Cutting Edge: In Vivo Blockade of Human IL-2 Receptor Induces Expansion of CD56 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<sup>bright</sup> regulatory NK cells in uveitis patients over time. The induced CD56<sup>bright</sup> regulatory NK cells from patients exhibited similar phenotype as those naturally occurring CD56<sup>bright</sup> cells. Patients with active uveitis had a significantly lower level of CD56<sup>bright</sup> NK cells compared with normal donors (p < 0.01). In addition, the induced CD56<sup>bright</sup> cells could secrete large amounts of IL-10 whereas CD56<sup>dim</sup> NK cells could not, suggesting that the induction of the CD56<sup>bright</sup> 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<sup>bright</sup> regulatory NK cells in autoimmune diseases. *The Journal of Immunology, 2005, 174: 5187–5191.*

**Materials and Methods**

Five patients with active non-infectious uveitis who underwent therapy with the humanized anti-IL-2R α-chain (CD25) mAb, daclizumab, 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 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<sup>CD3</sup><sup>−</sup> staining. CD56<sup>bright</sup> cells were gated based on CD56<sup>light</sup>CX3CR1<sup>−</sup>CD3<sup>+</sup> or CD56<sup>bright</sup>CX3CR3<sup>−</sup>CD3<sup>−</sup>. The results were represented by the percentage of the CD56<sup>bright</sup> subset in either lymphocyte or the CD56<sup>bright</sup> NK cell subpopulation. Student’s t-test was used for statistical analysis.
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-CD4 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 FACSAria sorter (BD Biosciences). The purified populations 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 × 10⁵/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 (National Institutes of Health blood bank) were used. Approximately 5 × 10⁵/ml of 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 anti-phospho-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 population 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 at 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 CD56<sup>bright</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...
uveitis and was shown to be required for the induction of oral tolerance to experimental uveitis (22, 23). A recent study demonstrated that the CD56bright NK cells were present in local lymph nodes participating in immune responses (24). Thus, our data implicate that the induced CD56bright 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 CD56bright NK cells by IL-2R blockade therapy shared the same phenotype with those from normal donors. Data showed that those CD56bright 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 CXCRI3, CD122, CD62L, CD94, and NKG2D, but they did not express CX3CR1 with low levels of CD16 (data not shown). However, the CD56bright NK cells induced in the patients after IL-2R blockade seemed to be CD161 low or negative whereas naturally occurring CD56bright 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 CD56bright NK cells induced by IL-2R blockade therapy and naturally occurring CD56bright NK cells in healthy individuals.

Long-term low-dose infusion of IL-2 cytokine resulted in selective expansion of the CD56bright NK subpopulation (19). It is intriguing that in vivo IL-2R blockade therapy can similarly induce in vivo expansion of the same subpopulation. Daclizumab is a humanized anti-IL-2R 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 CD56bright 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 CD56bright 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 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 after daclizumab binding.

In summary, we report here observations of in vivo expansion of the CD56bright NK subpopulation after IL-2R blockade therapy and significantly lower levels of the CD56bright 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 CD56bright NK subpopulation.

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Disclosures
The authors have no financial conflict of interest.

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

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