IL15 infusions induced expression changes on the surface of both NK-cell subsets, resulting in a previously undescribed and similar phenotype. These data suggest that IL15 infusions expand and arm CD56^{bright} NK cells that alone or in combination with tumor-targeting antibodies may be useful in the treatment of cancer. *Cancer Immunol Res; 5(10); 929–38. ©2017 AACR.*

Introduction

Natural killer (NK) cells were discovered by their ability to lyse tumor cells without prior sensitization (1–4). This ability was the basis of attempts to exploit NK cells in the treatment of patients with cancer (5). NK cells activated and expanded *in vitro* were used for adoptive transfer, or cytokines and activators were used to affect NK cells *in vivo*. Several problems limited the success of these treatments, such as limited survival of adoptively transferred NK cells and toxicities caused by cytokine treatments (5).

Three subsets are distinguished within human NK cells based on their expressions of CD56 and CD94 (6). CD56^{bright} and CD56^{dim} subsets are believed to represent sequential differentiation stages of the same lineage (7, 8). A transitional subset of CD56^{dim} NK cells expresses CD94 (9). The three subsets differ in their locations with high portions of CD56^{dim} NK cells present in blood and spleen and CD56^{bright} NK cells mostly residing in lymph nodes and endometrium (10). CD56^{dim} NK cells have a higher capacity to recognize and kill target cells, whereas CD56^{bright} NK cells respond to activation by cytokine production, suggesting an immunoregulatory role (11). This functional distinction can be overcome *in vitro* with the addition of cytokine cocktails containing IL12, IL15, and IL18 that induce regulatory functions in CD56^{dim} and cytotoxicity in CD56^{bright} NK cells (12, 13). Cytokine incubations also induce the expression of activation markers on NK cells (14, 15).

NK cells possess various ways of recognizing target cells and distinguishing them from nontransformed cells (16). The Fc receptor FcRγIII (CD16) is mainly expressed in the CD56^{dim} subset and mediates the deletion of target cells via antibody-dependent cellular cytotoxicity (ADCC). Both NK subsets recognize stress-receptor ligands such as the MHC class I–related chains MICA and MICB through their expression of NKG2D. NK cells sense MHC class I presence on target cells as it serves as an inhibitor of activation. This ability to respond to MHC class I absence requires, however, a "licensing" step during NK-cell development, and NK cells without KIR expression that are largely found within the CD56^{bright} subset are unable to kill target cells that lack MHC class I (17, 18). In general, the decision to kill results from a balance of inhibitory and activating signals.

The cytokine that governs most of NK-cell biology is IL15, deficiency of which causes NK-cell absence in mice (19). Mice injected with IL15 show expansions in populations of NK cells (20). IL15 is also necessary for NK-cell activation (21). As a consequence, IL15 was shown to increase the survival of mice that bear NK-cell–sensitive tumors either as single agent or in combination with several antitumor drugs (22, 23). We have previously reported clinical trials' data using recombinant human IL15 given to cancer patients that mainly showed expansions in populations of NK cells (24).

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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Here, we investigate functions, phenotypes, and population expansion rates of NK-cell subsets in cancer patients before and after IL15 infusions. We show that the main impacts of this treatment were observed within the CD56^{bright} subpopulation that responded with substantial expansion and augmented abilities to respond with cytotoxicity and cytokine production.

Materials and Methods

Patient samples

A cohort of 9 patients was enrolled in this study (NCT01572493) that included one with metastatic melanoma, two with metastatic non–small cell lung cancer one with osteogenic sarcoma, one with metastatic prostate cancer, one with metastatic uterine papillary serous clear cell cancer, one with metastatic pancreatic cancer, one with squamous cell carcinoma, and one with colon adenocarcinoma. All patients had received prior therapy. All patients had adequate organ functions. rhIL15 was produced as previously described (24). All patients received 2 μg/kg/d rhIL15 via continuous intravenous infusion for 10 days that was followed by 30 days without IL15 treatments. Five patients received a second 10-day cycle of the same IL15 infusion schedule. No additional therapy was administered.

Blood samples were taken the same day prior to the beginning and 1 day after the completion of infusion cycles. Cytometry analyses were done on fresh cells and repeated on frozen cells. To prepare frozen cell samples, peripheral blood mononuclear cells (PBMCs) were purified via Ficoll centrifugation and frozen at 15×10^6 cells per vial. After thawing, PBMCs were rested overnight at 37°C in RPMI/10% human AB serum (Mediatech). Sortings were done using FACSARIA FUSION after staining PBMCs with CD3, CD56, and CX3CR1 for use in cytotoxicity assays, or with CD3, CD56, and CD94 for proliferation assays. Dead cells were excluded using 7-AAD (BD Biosciences). Blood samples from normal donors were provided by the NIH blood bank and were handled as described for patient samples.

Cytometry

Antibodies used in this study are listed in Supplementary Table S1. PBMCs were blocked with human TruStain Fcx [Biolegend; 15 minutes, room temperature (RT)] and stained with antibodies recognizing surface proteins (45 minutes, 4°C). Erythrocytes in analyses of fresh samples were lysed with Cal-Lyse (Invitrogen). Cells were permeabilized with BD Cytofix/Cytoperm (BD Biosciences) to stain intracellular cytokines and cytolytic proteins, or with Transcription Factor Fixation Permeabilization (eBioscience) to stain Ki67. Stains for intracellular proteins were done with the provided buffers (30 minutes, RT). Analyses were done with FACS Verse and BD Fortessa.

Ex vivo activations and proliferations

For cytokine production analyses, thawed and rested PBMCs were stimulated with PMA (5 ng/mL; Sigma) plus Ionomycin (500 nmol/L; Sigma), or with human IL12 (10 ng/mL; Peprotech) plus human IL18 (50 ng/mL; R&D Systems). Alternatively, PBMCs were exposed to the target cell lines K562 (ATCC CCL-243) or to anti–CD20-coated Raji (ATCC CCL-86) that had been preincubated with 10 μg/mL of the antibody (30 minutes, 4°C) and washed. Stimulation and coincubations were done for 5 hours at 37°C with Brefeldin A present during the last 4 hours. For proliferation assays, sorted cells were carboxyfluorescein succinimidyl ester (CFSE)-labeled and cultured in IL15 at indicated dosages for 7 days.

Cytotoxicity assays

We used K562, CD20-coated Raji, and C1R-MICA (kindly provided by Dr. Groh-Spies, Fred Hutchinson Cancer Research Center) as target cells and uncoated Raji as control cells. Target and control cells were labeled at different CFSE concentrations (1 vs. 0.1 μmol/L; Invitrogen). We mixed target and control cells at equal numbers and added various numbers of sorted NK cells. Incubations were done in the presence of human IL15 (2 ng/mL, 18 hours, 37°C). The ratios of surviving propidium iodide–negative target and control cells with or without NK-cell exposures were determined by FACS and used to calculate the percentages of target cell lysis with the formula: $100 - T_{NK}/C_{NK} \div T_{con}/C_{con} \times 100$ (T = target, C = Control, $_{NK}$ = NK-cell presence, $_{CON}$ = NK-cell absence).

Statistical analyses

We used PRISM7. *P* values were calculated using the paired *t* test to compare cytometry analyses of patient samples before and after treatments. Unpaired *t* tests were used for cytotoxicity assays.

Results

Predominant expansions of CD56^{bright} NK-cell populations after IL15 infusions

We studied a cohort of 9 patients with metastatic cancer (24). Patients received two 10-day cycles of continuous infusions of recombinant human IL15 at 2 μg/kg/d that were interrupted by 30-day periods without IL15 treatments. We analyzed NK cells from blood samples that were taken immediately prior to the start of the infusion and 1 day after the completions of the infusion cycles.

Prior to infusions, NK-cell numbers and subset ratios were within the normal range (Fig. 1A). IL15 treatments caused an increase in the total number of NK cells (Fig. 1A) as has been reported before (24). The amplitudes of increase were greatest among CD56^{bright} NK cells. Their numbers increased on average more than 350-fold following both treatment cycles and returned to normal between treatments. Lesser increases were measured for CD56^{dim} NK cells (Fig. 1A). Successive treatment cycles limited their ability to expand with approximately 20- and 10-fold increases in cell numbers during the first and second cycles, respectively. NK-cell numbers of the CD56^{dim} subset stayed slightly elevated throughout the resting period. Thus, increased NK-cell numbers in response to IL15 infusions were predominantly caused by expansions of CD56^{bright} NK cells.

We analyzed Ki67 in *ex vivo* NK cells as a marker of their proliferation. Both NK-cell subsets responded to IL15 infusions with increased proliferation (Fig. 1B) with the highest portions of Ki67-expressing cells being found within the CD56^{bright} subset. Approximately 75% of these cells proliferated after IL15 treatments mirroring increases in cell numbers (see above). We observed proliferation of CD56^{dim} NK cells only after IL15 infusions with nearly half of all cells expressing Ki67 (Fig. 1B). IL15 infusions also caused increased expressions of the IL15 receptor signaling β-chain CD122 in both NK-cell subsets with total amounts higher in CD56^{bright} NK cells both before and after IL15 treatments. These data suggest that IL15 infusions were associated

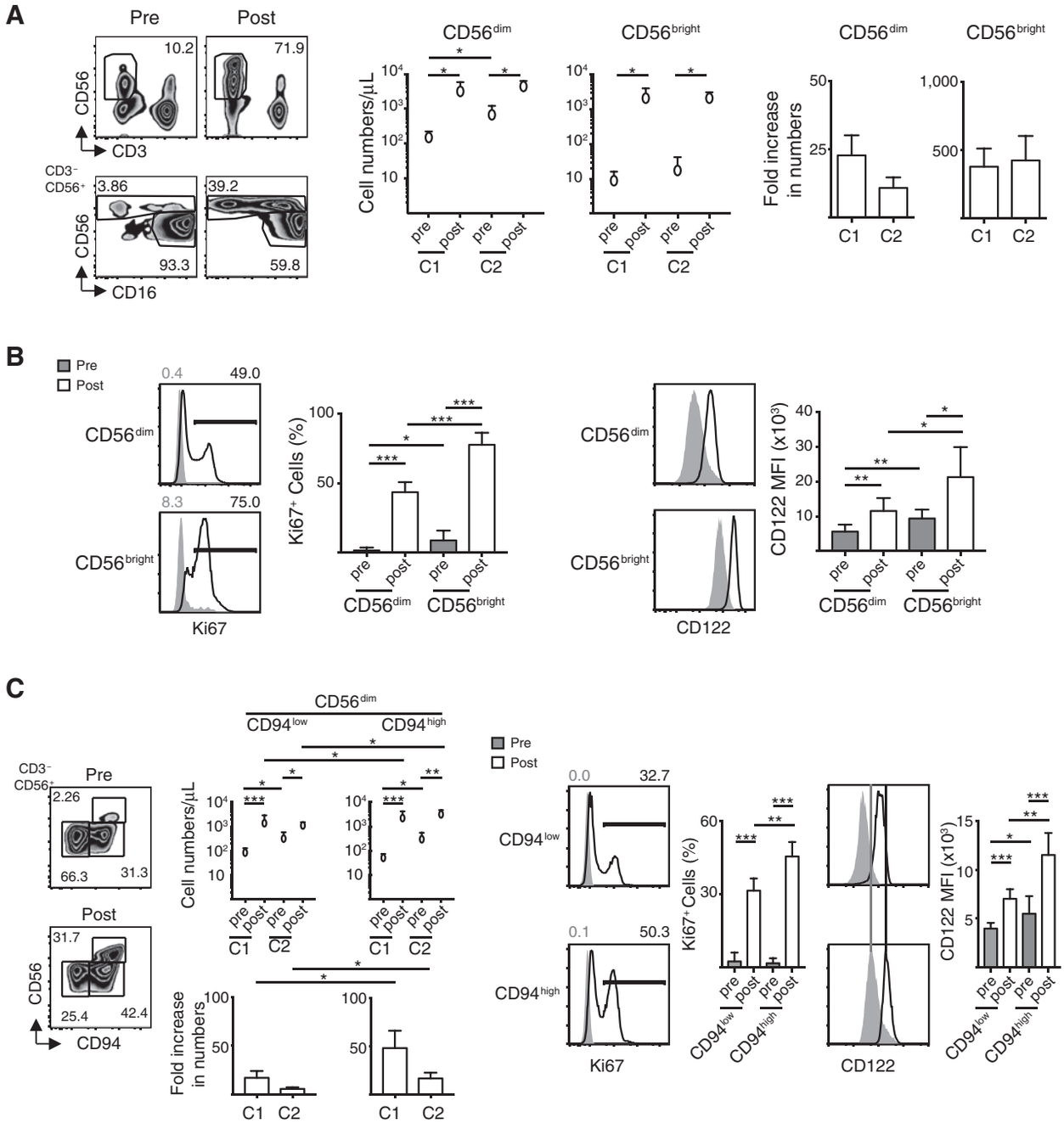


Figure 1.

NK-cell subsets proliferate in response to IL15 infusions. We investigated NK-cell numbers after one or two 10-day cycles of IL15 infusions in cancer patients. **A,** Left, the percentages of NK cells among PBMCs (top) and subsets of gated NK cells (bottom) before and after IL15 infusions. Middle graphs indicate the numbers of CD56^{dim} or CD56^{bright} NK cells before and after first and second IL15 infusion cycles (C1, C2) in blood. Right graphs depict increases comparing NK-cell numbers in blood before and after IL15 infusions for each cycle. **B,** IL15 infusions induced NK-cell proliferation. Left plots and graph show percentages of the proliferation marker Ki67^{+} NK cells among both subsets (CD3^{-}/CD16^{+}/CD56^{dim} or CD3^{-}/CD56^{bright}) before (pre) and after (post) IL15 infusions. Right plots and graph show that IL15 infusions were accompanied by increases of the IL15 receptor β-chain CD122 in both NK-cell subsets. Analyses were done with PBMCs from 7 patients. **C,** IL15 infusions preferentially expanded a CD94^{high} subpopulation among CD56^{dim} NK cells. Left plots and graph show percentages, numbers, and fold increases of CD56^{dim}/CD94^{low} versus CD56^{dim}/CD94^{high} NK cells before and after IL15 infusion cycles. Middle plots and graph show a higher percentage of proliferating cells, and right plots and graph show higher CD122 expression within CD56^{dim}/CD94^{high} when compared with CD56^{dim}/CD94^{low} NK cells. Analyses were done one time on each of 7 patients. Graphs depict means + SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

with upregulation of CD122 particularly within the $CD56^{bright}$ subset that sensitized NK cells to IL15 signaling and resulted in increased cell proliferation and population expansion.

Some studies suggest the existence of an intermediary population between both NK-cell subsets that can be identified by its expression of CD94 within $CD56^{dim}$ NK cells (9). We investigated the effect of IL15 infusions on this NK-cell subset. When compared with the two other subsets, the increase in numbers of $CD56^{dim}$ $CD94^{high}$ NK cells was lower than the population expansion evident for $CD56^{bright}$ progenitor cells but higher than that of $CD56^{dim}$ $CD94^{low}$ NK cells (Fig. 1C). The rank order of these three NK-cell subsets according to population expansion corresponded to rank order according to the percentage of proliferating cells that were positive for Ki67 (Fig. 1C). Moreover, IL15 infusions increased CD122 expression in $CD56^{dim}$ $CD94^{high}$ NK cells to levels lower than in $CD56^{bright}$ but higher than in $CD56^{dim}$ $CD94^{low}$ NK cells (Fig. 1C), further suggesting a direct correlation between CD122 expression and proliferation rates. We also studied whether the three NK-cell subpopulations do require different amounts of IL15 to respond with proliferation *in vitro* (Supplementary Fig. S1). We sorted the NK-cell populations from blood cells of normal donors and observed that the differentiation from $CD56^{bright}$ to $CD56^{dim}$/$CD94^{high}$ to $CD56^{dim}$/$CD94^{low}$ NK cells was accompanied by increasing requirements of IL15 to induce their proliferation. Together, these data suggest an inverse correlation between NK-cell differentiation and IL15 responsiveness that resulted in a preferential expansion of the least differentiated $CD56^{bright}$ subpopulation in response to IL15 infusion.

IL15 infusions alter phenotype of NK cells

We analyzed whether IL15 infusions altered the surface expressions of molecules that are involved in cytotoxicity, differentiation, cell adhesion, chemokine response, and mechanisms of inhibition or activation. Normally, $CD56^{dim}$ NK cells express more CD16 and CX3CR1 than do $CD56^{bright}$ NK cells. After IL15 infusions, $CD56^{bright}$ NK cells expressed amounts of CD16 and CX3CR1 similar to those expressed by $CD56^{dim}$ NK cells (Fig. 2A). Treatment of $CD56^{bright}$ NK cells with IL15 reduced expression of CCR7, CD27, and CD25. Conversely, $CD56^{dim}$ NK cells treated with IL15 acquired features normally observed in $CD56^{bright}$ NK cells in that expression of NKG2D, Trail (Fig. 2A), and IL18R (Fig. 3D) rose, subpopulations that expressed CD62L or CXCR3 appeared, and subpopulations that expressed CD57 disappeared (Fig. 2A). In $CD94^{low}$ and $CD94^{high}$ subpopulations, NKG2D and CD57 expression changed in both subpopulations. Increased expression of CD62L, CXCR3, and Trail was only seen in the intermediary $CD94^{high}$ cells (Fig. 2B). Common to all subsets were decreases in the surface amounts of CD2 and increases of NKp30, NKp46 (Fig. 2A), and CD122 (Fig. 1B). We found no changes in expression of KIRs, NKG2A/C, CD69, DNAM1, and other markers (Supplementary Fig. S2). Thus, IL15 infusions caused phenotypic changes in all subsets of NK cells. Expression of certain receptors associated with cytotoxicity, CD16, NKG2D, NKp30, and NKp46 was induced by IL15 treatments.

IL15 infusions sensitize $CD56^{bright}$ NK cells to increased cytokine production

NK cells respond to various stimulations with the production of cytokines (12, 25, 26). We investigated whether this ability was affected by IL15 infusions. We exposed total PBMCs from patients before and after IL15 infusions to combinations of PMA/Ionomycin, IL12/IL18, or to K562 or anti–CD20-coated target cells for 5 hours, and then assessed intracellular cytokine levels in gated $CD56^{dim}$ and $CD56^{bright}$ NK cells by FACS (Fig. 3). Although IL15 infusions were associated with increased cytokine production for all NK-cell types, the $CD56^{bright}$ subpopulation was most affected: We observed increases in production of IFNγ, TNFα, and GM-CSF in response to PMA/Ionomycin (Fig. 3A) or IL12/IL18 (Fig. 3B) within the $CD56^{bright}$ population of cells. In contrast, $CD56^{dim}$ cells only increased IFNγ production only in response to IL12/IL18. $CD56^{bright}$ NK cells responded to target cell exposures with the production of IFNγ and TNFα only after IL15 infusions; the same response was not affected by IL15 treatments in the $CD56^{dim}$ subset (Fig. 3C). Some of the augmented cytokine responses could be related to the increased IL18R expression in $CD56^{dim}$ NK cells after IL15 treatments, but cytokine expression was constitutively higher from $CD56^{bright}$ NK cells (Fig. 3). Expression of the IL12R was unchanged (Fig. 3). Together, these data show a sensitization of $CD56^{bright}$ NK cells to respond with cytokine production.

IL15 infusions augment cytotoxic responses in all subsets of NK cells

Immunotherapies target NK cells for their ability to recognize and lyse tumor cells. A main cytotoxic pathway involves degranulation of vesicles that contain granzymes A and B as well as perforin. We analyzed the intracellular amounts of these molecules in NK cells before and after IL15 infusions. $CD56^{dim}$ NK cells constitutively expressed all three molecules, and IL15 infusions were associated with diminished amounts of granzyme B and perforin (Fig. 4A, top). We observed opposite reactions from $CD56^{bright}$ NK cells, in which IL15 treatment resulted in increased amounts of all three cytolytic molecules (Fig. 4A, bottom). Thus, IL15 infusions appear to equip $CD56^{bright}$ NK cells to become cytotoxic.

We assessed the cytotoxic abilities of NK-cell subsets. We used three target cell lines, the killing of which involved different receptors: anti-CD20 (rituximab) antibody-coated RAJI, K562, and C1R-MICA. These cell lines are recognized by NK cells via CD16-mediating ADCC, natural cytotoxicity receptors (Nkp30 and NKp46), and NKG2D, respectively (27, 28). Target cells were mixed with control cells insensitive to NK cells (uncoated RAJI) and exposed to sorted NK cells for 18 hours *in vitro* (Supplementary Fig. S3). The ratio of surviving target to control cells was used to calculate the percentage of lysed target cells. Figure 4B shows that cytotoxic activities were augmented for both $CD56^{dim}$ and $CD56^{bright}$ subsets of NK cells from IL15-treated patients when compared with untreated donors in response to all three target cells. Changes in cytotoxicity were greater after IL15 infusions for $CD56^{bright}$ NK cells that reached cytotoxic response levels similar to $CD56^{dim}$ NK cells. Together, these data show that IL15 infusions enabled all subsets of NK cells to respond with increased cytotoxicity.

Discussion

NK-cell subpopulations have been defined in humans based on their expression of CD56: Cells that express this marker at low intensity are considered the cytotoxic NK-cell subset,

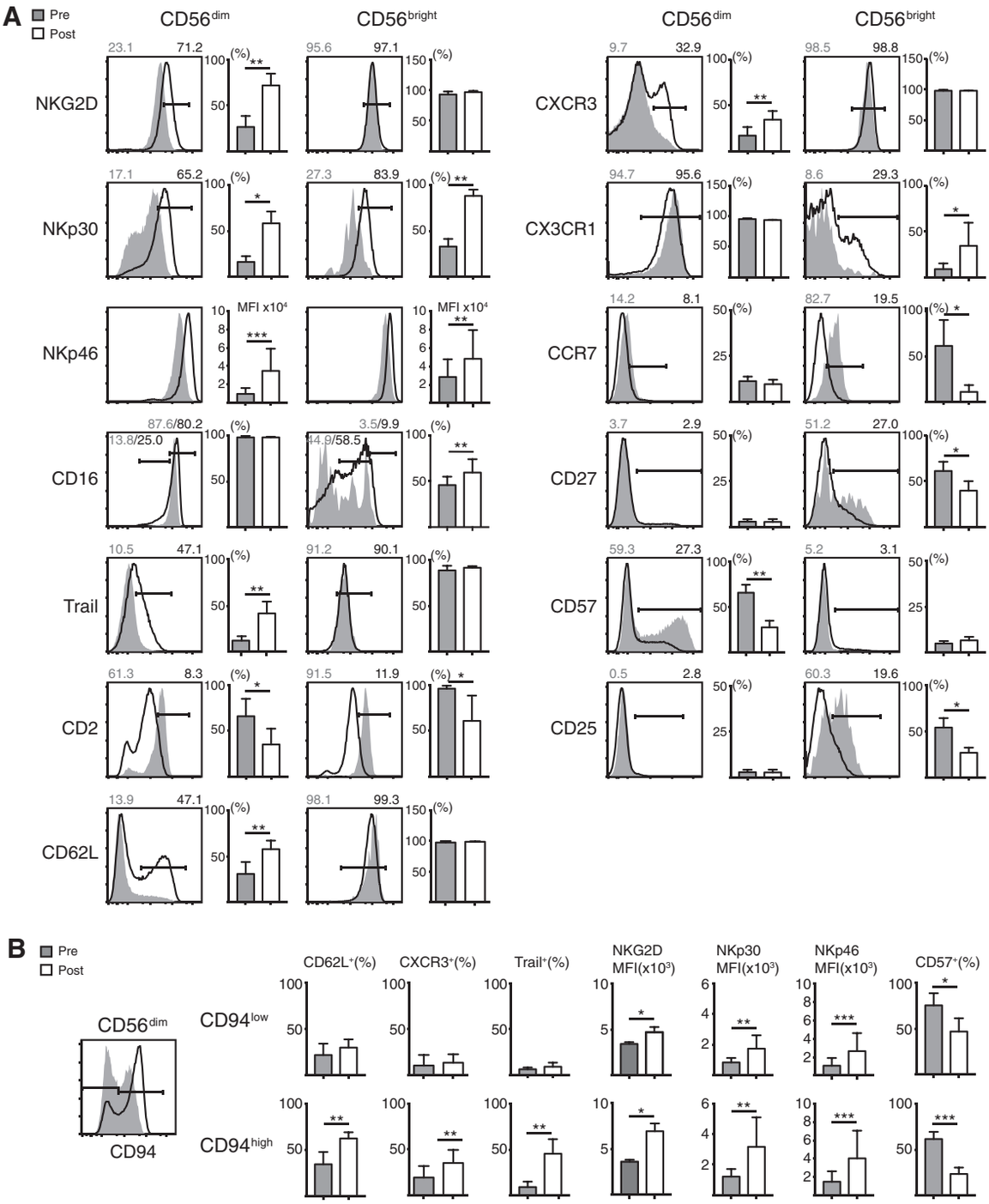
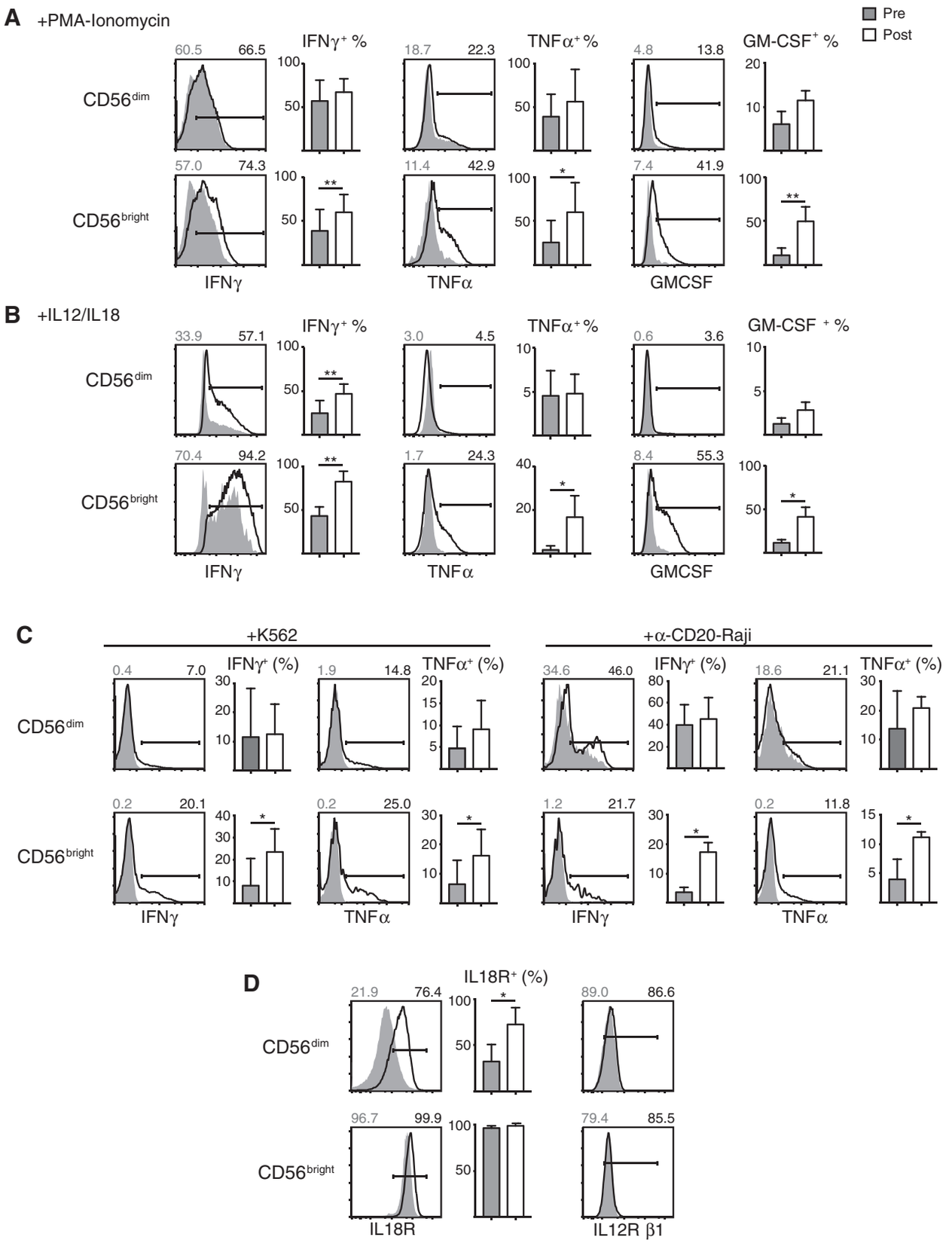


Figure 2.

IL15 infusions induce phenotypical surface marker changes on NK cells. **A,** Examples of expression changes before (gray areas) and after (black solid lines) IL15 infusions of gated CD56^{dim} (CD3^{-}/CD16^{+}/CD56^{dim}) and CD56^{bright} (CD3^{-}/CD56^{bright}) NK cells. Several surface proteins involved in cytotoxicity were induced in both subsets, NKG2D, NKp30, NKp46, and Trail on CD56^{dim} NK cells and NKp30, NKp46, and CD16 on CD56^{bright} NK cells. The adhesion molecule CD2 decreased in both, whereas CD62L increased in the CD56^{dim} subset only. We observed an increase of the expression of the chemokine receptor CXCR3 on CD56^{dim} NK cells, whereas CX3CR1 expression increased and CCR7 decreased on CD56^{bright} NK cells. The population of CD57^{+} cells decreased within CD56^{dim} NK cells, whereas CD27 and CD25 expression decreased on CD56^{bright} NK cells. Graphs on the right of plots show mean + SD for expressions before and after IL15 infusions. **B,** Plots and graphs show that expression differences in CD62L, CXCR3, and Trail that had been induced in the CD56^{dim} subset by IL15 infusions could be attributed to the CD56^{dim}/CD94^{high} subpopulation. Analyses were done once on each of 5 patients. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.



whereas CD56^{bright} cells are believed to be immunoregulatory actors (10, 11). Here, we present studies that characterize the effects of IL15 infusions on various aspects of NK-cell function in cancer patients. We show that IL15 infusions were associated with over 350-fold expansion of the CD56^{bright} NK-cell subpopulation and a 20-fold expansion of the CD56^{dim} NK subpopulation. The CD56^{bright} cells showed increased proliferation that may have been caused by higher CD122 expression. Population expansion rates were more than 10-fold higher in the CD56^{bright} subset following treatment when compared with other NK-cell subsets resulting in nearly equal numbers of both types of cells present in the peripheral blood after 10-day IL15 infusions. In addition, CD56^{bright} NK cells acquired the capability to respond to target cell activation by both increased cytokine production and cytotoxicity as a result of the treatment. These data suggest that CD56^{bright} NK cells generated by IL15 infusions have a potential to respond to tumors sensitive to NK cells.

We analyzed cytokine production of NK-cell subsets. CD56^{bright} NK cells respond to cytokine or PMA/Ionomycin stimulations with the generation of several cytokines (12, 25, 26, 29). We observed that this activity was even more pronounced after IL15 infusions. The effect could be explained by increased cytokine receptor expressions, but additional intracellular changes are likely to exist as suggested by the improved responses to PMA/Ionomycin that did not require receptor expression. In addition, cytokine generation was only observed after coincubations with NK target cells after IL15 infusions. This suggests that treatment induced expression of receptors, normally absent on CD56^{bright} NK cells, that could recognize ligands on tumor cells. In the case of antibody-coated target cells, this improved recognition may involve the Fc receptor CD16, expression of which was upregulated after IL15 infusion. IL15 treatment–induced CD56^{bright} NK cells also responded to K562 cells. The receptors that recognize this cell line appear to be more complex (27, 28) and may involve NKp30, NKp46, and NKG2D, expression of which had also been increased after IL15 infusions. Thus, multiple mechanisms are implicated in increased cytokine responses in IL15 infusion-induced CD56^{bright} NK cells.

We analyzed surface marker expression changes that were induced by IL15 infusions. We observed that changes in both subpopulations contributed to an increased phenotype similarity between them. We believe that this is most consistent with activities that were induced by higher concentrations of IL15 that had been achieved after infusions when compared with physiological steady-state levels. Such higher cytokine concentrations could have activated a signaling pathway with similar signaling strength in both cell types to result in similar expressions of some surface molecules. Infusions also caused an increase of CD62L^{-} and a decrease of CD57^{-}expressing populations among CD56^{dim} cells as well as a decrease of the CD27^{+} population among CD56^{bright} cells, suggesting that IL15 treatments induced expansion of less mature NK-cell populations (30–34).

Many phenotypic changes that were induced by IL15 infusions are also found after IL15-induced *ex vivo* cultures of NK cells (14, 15, 35–37). These include increases of NKp30, NKp46, NKG2D, Trail, and CXCR3, and decreases of CD57, CD27, and CCR7. One exception, however, exists. Cultured NK cells typically upregulate markers of activation including CD69, CD25, NKp44, and DNAM1 (14, 15, 38). We did not observe inductions of these markers after IL15 infusions. Because bona fide activating ligands are unlikely to be induced by culturing, the discrepancy between culture and infusions is most consistent with inhibitory mechanisms *in vivo* that are lost during culturing resulting in NK-cell activation *in vitro* only. A lack of NK-cell activation in IL15-infused cancer patients also suggests that no encounters between cancer and the analyzed blood NK cells had occurred that would have caused their activation.

The relationship between CD56^{dim} and CD56^{bright} NK cells remains a matter of debate. Most publications suggest CD56^{bright} NK cells represent a developmentally precursor stage (10). Others describe the ability to derive CD56^{bright} from CD56^{dim} via NK-cell activation (39, 40). Along this line, the increased presence of CD56^{bright} NK cells following IL15 treatments could have been caused by either different expansion rates of both cell subsets or a conversion of CD56^{dim} into CD56^{bright} NK cells. Most of our findings support the former: Analyses *ex vivo* showed increased proliferation rates in the CD56^{bright} subset (Fig. 1B). In addition, *in vitro* proliferation assays point to an increased ability of CD56^{bright} NK cells to proliferate in response to IL15 (Supplementary Fig. S1). Moreover, one would expect that descendants of a recent CD56^{dim} to CD56^{bright} conversion would retain some of the phenotypical markers of their precursor cells. Most of our data appear to suggest the opposite in that the majority of surface markers that are distinct between both subsets were expressed at similar levels on CD56^{bright} NK cells before and after treatments. These include CXCR3, CX3CR1, CD16, NKG2A, and CD158b (Fig. 2; Supplementary Fig. S2). CD25 and CCR7 are exceptions in that their expressions were downregulated on posttreatment CD56^{bright} NK cells to levels similar of CD56^{dim} NK cells. In summary, our data suggest that the predominant appearance of CD56^{bright} NK cells in response to IL15 treatments resulted mainly from a superior ability of this subset to proliferate.

An issue for all NK-cell–based cancer immunotherapy targeting solid tumors is their ability to migrate from blood to tumor sites. We had analyzed the expression of several chemokine receptors whose involvement in tumor-directed migration has been reported (5, 41, 42). No clear pattern emerged. Expression of CXCR3 and CX3CR1 increased, expression of CCR7 decreased, and expression of CXCR1 remained unchanged. Further studies are necessary to delineate migration patterns of IL15 infusion–induced NK cells.

Figure 3.

IL15 infusions sensitize NK cells to respond with cytokine production. PBMCs were stimulated with PMA/Ionomycin (**A**), IL12/IL18 (**B**), or coincubated with NK target cells (**C**), and intracellular cytokine amounts were determined by FACS on gated CD56^{dim} (CD3^{-}/CD16^{+}/CD56^{dim}) or CD56^{bright} (CD3^{-}/CD56^{bright}) NK cells. IL15 infusions caused increases of IFNγ, TNFα, and GM-CSF productions in PMA/Ionomycin- or IL12/IL18-responding NK cells within the CD56^{bright} subset. Within the CD56^{dim} subset, IFNγ production was increased after IL12/IL18 stimulation. CD56^{bright} NK cells also acquired the ability to respond to target cell exposure by cytokine production after IL15 infusions, whereas little change was seen for target cell–exposed CD56^{dim} NK cells. **D** shows that IL15 infusions had increased the expression of surface IL18 receptor on CD56^{dim} NK cells to levels lower than those on CD56^{bright} NK cells. We observed no IL12 receptor expression changes. Analyses were done once on each of 5 patients. Graphs depict mean + SD. *, $P < 0.05$ and **, $P < 0.01$.

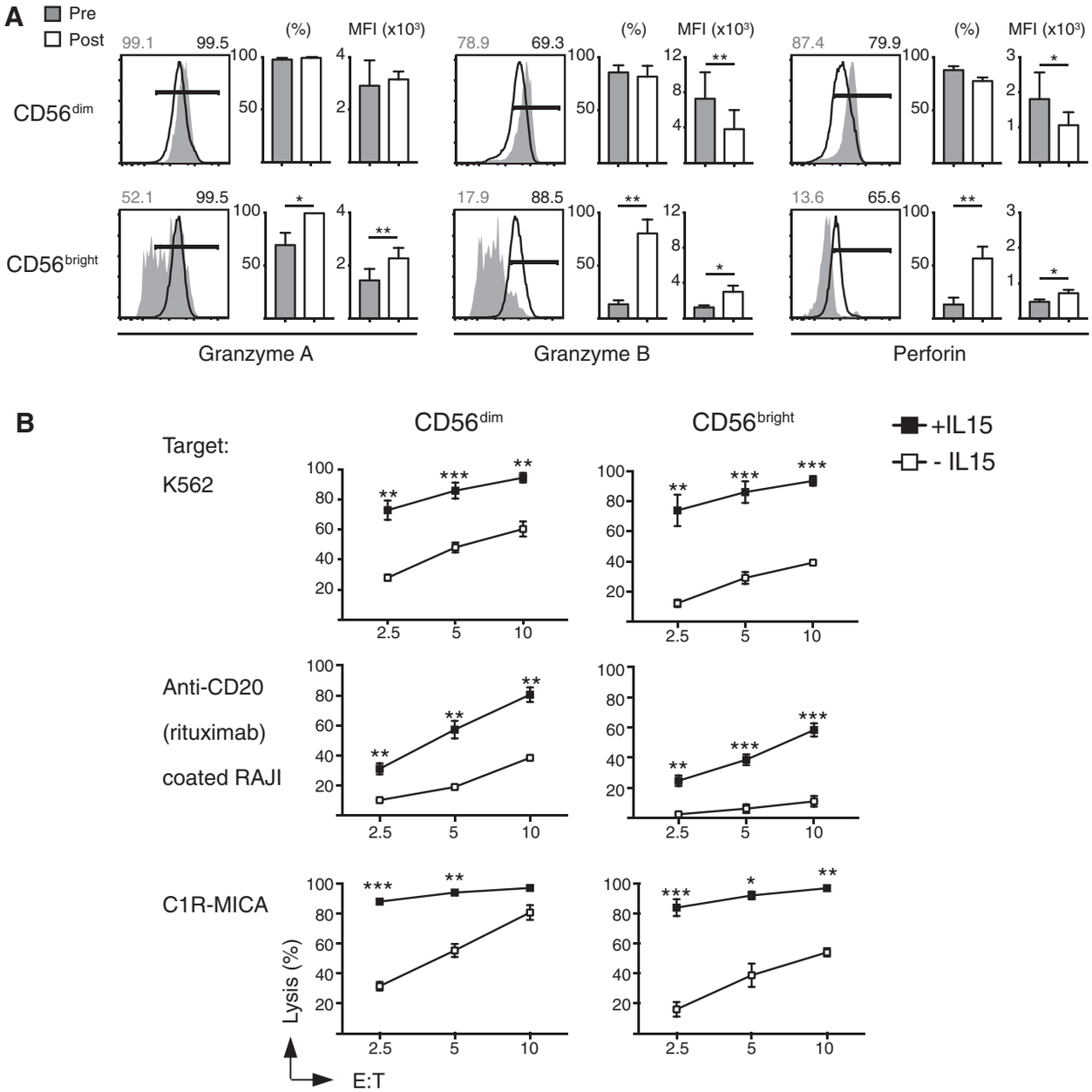


Figure 4.

IL15 infusions increase cytotoxic activities in both NK-cell subsets. **A,** Intracellular stains of gated NK subsets indicate decreases of cytotoxic molecules in CD56^{dim} NK cells, whereas the amounts of granzymes A, B, and perforin increased in CD56^{bright} NK cells after IL15 infusions. **B,** IL15 infusions augmented cytolytic activities for both sorted NK-cell subsets against three different target lines. Analyses were done once on each of 5 patients. Graphs depict mean ± SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

We observed improved cytotoxic activities for both CD56^{bright} and CD56^{dim} NK cells against diverse target cell lines with various recognition requirements. Several changes in CD56^{bright} cells that had been caused by IL15 treatments could account for this increase: Besides improved recognition of target cells, these cells had increased intracellular amounts of the molecules Granzyme A, Granzyme B, and Perforin that are used for killing target cells. This was not the case for CD56^{dim} NK cells that also showed increased killing activities despite lower amounts of granzymes and perforin. However, increased amounts of membrane-bound death receptor ligand Trail may contribute to stronger cytotoxic responses as may the improved recognition via NKG2D for MICA-expressing targets, NKp30 and NKp46 for K562. Thus, the goal of inducing augmented cytotoxic NK-cell activities by IL15 infusions was achieved that may suggest a potential utility in cancer immunotherapy.

In summary, continuous infusions of IL15 in cancer patients is accompanied by a preferential expansion of populations of phenotypically immature CD56^{bright} NK cells with increased abilities to recognize tumor cells and react by cytokine production and cytotoxicity. On the basis of the present study, IL15, by increasing NK-cell numbers and their cytotoxic capacity, may increase the efficacy of anticancer therapies. IL15 could be useful in cases of tumors that have deleted Class I MHC where direct NK cytotoxicity may be effective. IL15 may be useful in combination with therapies that are based on antitumor antibodies of which the effects include tumor killing via ADCC. To translate this opportunity in the treatment of leukemia, we have initiated a clinical trial using IL15 with anti-CD52 alemtuzumab (NCT02689453), and we are planning to combine IL15 with anti-CCR4 mogamulizumab in patients with HTLV-1–associated adult T-cell leukemia/lymphoma (43).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Dubois, J.R. Müller, T.A. Waldmann
Development of methodology: S. Dubois, J.R. Müller
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Dubois, K.C. Conlon, J. Hsu-Albert, T.A. Waldmann
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Dubois, J.R. Müller
Writing, review, and/or revision of the manuscript: S. Dubois, K.C. Conlon, J.R. Müller, T.A. Waldmann
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Beltran, B.R. Bryant
Study supervision: T.A. Waldmann

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References

1. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer 1975; 16:230–9.
2. Jondal M, Pross H. Surface markers on human b and t lymphocytes. VI. Cytotoxicity against cell lines as a functional marker for lymphocyte subpopulations. Int J Cancer 1975;15:596–605.
3. Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Immunol 1975;5:117–21.
4. Pross HF, Jondal M. Cytotoxic lymphocytes from normal donors. A functional marker of human non-T lymphocytes. Clin Exp Immunol 1975;21:226–35.
5. Cantoni C, Grauwet K, Pietra G, Parodi M, Mingari MC, Maria AD, et al. Role of NK cells in immunotherapy and virotherapy of solid tumors. Immunotherapy 2015;7:861–82.
6. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol 1986; 136:4480–6.
7. Chan A, Hong DL, Atzberger A, Kollnberger S, Filer AD, Buckley CD, et al. CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. J Immunol 2007;179:89–94.
8. Yu J, Freud AG, Caligiuri MA. Location and cellular stages of natural killer cell development. Trends Immunol 2013;34:573–82.
9. Yu J, Mao HC, Wei M, Hughes T, Zhang J, Park IK, et al. CD94 surface density identifies a functional intermediary between the CD56bright and CD56dim human NK-cell subsets. Blood 2010;115:274–81.
10. Melsen JE, Lugthart G, Lankester AC, Schilham MW. Human circulating and tissue-resident CD56(bright) natural killer cell populations. Front Immunol 2016;7:262.
11. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001;22:633–40.
12. Fehniger TA, Shah MH, Turner MJ, VanDeusen JB, Whitman SP, Cooper MA, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol 1999;162: 4511–20.
13. Nielsen N, Odum N, Urso B, Lanier LL, Spee P. Cytotoxicity of CD56 (bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. PLoS One 2012;7:e31959.
14. Anguille S, Van Acker HH, Van den Bergh J, Willemen Y, Goossens H, Van Tendeloo VF, et al. Interleukin-15 dendritic cells harness NK cell cytotoxic effector function in a contact- and IL-15-dependent manner. PLoS One 2015;10:e0123340.
15. Romee R, Leong JW, Schneider SE, Sullivan RP, Fehniger TA. IL-15 primes a highly potent anti-leukemia response by CD56bright NK cells. Blood 2013;122:2283.
16. Guillerey C, Huntington ND, Smyth MJ. Targeting natural killer cells in cancer immunotherapy. Nat Immunol 2016;17:1025–36.
17. Moretta L. Dissecting CD56dim human NK cells. Blood 2010;116: 3689–91.
18. Orr MT, Lanier LL. Natural killer cell education and tolerance. Cell 2010; 142:847–56.
19. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med 2000;191:771–80.
20. Dubois S, Patel HJ, Zhang M, Waldmann TA, Muller JR. Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8+/CD44high T cells and its antitumor action. J Immunol 2008;180:2099–106.
21. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity 2007;26:503–17.
22. Zhang M, Ju W, Yao Z, Yu P, Wei BR, Simpson RM, et al. Augmented IL-15Ralpha expression by CD40 activation is critical in synergistic CD8 T cell-mediated antitumor activity of anti-CD40 antibody with IL-15 in TRAMP-C2 tumors in mice. J Immunol 2012;188:6156–64.
23. Zhang M, Yao Z, Dubois S, Ju W, Muller JR, Waldmann TA. Interleukin-15 combined with an anti-CD40 antibody provides enhanced therapeutic efficacy for murine models of colon cancer. Proc Natl Acad Sci U S A 2009;106:7513–8.
24. Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, Morris JC, et al. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. J Clin Oncol 2015;33:74–82.
25. Barakonyi A, Rabot M, Marie-Cardine A, Aguerre-Girr M, Polgar B, Schiavon V, et al. Cutting edge: engagement of CD160 by its HLA-C

physiological ligand triggers a unique cytokine profile secretion in the cytotoxic peripheral blood NK cell subset. J Immunol 2004;173:5349–54.
26. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood 2010;115:2167–76.
27. Bae DS, Hwang YK, Lee JK. Importance of NKG2D-NKG2D ligands interaction for cytolytic activity of natural killer cell. Cell Immunol 2012;276:122–7.
28. Mavoungou E, Bouyou-Akotet MK, Kremsner PG. Effects of prolactin and cortisol on natural killer (NK) cell surface expression and function of human natural cytotoxicity receptors (NKp46, NKp44 and NKp30). Clin Exp Immunol 2005;139:287–96.
29. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 2001;97:3146–51.
30. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood 2010;116:3853–64.
31. Juelke K, Killig M, Luetke-Eversloh M, Parente E, Gruen J, Morandi B, et al. CD62L expression identifies a unique subset of polyfunctional CD56dim NK cells. Blood 2010;116:1299–307.
32. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood 2010;116:3865–74.
33. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. J Immunol 1989;143:3183–91.
34. Vossen MT, Matmati M, Hertoghs KM, Baars PA, Gent MR, Leclercq G, et al. CD27 defines phenotypically and functionally different human NK cell subsets. J Immunol 2008;180:3739–45.
35. Lum JJ, Schnepple DJ, Nie Z, Sanchez-Dardon J, Mbisa GL, Mihowich J, et al. Differential effects of interleukin-7 and interleukin-15 on NK cell anti-human immunodeficiency virus activity. J Virol 2004;78:6033–42.
36. Moustaki A, Argyropoulos KV, Baxevanis CN, Papamichail M, Perez SA. Effect of the simultaneous administration of glucocorticoids and IL-15 on human NK cell phenotype, proliferation and function. Cancer Immunol Immunother 2011;60:1683–95.
37. Vukicevic M, Chalandon Y, Helg C, Matthes T, Dantin C, Huard B, et al. CD56bright NK cells after hematopoietic stem cell transplantation are activated mature NK cells that expand in patients with low numbers of T cells. Eur J Immunol 2010;40:3246–54.
38. Hromadnikova I, Pirkova P, Sedlackova L. Influence of in vitro IL-2 or IL-15 alone or in combination with Hsp-70-derived 14-mer peptide (TKD) on the expression of NK cell activatory and inhibitory receptors. Mediators Inflamm 2013;2013:405295.
39. Loza MJ, Perussia B. The IL-12 signature: NK cell terminal CD56+high stage and effector functions. J Immunol 2004;172:88–96.
40. Muller JR, Waldmann TA, Dubois S. Loss of cytotoxicity and gain of cytokine production in murine tumor-activated NK cells. PLoS One 2014;9:e102793.
41. Park MH, Lee JS, Yoon JH. High expression of CX3CL1 by tumor cells correlates with a good prognosis and increased tumor-infiltrating CD8+ T cells, natural killer cells, and dendritic cells in breast carcinoma. J Surg Oncol 2012;106:386–92.
42. Wendel M, Galani IE, Suri-Payer E, Cerwenka A. Natural killer cell accumulation in tumors is dependent on IFN-gamma and CXCR3 ligands. Cancer Res 2008;68:8437–45.
43. Nakagawa M, Schmitz R, Xiao W, Goldman CK, Xu W, Yang Y, et al. Gain-of-function CCR4 mutations in adult T cell leukemia/lymphoma. J Exp Med 2014;211:2497–505.