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Cutting Edge: In Vivo Blockade of Human IL-2 Receptor Induces Expansion of CD56\textsuperscript{bright} Regulatory NK Cells in Patients with Active Uveitis

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In vivo blockade of the human IL-2R by mAb has been used for immunosuppression in transplantation, therapy for leukemia, and autoimmune diseases. In this study, we report that administration of a humanized IL-2R blocking Ab induced a 4- to 20-fold expansion of CD56\textsuperscript{bright} regulatory NK cells in uveitis patients over time. The induced CD56\textsuperscript{bright} regulatory NK cells from patients exhibited similar phenotype as those naturally occurring CD56\textsuperscript{bright} cells. Patients with active uveitis had a significantly lower level of CD56\textsuperscript{bright} NK cells compared with normal donors (p < 0.01). In addition, the induced CD56\textsuperscript{bright} cells could secrete large amounts of IL-10 whereas CD56\textsuperscript{dim} NK cells could not, suggesting that the induction of the CD56\textsuperscript{bright} cells may have a beneficial effect on the remission of active uveitis. Our observation may have implications to IL-2R blockade therapy and for the potential role of CD56\textsuperscript{bright} regulatory NK cells in autoimmune diseases. The Journal of Immunology, 2005, 174: 5187–5191.

Blockade of the human IL-2R by mAb has been used in immunosuppressive regimen for transplantation (1–3), in the treatment of T cell adult leukemia (4, 5), and to treat refractory anaplastic large-cell lymphoma (6). IL-2R blockade therapy has also been used for treating autoimmune diseases (7). We reported its effectiveness in treating non-infectious ocular inflammatory disease, or uveitis (8, 9), a presumed autoimmune inflammatory disease mediated primarily by Th1 lymphocytes (10). Recently, IL-2R blockade has also been reported effective in treating multiple sclerosis (11, 12). However, its mechanism is not completely clear.

Human NK cells have been typically identified as CD56\textsuperscript{CD3} lymphocytes (13). Early studies demonstrated two subsets of human NK cells based on expression levels of cell surface CD56, CD56\textsuperscript{dim}, and CD56\textsuperscript{bright} (14, 15). CD56\textsuperscript{bright} NK cells have been proposed to play a regulatory role in immune responses based on their lower cytotoxic potential, higher secretion of cytokines and unique surface receptor expression profile (14, 16–18). Previous studies demonstrated that low dose infusion of recombinant human IL-2 selectively induced the CD56\textsuperscript{bright} NK subset (19). This IL-2 induced expansion of the CD56\textsuperscript{bright} subset was attributed to enhanced NK cell differentiation from bone marrow progenitors combined with delayed NK cell death (20). However, much remains unclear regarding the development, differentiation, and roles of this subset of NK cells under physiological and pathological conditions. This study was intended to address the following: 1) Would continuous infusion of an IL-2 antagonist, daclizumab, a humanized anti-IL-2R α-chain (CD25) mAb, have an effect on the human CD56\textsuperscript{bright} NK cell population in vivo? 2) Was there a significant difference in the CD56\textsuperscript{bright} subset of lymphocytes in autoimmune uveitis patients compared with normal human donors?

Materials and Methods

Five patients with active non-infectious uveitis who underwent therapy with the humanized anti-IL-2R α-chain (CD25) mAb, daclizumab (Protein Design Laboratories), were studied. All the patients received an i.v. infusion of daclizumab (8 mg/kg) at day 0 followed by another infusion (4 mg/kg) at day 14. Patients who did not develop any end points received further therapy with a reduced dosage of 2 mg/kg. The follow up of these patients ranged from 6 to 28 wk.

Patient consent and Institutional Review Board approval were obtained before sample collection. Whole blood samples from patients were collected immediately before starting daclizumab infusion and at different time points after the beginning of therapy. At least six time points were examined for each patient. Blood cells were stained for CD3 and CD56 in combination with other surface markers using a 4-color whole blood lysing-washing protocol (21) and analyzed by a FACSCaliber flow cytometer (BD Biosciences). Blood samples from five normal donors (National Institutes of Health blood bank) were used as normal controls. All Abs were from BD Pharmingen except CX3CR1 (MBL International) and NKG2D (R&D Systems). Flow cytometry data were analyzed by FlowJo (TreeStar). Briefly, the lymphocyte population was gated based on forward scatter and side scatter characteristics. NK cells were gated based on CD56\textsuperscript{CD3} staining. CD56\textsuperscript{bright} cells were gated based on CD56\textsuperscript{bright} CX3CR1\textsuperscript{CD3} or CD56\textsuperscript{bright}CXCR3\textsuperscript{CD3}. The results were represented by the percentage of the CD56\textsuperscript{bright} subset in either lymphocyte or the CD56\textsuperscript{CD3} NK cell subpopulation. Student’s t test was used for statistical analysis.

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For cell sorting and cytokine analysis experiments, PBMCs were isolated from patients’ whole blood using a gradient centrifugation procedure as previously described (21). Cells were then stained with FITC-CD3, PE-CX3CR1, PerCP-Cy5.5, and allophycocyanin-CD56 Abs (BD Biosciences). CD56bright CD3−CX3CR1−CD4− and CD56dimCD3−CXCR3+ CD4− cells were purified by a 4-way high speed sorting protocol using a FACS Aria sorter (BD Biosciences). The purity for downstream analysis were all of >95% purity based on flow cytometry analysis. Cells were then plated in 96-well tissue culture plates in duplicates (1–4 × 10^5/ml density) in RPMI 1640 medium with 10% FBS and stimulated with or without cytokine cocktails, IL-12 (20 ng/ml plus IL-15 100 ng/ml; PeproTech). After 72 h of stimulation, duplicates of culture supernatants were pooled and cytokine levels were measured by a multiplex cytokine array assay (Pierce).

For the STAT5 phosphorylation study, PBMCs from normal donors (Natalie Laboratories of Health blood bank) were used. Approximately 5 × 10^5 cells were either treated or untreated with the IL-2R blocking Ab (100 μg/ml) or with 20 ng/ml recombinant human IL-2 (PeproTech). Cells were immediately lysed with radioimmunoprecipitation assay buffer starting from 10 min up to 120 min. Cell lysates were subjected to Western analysis using antiphospho-STAT5-specific Ab (Cell Signaling Technology).

**Results and Discussion**

Although CD56bright NK cell subpopulation has been commonly defined either by its intensity of CD56 staining in CD56+CD3− population or by double staining as CD56bright and CD16 low or negative (15), we found that the above criteria could result in ambiguous conclusion if the CD56bright NK cell subpopulation is relatively small. In our experience, the CD56bright NK cell subpopulation can be more readily and consistently defined by double staining of either CD56brightCX3CR1− or CD56brightCXCR3− in the lymphocytes or in the CD56−CD3− NK cell population. Therefore, we used CD56brightCD3−CX3CR1+ as a primary criteria to define the CD56 regulatory NK cell subpopulation throughout this study. As shown in Fig. 1A, all five patients who received anti-IL-2R therapy demonstrated a dramatic expansion of their CD56bright NK cell subpopulation in their peripheral blood compared with their baseline levels. The expansion ranged from 4-fold to as much as 20-fold. Analysis of the time course of the expansion revealed that the expansion started as early as 1 wk after administration of the anti-IL-2R blocking Ab (Fig. 1B). Continued therapy resulted in a further expansion of the CD56bright NK subpopulation, as confirmed by flow cytometry. By wk 6–28, all patients demonstrated a 4- to 20-fold expansion of the CD56bright NK cell subpopulation. In one patient, the CD56bright NK cells made up 50% of the total NK cell population 18 wk after anti-IL-2R therapy (Fig. 1B).

All five patients had active uveitic disease before daclizumab therapy. Interestingly, they all had significantly lower levels of CD56bright NK cells (p < 0.01) compared with normal healthy donors (Fig. 1C). It was evident that patients showed variable responses to anti-IL-2R therapy in terms of the induction of CD56bright NK cells, but all patients had CD56bright NK expansion after 4–6 wk of Ab infusion, which coincided with the onset of a clinical therapeutic effect (data not shown). To investigate the hypothesis that the induction of CD56bright NK cells may have a beneficial effect on active uveitis due to the treatment with daclizumab, we compared the cytokine profiles between the induced CD56bright NK cell subpopulation and active uveitis patients before daclizumab therapy represented either by CD56brightCX3CR1− or CD56brightCXCR3− in the CD56−CD3− NK population were plotted against those from normal donors. The solid bars represent data from normal donors (NL) whereas the open bars represent data from patients before daclizumab infusion (p < 0.01).
the CD56<sup>dim</sup> NK cell subpopulation from the same patient. The induced CD56<sup>bright</sup> and the CD56<sup>dim</sup> NK cell populations were separated by cell sorting and 14 cytokines, including IL-2, -4, -5, -6, -10, and -18, IFN-γ, IL-1α, TNF-α, IFN-α, GM-CSF, G-CSF, RANTES, and lymphotoxin were analyzed by a multiplex cytokine array assay. The cytokines produced by the induced CD56<sup>bright</sup> NK cells and the CD56<sup>dim</sup> NK cells can be classified into three groups, those not secreted by both populations (IL-4, IL-5, IL-1α, IFNα, and G-CSF); those secreted at low levels by both groups (IL-6 and IL-18), and those secreted abundantly only by the induced CD56<sup>bright</sup> NK cells. As shown in Fig. 2A, the induced CD56<sup>bright</sup> NK cells were capable of producing an array of cytokines (IL-10, IL-2, IFN-γ, TNF-α, GM-CSF, and lymphotoxin) upon activation. But the CD56<sup>dim</sup> NK cells produced minimum or undetectable cytokines. This data is consistent with previous studies in healthy normal donors that the CD56<sup>bright</sup> NK cells were more prone to secreting multiple cytokines than CD56<sup>dim</sup> NK cells, hence the name of “regulatory NK cells” (18). Moreover, it is intriguing that the induced CD56<sup>bright</sup> NK cells from daclizumab-treated patients secreted large amounts of IL-10, an immunosuppressive cytokine whereas the CD56<sup>dim</sup> NK cells did not (Fig. 2A). IL-10 has been demonstrated to be protective for

**FIGURE 2.** Phenotypic and functional analysis of the CD56<sup>bright</sup> NK cells from patients receiving daclizumab therapy. A, Comparison of cytokine profiles between the induced CD56<sup>bright</sup> NK subpopulation and CD56<sup>dim</sup> NK subpopulation. Sorted CD56<sup>bright</sup> NK and CD56<sup>dim</sup> NK cells were stimulated with IL-12 and IL-15 for 72 h, and culture supernatants were analyzed for multiple cytokine levels as described in Materials and Methods. Data were representative of two independent sorting and cytokine array analysis experiments. B, The in vivo expanded CD56<sup>bright</sup> NK cells after daclizumab therapy appeared to have much lower expression of CD161 compared with those from normal donors. Representative data are from one of five patients and one of five normal donors. The gated cells are the CD56<sup>bright</sup> NK subset. C, Daclizumab does not trigger downstream signaling events. Purified PBMCs from normal human donors were treated with either daclizumab (100 μg/ml) or recombinant human IL-2 (20 ng/ml). Cells were lysed over time by radioimmunoprecipitation assay buffer, and phosphorylated STAT5 was examined by Western blot analysis using a phospho-STAT5-specific Ab. Arrow indicates band that corresponds to phosphorylated STAT5.
uveitis and was shown to be required for the induction of oral tolerance to experimental uveitis (22, 23). A recent study demonstrated that the CD56\textsuperscript{bright} NK cells were present in local lymph nodes participating in immune responses (24). Thus, our data implicate that the induced CD56\textsuperscript{bright} NK cells might play a beneficial role in the remission of active uveitis by secreting substantial amounts of the immunosuppressive cytokine, IL-10.

We further investigated whether the induced CD56\textsuperscript{bright} NK cells by IL-2R blockade therapy shared the same phenotype with those from normal donors. Data showed that those CD56\textsuperscript{bright} NK cells induced in the patients indeed shared most of the phenotypic markers observed in normal donors. They were typically high in their expression of CXCR3, CD122, CD62L, CD94, and NKG2D, but they did not express CX3CR1 with low levels of CD16 (data not shown). However, the CD56\textsuperscript{bright} NK cells induced in the patients after IL-2R blockade seemed to be CD161 low or negative whereas naturally occurring CD56\textsuperscript{bright} NK cells from healthy donors were CD161 high (Fig. 2B). CD161 is an activating NK molecule and may be involved in the regulation of NK maturation (25–27). It is also proposed as an early marker for NK cells during NK development (28). Our data indicate that there might also be significant differences between the CD56\textsuperscript{bright} NK cells induced by IL-2R blockade therapy and naturally occurring CD56\textsuperscript{bright} NK cells in healthy individuals.

Long-term low-dose infusion of IL-2 cytokine resulted in selective expansion of the CD56\textsuperscript{bright} NK subpopulation (19). It is intriguing that the in vivo IL-2R blockade therapy can similarly induce in vivo expansion of the same subpopulation. Daclizumab is a humanized anti-IL2R α-chain mAb (anti-Tac). Earlier studies showed that this blocking Ab was an antagonist for human IL-2 on T cells (29, 30). Thus, it is provocative to observe that an IL-2 antagonist can similarly induce in vivo expansion of the CD56\textsuperscript{bright} NK subpopulation as IL-2 does. It seems unlikely that IL-2 infusion and IL-2R blockade would share the same molecular mechanism in inducing in vivo expansion of the CD56\textsuperscript{bright} NK subpopulation. To rule out that daclizumab may still be able to trigger downstream signaling events upon binding to the IL-2R α-chain, we examined STAT5 phosphorylation after daclizumab binding. As shown in Fig. 2C, although there was evidence for strong STAT5 phosphorylation after IL-2 stimulation, there was no STAT5 phosphorylation with daclizumab binding, demonstrating that daclizumab could not trigger downstream signaling events upon binding to IL-2R α-chain. Similarly, there was no phosphorylation for STAT1, STAT2, and STAT6 proteins (data not shown). We also examined whether daclizumab could directly up-regulate CD56 expression. Human PBMCs were cultured in the absence and presence of daclizumab as high as 100 μg/ml and surface CD56 intensity was examined by flow cytometry. Similarly, we did not see a direct effect of daclizumab in up-regulating CD56 expression (data not shown). Taken together, our in vitro studies suggest that daclizumab was not able to exert a direct effect on human blood cells. Therefore, it is likely that the in vivo up-regulation of the CD56\textsuperscript{bright} NK subpopulation is an indirect effect upon blockade of IL-2R.

In summary, we report here observations of in vivo expansion of the CD56\textsuperscript{bright} NK subpopulation after IL-2R blockade therapy and significantly lower levels of the CD56\textsuperscript{bright} NK subpopulation in patients with active inflammatory eye disease (uveitis). Since IL-2R blockade therapy has been widely and successfully used clinically, the data recorded here may have significance to help elucidating molecular mechanisms of IL-2R blockade therapy as well as biological roles of the CD56\textsuperscript{bright} NK subpopulation.

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Disclosures

The authors have no financial conflict of interest.

References


anisms of human natural killer cell expansion in vivo during low-dose IL-2 therapy.


