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# Regulatory NK Cells Suppress Antigen-Specific T Cell Responses[1]

**Gunnur Deniz,[2]*‡ Gaye Erten,*‡ Umut Can Kücüksezer,*‡ Dilara Kocacik,†‡ Christian Karagiannidis,‡ Esin Aktas,* Cezmi A. Akdis,‡ and Mubeccel Akdis‡**

**The immune system has a variety of regulatory/suppressive processes, which are decisive for the development of a healthy or an allergic immune response to allergens. NK1 and NK2 subsets have been demonstrated to display counterregulatory and provocative roles in immune responses, similar to Th1 and Th2 cells. T regulatory cells suppressing both Th1 and Th2 responses have been the focus of intensive research during the last decade. In this study, we aimed to investigate regulatory NK cells in humans, by characterization of NK cell subsets according to their IL-10 secretion property. Freshly purified IL-10-secreting NK cells expressed up to 40-fold increase in IL-10, but not in the FoxP3 and TGF-$\beta$ mRNAs. PHA and IL-2 stimulation as well as vitamin D3/dexamethasone and anti-CD2/CD16 mAbs are demonstrated to induce IL-10 expression in NK cells. The effect of IL-10$^+$ NK cells on Ag-specific T cell proliferation has been examined in bee venom major allergen, phospholipase $A_2$- and purified protein derivative of *Mycobecterium bovis*-induced T cell proliferation. IL-10$^+$ NK cells significantly suppressed both allergen/Ag-induced T cell proliferation and secretion of IL-13 and IFN-$\gamma$, particularly due to secreted IL-10 as demonstrated by blocking of the IL-10 receptor. These results demonstrate that a distinct small fraction of NK cells display regulatory functions in humans.** ***The Journal of Immunology,*** **2008, 180: 850–857.**

Natural killer cells mediate the early, nonadaptive responses against virus-, intracellular bacteria-, and parasite-infected cells, and modulate the activity of other effector cells of the adaptive and innate immune system (1–3). They mediate these effects through production of cytokines and direct killing of transformed or infected cells (4, 5). Distinct types of immune response is controlled by type 1 (Th1) and type 2 (Th2) subpopulations of T cells, discriminated on the basis of their cytokine secretion (6, 7). Similar to Th1 and Th2 cells, human NK cells cultured in the presence of IL-12 or IL-4 differentiate into cell populations with distinct patterns of cytokine secretion (8, 9). The in vivo existence of human NK cell subsets similar to Th1 and Th2 cells was demonstrated in freshly isolated IFN-$\gamma$-secreting and IFN-$\gamma$-nonsecreting NK cells (10). The IFN-$\gamma$-secreting NK cell subset showed a typical cytokine pattern with predominant expression of IFN-$\gamma$, but almost no IL-4, IL-5, and IL-13 (10). In contrast, IFN-$\gamma$-nonsecreting NK cells mainly produce IL-13 and contribute to IgE production by B cells (11, 12). Although the production and role of TGF-$\beta$ and IL-10 in human peripheral blood NK cells have been demonstrated (12–16), regulatory subsets of NK cells remained to be elucidated.

NK cell function is tightly regulated by a fine balance of inhibitory and activatory signals that are delivered by a diverse array of cell surface receptors. Killer cell Ig-like receptor (KIR)[3] binds to HLA class I molecules on the surface of the target cell, and it confers inhibitory signals to NK cells (17–19). Upon its ligation by HLA class I molecules, KIR can deliver inhibitory signals via the immune-receptor tyrosine-based inhibitory motif. Therefore, NK cells can recognize the cells that do not express HLA class I molecules as cytotoxic target cells, and KIR plays a role in the cytotoxic target discrimination of NK cells (4, 18). Among inhibitory receptors, some are specific for different groups of MHC class I alleles, while others are still orphan receptors. On the contrary, various activating receptors are involved in the triggering of NK cell-mediated natural cytotoxicity (17). In addition, the expression of CD16 (Fc$\gamma$RIII), the low-affinity receptor for IgG and CD56, isoform of neural cell adhesion molecule, to assess the NK cell activation state CD25, CD69, CD49d, activatory/inhibitory KIR: CD94 (KLRD1), activatory KIR: CD161 (NKR-P1A), and inhibitory KIRs: CD158a (NKAT1, KIR2DL1) and CD158b (NKAT2, KIR2DL3) by IL-10-secreting NK cells are analyzed in this study (20–22).

Recently, additional subtypes of T cells, with immunosuppressive function and cytokine profiles distinct from either Th1 or Th2 cells, termed T regulatory ($T_{Reg}$) cells, have been described (23, 24). $CD4^+CD25^+$ $T_{Reg}$ cells express FoxP3 and have been shown to be effective in several models of allergy, autoimmunity and transplantation tolerance (23, 25, 26). In addition, inducible type 1 $T_{Reg}$ cells ($T_R1$ cells) have been shown to be regulatory by high IL-10 secretion (27–29). In the present study, the existence of regulatory NK cells in humans, NK cell subsets, are characterized

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[3] Abbreviations used in this paper: KIR, killer cell Ig-like receptor; $T_{Reg}$, T regulatory cell; PLA, phospholipase $A_2$; PPD, purified protein derivative.

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according to their IL-10 secretion, and IL-10-secreting and IL-10-nonsecreting NK cells are purified by magnet-activated cell sorting. The effect of IL-10-secreting NK cells on Ag-specific T cell proliferation is examined in bee venom major allergen, phospholipase $A_2$ (PLA)- and purified protein derivative of *Mycobacterium bovis* (PPD)-induced T cell proliferation. These data support that a small fraction of NK cells display regulatory functions similar to T regulatory cells in humans.

## Materials and Methods

### *Purification of IL-10- and IFN-γ-secreting NK cell subsets*

PBMCs from healthy individuals are isolated by Ficoll density gradient centrifugation of peripheral venous blood (Sigma-Aldrich). NK cells are purified by MACS (Miltenyi Biotec). In brief, NK cells are isolated from PBMC by immunomagnetic depletion of T cells, B cells, monocytes, and other myeloid cells, such as basophils and dendritic cells, according to expression of CD3, CD4, CD19, and CD33. The purity of NK cells was >96%, as assessed by flow cytometric analysis of cells stained with FITC-labeled anti-CD16, rhodamine-labeled anti-CD56 and PE-Texas red-X (ECD)-labeled anti-CD3 (EPICS XL, Beckman Coulter). CD3 positive T cell contamination in purified NK cells was <2%. For the isolation of IL-10- and IFN-γ-secreting and nonsecreting NK cell subsets, freshly purified NK cells are stimulated with 5 μg/ml PHA (Sigma-Aldrich) overnight. Cells are washed and labeled for 10 min at a concentration of $10^7$ cells/ml in ice cold medium with 50 μg/ml anti-IL-10/CD45 or -IFN-γ/CD45 Ab-Ab conjugates (Miltenyi Biotec) (10). Cells are resuspended in 37°C warm medium to a final concentration of $5 \times 10^5$ cells/ml and are allowed to secrete IL-10 or IFN-γ for 40 min at 37°C. After capturing secreted cytokines on their surface, cells are stained with 5 μg/ml PE-conjugated anti-IL-10 or -IFN-γ for 10 min at 4°C. Cytokine-secreting and -nonsecreting NK cells are analyzed by flow cytometry before enrichment. For the enrichment procedure, the cells are washed and resuspended in 80 μl buffer and magnetically labeled for 15 min at 4°C with 20 μl microbead-labeled anti-PE mAb and enriched by positive or negative selection (10). The frequency of IL-10- and IFN-γ-secreting NK cells was calculated by division of the numbers of cytokine-secreting or nonsecreting NK cells with the total number of NK cells. For the isolation of naive and memory T cells, first pure T cells are isolated with human pan T cell isolation kit (Miltenyi Biotec AG, Bergisch Gladbach, Germany). In brief, anti-CD14, anti-CD16, anti-CD19, anti-CD56, anti-CD36, anti-CD123, and anti-CD235a are added to PBMCs for the depletion of B cells, NK cells, dendritic cells, monocytes, granulocytes, and erytroid cells. For the negative selection of $CD45RA^+$ naive T cells, anti-CD45RO microbeads are used in the second step. For the negative selection of $CD45RO^+$ memory T cells, anti-CD45RA microbeads are used in the second step. Monocytes are isolated by using anti-CD14 microbeads and the same method.

The purity of the cell subsets analyzed by flow cytometry was >96%.

### *Cell cultures, proliferation, and quantitation of cytokines*

Ag-specific T cell proliferative response is determined by stimulation of $2 \times 10^5$ PBMCs alone or together with freshly purified cytokine-secreting NK cell subsets for 5 days with Ag in 200 μl of RPMI 1640 medium with 10% FCS in 96-well flat-bottom tissue culture plates in triplicates (10). PPD was from the Serum Institute and PLA is from Sigma-Aldrich. Cells are pulsed with 1 μCi/well [$^3$H]thymidine (Dupont and NEN Life Science Products), and incorporation of labeled nucleotide is determined after 8 h in a betaplate reader (Wallac and Amersham Biosciences). For polyclonal activation of T cells, plates are coated with 10 μg/ml anti-CD3 (Sigma-Aldrich) for 2 h at 37°C [$^3$H]thymidine incorporation was detected at day 3. Cell proliferation is additionally measured by CFSE labeling. Cells are labeled with CFSE (5 μM, Molecular Probes) and washed twice with medium before being subjected to the various stimulations (anti-CD3, PPD and PLA). After 3 days for anti-CD3 and after 5 days for PPD and PLA stimulations, $CD4^+$ cells are counterstained with Pc5-labeled anti-CD4 mAb (BD Pharmingen) and analyzed by flow cytometry (30). IL-10 is neutralized in cultures with 4 μg/ml anti-IL-10R mAb (provided by K. Moore, DNAX Research Institute, Palo Alto, CA) (26, 31). The solid-phase sandwich ELISAs for IFN-γ, IL-10, and IL-13 are performed in supernatants obtained at day 5, as described previously (32).

### *Flow cytometric analysis and ELISPOT assay*

Before purification, $5 \times 10^4$ cells are stained with FITC-conjugated anti-CD8, anti-CD16, anti-CD25, anti-CD49d, anti-CD56, anti-CD69 (Immunotech), anti-CD94, anti-CD158a, anti-CD161, and anti-CD158b (BD Pharmingen). Stained cells are fixed in 2% paraformaldehyde and flow cytometric analysis is performed with an EPICS XL (Beckman Coulter).

In brief, $10^6$ cells/ml NK cells from healthy donors are stimulated with 5 μg/ml PHA in 200 μl of medium containing 96-well flat-bottom ELISPOT plates for 18 h (R&D Systems). Locally produced IFN-γ and IL-10 are captured by specific mAbs. After cell lysis, captured cytokine molecules are revealed by a secondary biotinylated detection Ab, which in turn is recognized by streptavidin-conjugated alkaline phosphatase. Colored "purple" spots developed after addition of the substrate (5-bromo-4-chloro-3-indolyl phosphate/NBT) are counted (ImmunoSpot; Cellular Technology Ltd.). Eighteen hours has been found to be the optimal time for determination of the frequency of cytokine-secreting cells, as it is the time point for highest cytokine secretion before NK cell proliferation starts.

### *Real-time quantitative PCR analysis*

For the stimulation of NK cells and other leukocyte subsets PHA was used at 5 μg/ml, IL-2 was 25 ng/ml, phorbol myristate acetate was 10 ng/ml, and ionomycine was 250 ng/ml (all from Sigma-Aldrich), vitamin D3 was at $4 \times 10^{-8}$ M (Biomol Research Laboratories), and dexamethasone was 0.1 μM, 10 μg/ml plate bound, for 2 h at 37°C anti-CD2 mAbs (clones 4B2 and 6G4, Sanquin) anti-CD16 mAb (BD Pharmingen). Five $\times 10^5$ cells are stimulated for indicated time points. RNA is isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Reverse transcription is performed with TaqMan reverse transcription reagents (Applied Biosystems) with random hexamers according to the manufacturer's protocol. The following PCR primers are used: IL-10 forward primer, 5′GGC GCT GTC ATC GAT TTC TT 3′; IL-10 reverse primer, 5′TTG GAG CTT ATT AAA GGC ATT CTT C 3′; FoxP3 forward primer, 5′CCC GGC CTT CCA CAG AA 3′; FoxP3 reverse primer, 5′CAC CCG CAC AAA GCA CTT G 3′; TGFβ1 forward primer, 5′AAA TTG AGG GCT TTC GCC TTA 3′ and TGFβ1 reverse primer, 5′GAA CCC GTT GAT GTC CAC TTG 3′, IL-13 forward primer A 5′ GCC CTG GAA TCC CTG ATC A 3′, IL-13 reverse primer A 5′ GCT CAG CAT CCT CTG GGT CTT 3′, IFN-γ forward primer B 5′ TCT CGG AAA CGA TGA AAT ATA CAA GTT AT 3′, IFN-γ reverse primer B 5′ GTA ACA GCC AAG AGA ACC CAA AA 3′. For 18 s, rRNA commercially available primers are purchased (Applied Biosystems). Primers are spanning exon-exon borders to eliminate potential amplification of contaminating genomic DNA. The prepared cDNAs are amplified using SYBR-PCR Master Mix (Applied Biosystems) according to the recommendations of the manufacturer in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Relative quantification and calculation of the range of confidence is performed using the comparative ΔΔCT method as described (33). All analyses are conducted in triplicates.

### *NK cell cytotoxic activity*

Cytotoxic activity of NK cell subsets is determined by using green fluorescent-labeled K562 target cells (Orphogen Pharma) (34). In brief, cells are incubated 6 h in a humidified $CO_2$ incubator at desired effector/target ratios. Tubes are placed on ice until flow cytometric analysis and percentage of specific NK cell cytotoxicity on gated green K562 cells is analyzed by exclusion of 0.001% 7-amino acridinium by flow cytometry.

### *Statistical interpretation*

Data are expressed as mean ± SD. Statistical analysis is performed by Student's *t* test and Mann-Whitney *U* test.

## Results

### *A small fraction of NK cells secrete IL-10*

To explore potential immune regulatory/suppressor subsets of NK cells, we first analyzed the expression of IL-10 in purified NK cell population. For the demonstration of IL-10-secreting and nonsecreting NK cell subsets, freshly purified NK cells are stimulated with PHA and allowed to secrete IL-10, and secreted IL-10 is captured on their surface. After capturing secreted IL-10 on their surface, cells were stained with PE-conjugated anti-IL-10 and analyzed by flow cytometry.

Lymphocyte subset-specific receptors, activation markers, and activatory and inhibitory NK receptors are counterstained in IL-10-secreting NK cells. In the whole $CD16^+$ NK cells, the percentage of IL-10-secreting and $CD56^+$ NK cells is ~6.8% (4.9–9.0%) (Fig. 1*A*). Two-third of NK cells express CD8 and IL-10 is expressed in 5.8% of CD8-positive and 2.0% CD8-negative NK

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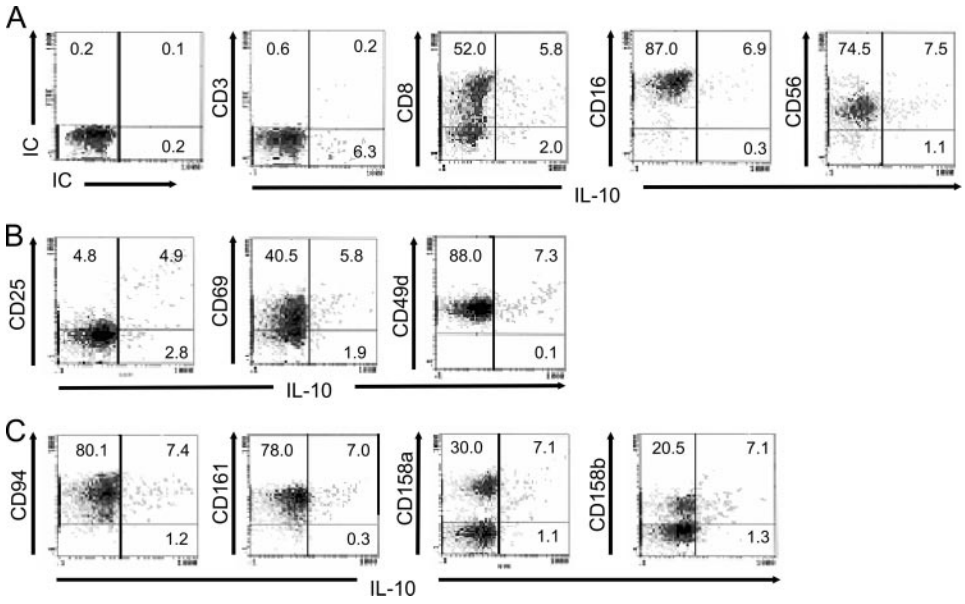



**FIGURE 1.** NK cells express IL-10 secreting subsets. Purified NK cells from healthy individuals are stimulated for overnight with PHA and analyzed by using a dual-specific, anti-CD45/anti-IL-10 mAb, and PE-labeled anti-IL-10 mAb together with surface receptors (*A*), activation molecules (*B*), inhibitory and activatory receptors (*C*) are stained with FITC-labeled mAbs for surface receptors and analyzed by flow cytometry. Results shown are one representative from five healthy individuals tested (IC: isotype control mAb).

cells. IL-10-secreting NK cells showed two distinct populations according to CD25 expression, one with very high and the other with low CD25 on their surface. IL-10 secreting and nonsecreting NK cells did not show a specific predominance according to CD69 expression. All of the NK cells expressed CD49d with ~7.0% of them secreting IL-10 (Fig. 1*B*). NK receptors CD94 and CD161 are expressed on almost all of NK cells, again ~7.0% of these cells expressed IL-10. In contrast, inhibitory KIRs, CD158a and CD158b are expressed in a subset of NK cells (~37.1% and 27.6% of all NK cells, respectively). Interestingly, the majority of the IL-10-secreting NK cells are confined to the inhibitory KIR-expressing NK cell subsets (Fig. 1*C*).

### *Purification and characteristics of IL-10-secreting and IL-10-nonsecreting NK cells*

IL-10-secreting and IL-10-nonsecreting NK cells are purified to further characterize this tiny subset of NK cells. After overnight PHA stimulation, 6.8% (4.9–9.0%) of NK cells showed IL-10 secretion before purification. After purification, the IL-10-secreting NK subset is enriched to >84.3% (84.3–88.9%) (Fig. 2*A*). Both of

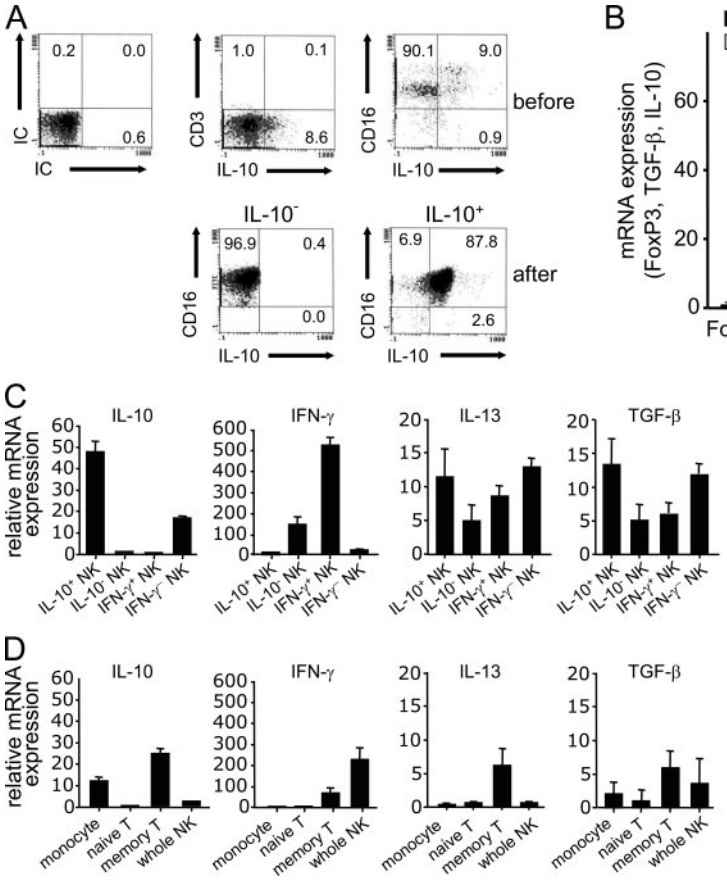



**FIGURE 2.** Purification and characteristics of IL-10-secreting and IL-10-nonsecreting NK cells. *A*, IL-10-secreting and IL-10-nonsecreting NK cells are purified by magnet-activated cell sorting and counterstained with FITC-labeled anti-CD3 and -CD16 before and after purification. Results shown are one representative of three healthy individuals tested (IC: isotype control mAb). *B*, The expression of IL-10, TGF-$\beta$, and FoxP3 mRNAs are analyzed by quantitative RT-PCR in IL-10-secreting and IL-10-nonsecreting NK cells immediately after purification. *C*, Comparison of cytokine mRNA expression between IL-10- and IFN-$\gamma$-secreting and nonsecreting NK cell subsets immediately after purification. *D*, Comparison of cytokine mRNA expression of whole NK cells with monocytes, naive, and memory T cells immediately after purification.

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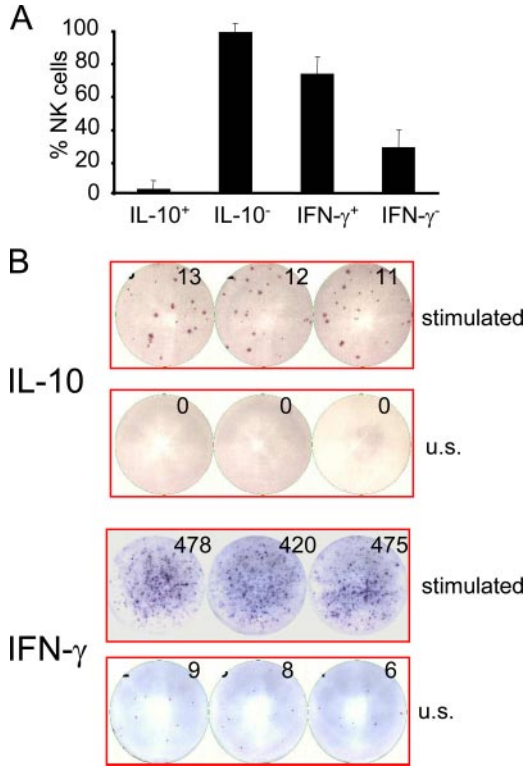



**FIGURE 3.** Frequency of IL-10-secreting NK cells. *A*, Frequency of IL-10- and IFN-γ-secreting NK cells from ten healthy individuals. *B*, One of three representative frequency analysis of IL-10- and IFN-γ-secreting NK cells in triplicates by ELISPOT assay.

the CD16$^{bright}$ and CD16$^{dim}$ populations secreted IL-10 by PHA stimulation. To support these findings, relative mRNA expressions of IL-10, FoxP3, and TGF-β are determined in IL-10-secreting and nonsecreting NK cells. IL-10-secreting NK cells from healthy individuals showed 40-fold increased IL-10 mRNA without any further stimulation compared with NK cells, which do not secrete any IL-10 (Fig. 2*B*). FoxP3 was not expressed both in IL-10-secreting and nonsecreting NK cells. IL-10- and IFN-γ-secreting and -nonsecreting NK cells are compared for cytokine mRNA expression after PHA stimulation (Fig. 2*C*). Although there can be a small overlap, the data demonstrate that IL-10-secreting and IFN-γ secreting fractions rather represent distinct subsets of NK cells. IL-10 mRNA expression is confined to IL-10-secreting NK cells and IFN-γ-nonsecreting cells and IFN-γ mRNA expression is high in IL-10-nonsecreting NK cells, in addition to IFN-γ-positive NK cells. IL-13 and TGF-β are expressed in relatively low amounts, however slightly higher in IL-10-positive and IFN-γ-negative NK cells. Then, the magnitude of cytokine mRNA expression is compared in monocytes, naive, and memory T cells and whole NK cells immediately after purification (Fig. 2*D*). IL-10 is highly expressed in memory T cells and monocytes and was detectable in unfractionated NK cells. Memory T cells and NK cells were the main source of IFN-γ. IL-13 is highly expressed by memory T cells. TGF-β mRNA is expressed in all cells without showing any predominance. It has to be noted here that upon enrichment, IL-10-secreting NK cells showed much higher IL-10 expression compared with unfractionated NK cells.

The frequency of IL-10-secreting and nonsecreting NK cells is investigated by two methods and compared with IFN-γ secreting NK cells. Direct calculation of the two subsets after purification demonstrated low frequency (2–9%) compared with IFN-γ-secreting NK cells (61–89%) (Fig. 3*A*). For comparison, IFN-γ-secreting NK cells were 78 ± 13% and IFN-γ-nonsecreting NK cells were 22 ± 8%. ELISPOT is used as an alternative method in NK cell cultures and demonstrated a similar distribution for IL-10- and IFN-γ-secreting NK cells. The frequency of IFN-γ-secreting NK cells in ELISPOT was 40-fold higher compared with IL-10-secreting NK cells (Fig. 3*B*). Although there was a small population of spontaneously IFN-γ-secreting NK cells, there was no spontaneous IL-10 secretion in unstimulated condition.

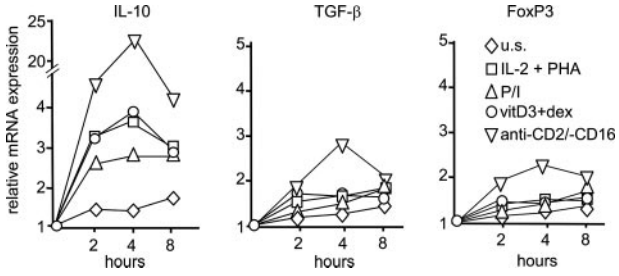



**FIGURE 4.** Induction of IL-10, TGF-β, and FoxP3 mRNA expression in NK cells. Freshly isolated NK cells are stimulated with IL-2 plus PHA, phorbol ester and ionomycine (P/I), or vitamin D3 and dexamethasone (vitD3+dex), plate-bound anti-CD2/CD16 mAbs for indicated time points and IL-10, TGF-β, and FoxP3 mRNA expressions are analyzed by quantitative RT-PCR. Data represent one of three experiments with similar results.

### *Induction of IL-10 in NK cells*

It has previously been shown that the combination of vitamin D3 and dexamethasone induces IL-10 production in T cells (35). Accordingly, we investigated factors that induce IL-10 mRNA and compared with regulation of TGF-β and FoxP3 mRNA in NK cells (Fig. 4). IL-10 mRNA is up-regulated in activated NK cells within 2 h of IL-2 and PHA, phorbol ester, and ionomycine. A strong stimulus for IL-10 production by NK cells was the combination of anti-CD2 and anti-CD16 mAbs, which also induced highest TGF-β and FoxP3 mRNA expression after 4 h. Interestingly, vitamin D3 and dexamethasone combination induced IL-10 mRNA expression in equal strength compared with IL-2 and PHA or PMA/I. TGF-β and FoxP3 mRNAs did not show any significant change with the same stimuli.

### *Natural cytotoxicity of IL-10-secreting and IL-10-nonsecreting NK cells*

The cytotoxic potential of IL-10-secreting and nonsecreting NK cells is analyzed by direct cytotoxic activity on NK-sensitive K562

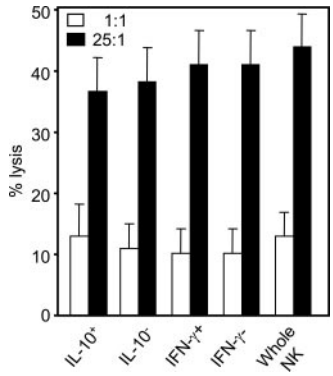



**FIGURE 5.** Same cytotoxic activity of freshly purified IL-10- and IFN-γ-secreting and -nonsecreting NK cells. NK cell subsets are cocultured 6 h with K562 cells immediately after purification. Percent death of K562 cells is analyzed at E:T ratios of 1:1 and 25:1 by flow cytometry. The results are mean ± SD of three experiments.

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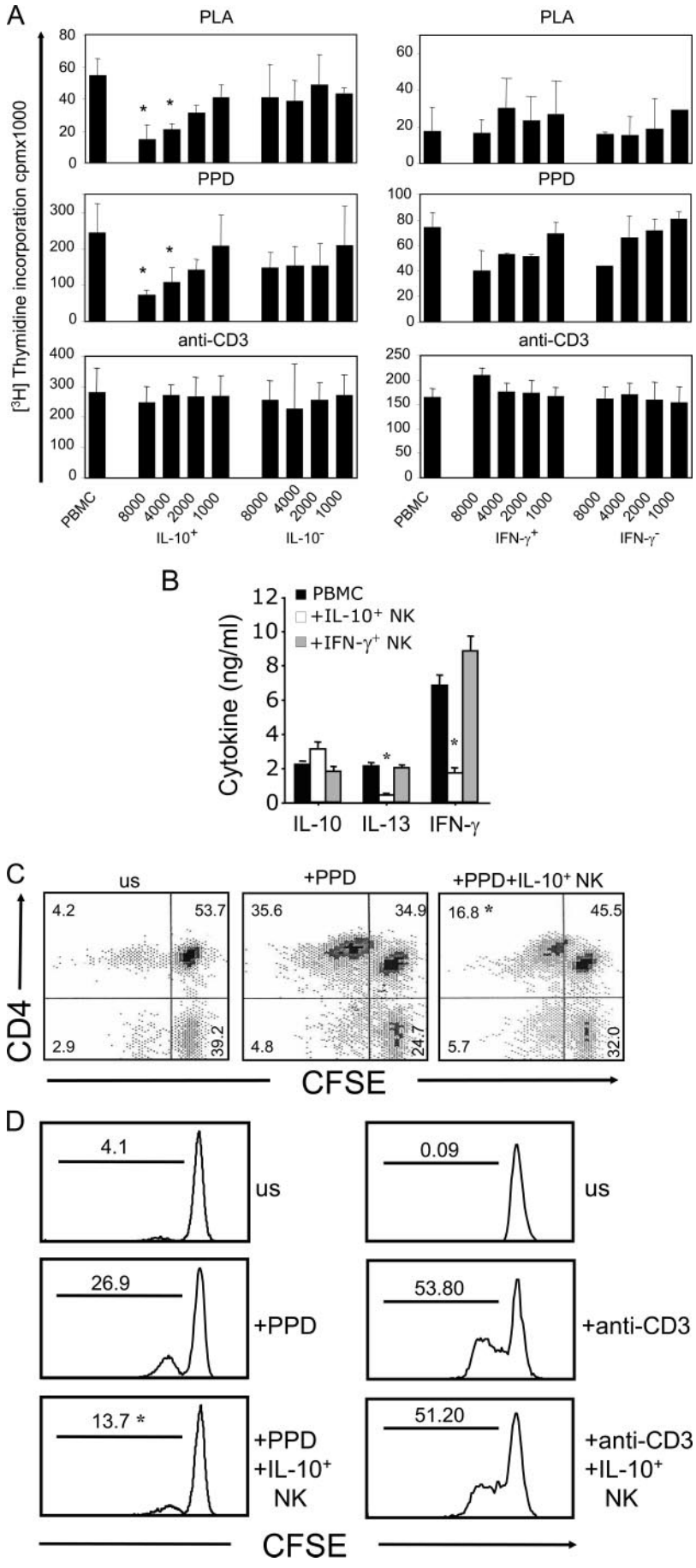



**FIGURE 6.** Suppressive effect of IL-10-secreting NK cells on Ag-specific $CD4^+$ T cell proliferation. IL-10- and IFN-γ-secreting and -nonsecreting NK cells are purified and added to autologous PLA- (5 μg/ml) and PPD- (1 μg/ml) stimulated PBMC ($2 \times 10^5$) cultures at indicated numbers. Two $\times 10^5$ PBMCs are stimulated with 10 μg/ml plate-bound anti-CD3 in the presence of different amounts of IL-10- and IFN-γ-secreting and nonsecreting NK cells. Bee venom hyperimmune individuals (bee keepers) are used for PLA stimulations and PPD-responsive healthy donors are used for PPD stimulations. Due to a limited number of NK cells, either IL-10- or IFN-γ-secreting and nonsecreting cells are purified from each donor. *A*, $[^3H]$Thymidine incorporation was determined after 5 days. *B*, IL-10, IL-13, and IFN-γ were determined from day 5 supernatants of parallel cultures. *C*, PBMCs were labeled with CFSE from the start $8 \times 10^3$ IL-10-secreting NK cells are added to $2 \times 10^5$ PBMC. $CD4^+$ T cells are stained and flow cytometric analysis is performed on day 5. *D*, $CD3^+CD4^+$ T cells are gated and CFSE dilution is analyzed on day 5 for PPD stimulation and day 3 for anti-CD3 stimulation. Results shown are one representative of three different healthy individuals tested for each stimulus. *, $p < 0.01$.

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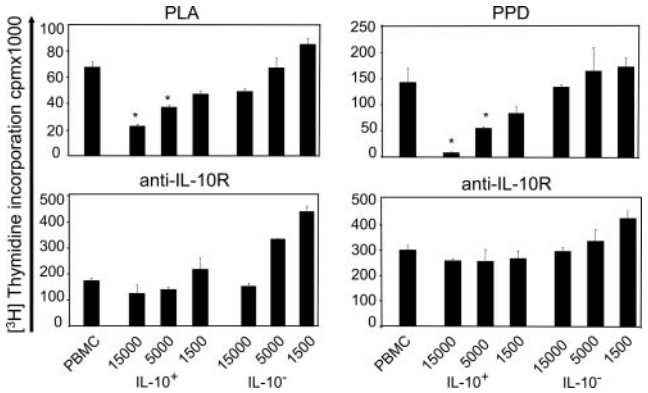



**FIGURE 7.** Blocking of the IL-10R inhibited the suppressive activity of IL-10-secreting NK cells. IL-10R is blocked in PLA- and PPD-stimulated PBMC of healthy individuals. Two $\times 10^5$ PBMCs are stimulated with PLA or PPD in the presence of different amounts of IL-10- and IFN-$\gamma$-secreting and nonsecreting NK cells. [$^3$H]Thymidine incorporation is determined after 5 days. Same results are obtained in three independent experiments. *, $p < 0.01$.

cells. A flow cytometry-based assay, which correlates well with the standard chromium-release assay is used (34). Freshly purified whole NK cells, IL-10-, and IFN-$\gamma$-secreting and nonsecreting NK cells are cocultured with K562 cells for 6 h (Fig. 5). There was no difference between purified IL-10- and IFN-$\gamma$-secreting and -nonsecreting NK cells and whole NK cells in natural cytotoxic activity.

### *Suppression of Ag-specific CD4$^+$ T cell proliferation by IL-10-secreting NK cells*

To investigate whether IL-10-secreting NK cells suppressed Ag-specific T cell proliferation, we cultured IL-10- and IFN-$\gamma$-secreting NK cells with autologous PBMC in the presence of either PLA or PPD (Fig. 6*A*). As few as $4 \times 10^3$ IL-10-secreting NK cells significantly suppressed both PLA- and PPD-induced T cell proliferation in $2 \times 10^5$ PBMC. However, IFN-$\gamma$-secreting NK cells and unfractionated NK cells (data not shown) did not show any suppression. Either IL-10- or IFN-$\gamma$-secreting NK cells did not exert any suppressive effect on anti-CD3 stimulation. In parallel cultures, IL-10 and IL-13 are determined in PLA-stimulated PBMC of bee venom sensitized individuals and IFN-$\gamma$ is detected in PPD-stimulated PBMC (Fig. 6*B*). IL-10-secreting NK cells significantly suppressed IL-13 and IFN-$\gamma$ production, whereas IFN-$\gamma$-secreting NK cells did not exert any suppressive effect.

To further investigate which cell subset is proliferating and inhibited by IL-10-secreting NK cells, we labeled PBMC with CFSE and stimulated with PPD and anti-CD3 mAbs. IL-10-secreting NK cells significantly suppressed the proliferation of CD4$^+$ T cells stimulated by PPD, but not by anti-CD3 (Fig. 6, *C* and *D*). Suppression of Ag-specific T cell proliferation by IL-10-secreting NK cells is not any more observed when the activity of IL-10 is inhibited by a receptor blocking mAb (Fig. 7). These data demonstrate that the suppressor activity of IL-10-secreting NK cells on Ag-specific T cell proliferation may play a regulatory role similar to T regulatory cells in humans.

## Discussion

Direct purification of IL-10-secreting and nonsecreting NK cell subsets from peripheral blood enabled to demonstrate the in vivo existence for a regulatory NK cell subset in the present study. The frequency of IL-10-secreting NK cells was relatively low compared with IFN-$\gamma$-secreting cells. We and others recently showed that NK cells can differentiate into cells with NK1 and NK2 phenotypes, similar to that described in T cells (8–12). Although effector and regulatory T cells have been demonstrated in several inflammatory and healthy conditions, there has been no report about any regulatory NK cell subset in peripheral blood.

Ag-specific T cell suppression by IL-10, a known suppressive cytokine of T cell proliferation and cytokine production, is essential in peripheral tolerance to allergens, autoantigens, transplantation Ags, and tumor Ags. IL-10 is a suppressor cytokine of T cell proliferation in both Th1 and Th2 cells. It was originally thought to be produced by Th2 cells only, however, it is in fact produced particularly by $T_R1$ cells, but also by Th0, Th1, and Th2 cells as well as B cells, monocytes, and keratinocytes (36, 37). $T_R1$ cells, also known as inducible $T_{Reg}$ cells, are defined by their ability to produce high levels of IL-10 and TGF-$\beta$ (27, 38). IL-10 is produced after stimulation by T cells or monocytes/macrophages (26, 35, 36, 38). Several reports showed that both CD56$^{bright}$ and CD56$^{dim}$ NK cells can produce TGF-$\beta$ and IL-10 (39, 40). In addition, freshly separated NK cells from chronic hepatitis C virus-infected patients showed significant production of IL-10 and normal concentrations of IFN-$\gamma$ upon cell-mediated triggering (41). Evidence for the presence of these NK cell subsets was also supported in mice, which showed that mouse IL-2-activated NK cells can be subdivided based on expression of high or low levels of the IL-12R$\beta$2 (42). It has been reported that populations of peripheral blood IL-10-producing (NKr1)-CD56$^{bright}$ and CD56$^{dim}$ NK cells in early pregnancy women were increased compared with spontaneous abortion cases (43). Veenstra van Nieuwenhowen et al. (44) reported a mild increase in the IL-10 production of blood NK cells in pregnancy, and these findings suggest that regulatory NK cells in peripheral blood and in deciduas of early pregnancy might play some important roles in the maintenance of pregnancy by the regulation of maternal immune function.

The investigation of several cell surface markers in NK cells enabled us to further characterize the IL-10-secreting NK cell subsets. The expression of CD94 and CD161 was higher in purified NK cells compared with CD158a and CD158b. Activatory and inhibitory KIRs are expressed differently in NK cell subsets. It has recently been shown that KIR, CD158b (NKAT2), and GL183 receptor expressions were significantly increased in NK2 cells compared with NK cells in atopic dermatitis, suggesting that NK1 and NK2 cell subsets may show differences in KIR and lectin-like receptor expressions (11). In this study, approximately one-third of NK cells expressed the inhibitory NK receptor CD158a and one-fourth of NK cells expressed the inhibitory NK receptor CD158b. Interestingly, a majority of the IL-10-secreting NK cells were confined to inhibitory NK receptor-expressing subsets, suggesting a cooperation between inhibitory receptors on NK cells and IL-10 secretion, which might have an impact on regulatory activity.

Secretion of suppressive cytokines IL-10 and TGF-$\beta$ by regulatory T cells suggests that generation of these cells might play a role in active immune suppression in inflammatory conditions (26, 45–48). Purified NK cells increased IL-10 mRNA up to 20-fold with anti-CD2 and CD16 mAbs and 4-fold by PHA and IL-2 as well as the combination of vitamin D3 and dexamethasone. It has been reported that vitamin D3 and dexamethasone induced development of IL-10-secreting T regulatory cells (35). It appears that similar mechanisms for T cells may function for IL-10-secreting regulatory NK T cells. Interestingly, IL-10-secreting NK cells did not express the $T_{Reg}$ cell transcription factor FoxP3 and expressed the suppressive cytokine TGF-$\beta$ in relatively low quantities. They seemed to be much like IL-10-secreting Tr1 cells, but not the CD4$^+$ CD25$^+$ FoxP3$^+$ $T_{Reg}$ cells and TGF-$\beta$-secreting Th3 cells (49).

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The studies on NK cells have demonstrated that they are a specific cell subset ready to secrete IFN-γ and initiate inflammation and also direct cytotoxic response against cells that do not express self MHC class I molecules (50). Indeed, this was demonstrated by comparison of unfractionated and fractionated NK cells with freshly purified monocytes, naive, and memory T cells. IL-10 mRNA expression is much less compared with IFN-γ in whole NK cells. However, upon purification, the IL-10-secreting subset showed high IL-10 expression. The comparison of cytokine profiles of IL-10 and IFN-γ-secreting NK cells demonstrated that these subsets rather represent distinct fractions, because IL-10 is expressed more in IFN-γ-negative NK cells and IFN-γ is expressed in IL-10-negative NK cells. The question was posed, how the in vivo frequency of IL-10-secreting NK cells compares with the IFN-γ-secreting NK cell frequency. Apparently, the IL-10-secreting NK cells represented a tiny population, which is 8 to 40 times less than the IFN-γ-secreting NK cells. ELISPOT of purified NK cells for cytokines and calculation of purified cytokine-secreting NK cells gave similar frequency ratios. IL-10-secreting NK cell frequency determined by cytokine secretion assay was relatively high compared with ELISPOT. This could be particularly due to the relatively high sensitivity of the secretion assay and different kinetics of the two assays. ELISPOT data represents a relatively longer time frame of cytokine production and secretion, whereas secretion assay represents an early and short time frame.

IL-10 has been shown to suppress both cytokine production and Ag-specific proliferation of Th1 and Th2 cells. In this study, we show that IL-10-secreting NK cells suppress both allergen-stimulated T cells and PPD-stimulated T cells, whereas IFN-γ-secreting NK cells did not show any suppression. CFSE-labeling and counterstaining of the cells demonstrated that specifically the $CD4^+$ T cell proliferation has been suppressed. Experiments by blocking the IL-10 receptor demonstrated that the suppressive effect was due to secreted IL-10. The molecular mechanisms of T cell suppression by IL-10 have been investigated in several models. IL-10 inhibits the proliferative T cell response in PBMC to various Ags, and the superantigen staphylococcal enterotoxin B. This is particularly due to suppression of CD28 costimulation (30). IL-10 particularly suppresses CD28 and ICOS costimulations, however, as shown in the present study, IL-10 does not affect the proliferative responses of T cells that are stimulated by anti-CD3 (30). In addition, IL-10 down-regulates the Ag-presenting capacity, such as HLA-DR expression, costimulatory molecules, and several cytokines in dendritic cells and monocytes/macrophages (51). Various studies have suggested that the timing and possibly the localization of IL-10 production critically affects its immune regulatory functions (35, 43–45, 51–53). Taken together, direct purification of IL-10-secreting and nonsecreting NK cell subsets from peripheral blood of healthy individuals enabled to demonstrate the in vivo existence of a regulatory NK cell subset, which may play an immune regulatory and suppressor role.

## Disclosures

The authors have no financial conflict of interest.

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