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Production of IL-10 by Human Natural Killer Cells Stimulated with IL-2 and/or IL-12

Priti T. Mehrotra,* Raymond P. Donnelly,† Susan Wong,* Hirokazu Kanegane,‡ Amare Geremew,* Howard S. Mostowski,* Keizo Furuke,* Jay P. Siegel,* and Eda T. Bloom‡*

Human NK cell activity can be augmented in vitro by stimulation with IL-2 or IL-12, both of which also induce the production of IFN-γ, TNF-α, and granulocyte-macrophage CSF by NK cells. For the first time, we demonstrate that freshly purified NK cells stimulated with IL-2 proliferated and produced IL-10 in a dose-dependent manner. IL-10 mRNA expression, as detected by semiquantitative reverse transcription-PCR, reached peak levels at 24 h. IL-10 protein was detectable on day 2 and further increased on days 3 and 6 as measured by ELISA. However, IL-12 alone induced neither substantial proliferation nor detectable IL-10 production by fresh NK cells, but it synergized with IL-2 in inducing IL-10 mRNA expression and protein synthesis. IL-10 production by activated NK cells was confirmed by intracytoplasmic cytokine staining by three-color immunofluorescence of CD16+ and/or CD56+ NK cells with anti-IL-10 antibody. IL-10 production by NK cells was further confirmed in the NK-like cell line, YT, which constitutively expressed IL-10 mRNA and protein. IL-12 alone did not induce NK proliferation, but it inhibited IL-2-induced proliferation. Neutralization of endogenously produced IL-10 with anti-IL-10 antibodies did not overcome the inhibition of IL-2-induced proliferation by IL-12. Together, these results demonstrate that IL-2 and IL-12 synergize to induce IL-10 production by human NK cells and that IL-12 inhibits IL-2 induced NK cell proliferation by an IL-10-independent mechanism. The Journal of Immunology, 1998, 160: 2637–2644.

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atural killer cells, initially identified by their ability to lyse selected target cells without prior apparent sensitization (1–3), play an important role in mediating the host immune response against tumors (4, 5), virus-infected cells (6), and bacterial infection (7, 8) and in regulating hemopoiesis (9). IL-2 augments NK activity, leading to increased cytolytic activity (10–12) and production of various cytokines, such as IFN-γ, TNF-α (13), GM-CSF,IL-3 (14), and IL-5 (15). NK cells are also regulated by numerous other cytokines (16). IL-12 up-regulates lytic activity and IFN-γ production by both NK and T cells (17–19) and facilitates the development of Th1-type responses by T cells (20, 21).

The immunoregulatory cytokine, IL-10, inhibits the Ag-dependent proliferation of T cells (22, 23). Recent evidence has suggested that mononuclear cells activated with mitogen rapidly produce IFN-γ, TNF-α, GM-CSF, IL-1, and IL-12 (24, 25). IL-10 is produced later after stimulation by T cells or monocytes/macrophages in the population (24) and down-regulates the production of other cytokines (25, 26). However, production of IL-10 by other cell types is poorly defined. Recently, it has been shown that IL-12 can prime T cells for high IL-10 production (27, 28), which then inhibits IL-12-induced T cell responses (29). In this study we analyzed the effects of IL-2 and IL-12 on IL-10 production by human NK cells. We found that IL-2 stimulates freshly purified human peripheral blood NK cells to produce low levels of IL-10. In contrast to T cells, IL-12 did not stimulate detectable IL-10 production by NK cells, but synergized with IL-2 to enhance IL-10 production.

Materials and Methods

Reagents

Human rIL-2 was provided by Chiron Corp. (Emeryville, CA), human rIL-12 was supplied by Hoffmann-La Roche, Inc. (Nutley, NJ), and rIL-10 was provided by Schering Corp. (Kenilworth, NJ). Mennosin was kindly provided by Ms. Jill Johnson (National Cancer Institute, National Institutes of Health, Bethesda, MD). Anti-CD5 and anti-CD3 Abs were produced and purified as previously described (19, 30). OKM5 (anti-CD36) was purchased from Ortho (Raritan, NJ), and Leu 14 (anti-CD22) was obtained from Becton Dickinson (Mountain View, CA). FITC and phycoerythrin (PE)-conjugated Abs to cell surface markers were obtained from Becton Dickinson. Cytochrome 5PE (Cy5)-conjugated anti-CD56 was provided by Dr. Calman Prussin (National Institute of Allergy and Infectious Diseases, National Institutes of Health). Anti-IL-10-PE, isotype control rat IgG-PE and unconjugated anti-IL-10 Abs were purchased from PharMingen (San Diego, CA).

Preparation of cells

Highly purified human NK cells were isolated from either buffy coats or leukapheresis products obtained from healthy volunteers (Department of Transfusion Medicine, National Institutes of Health). PBMC were separated by density gradient centrifugation over Lymphocyte Separation Medium (Organon Teknika Corp., Durham, NC). PBMC (10⁶ cells) were treated with sterilized carbonyl iron (100 mg; Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. The cell suspension was then subjected to

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a magnet (MCP-6, Dynal A.S., Oslo, Norway) to deplete monocytes, and unbound cells were layered over 47.5% Percoll to recover high density cells. The NK-enriched low density cells were incubated with goat anti-mouse IgG-coated magnetic beads (PerSeptive Diagnostics, Inc., Cambridge, MA) and exposure to a magnet to remove residual T cells, B cells, and monocytes. Unbound cells were treated with a second cycle of goat anti-mouse IgG-coated Dynal magnetic beads at a 1:1 ratio (Dynal) and exposed to a magnetic field. In replicate experiments, the purity of NK cells (CD16⁺ and/or CD56⁺) was approximately 90 to 95%, with <1% monocytes, 1 to 3% T cells (often positive for CD56 Ag), and 1 to 2% B cells as analyzed by FACScan (Becton Dickinson).

**Cell culture**

NK cells were cultured in RPMI 1640 plus 10% FCS (BioWhittaker, Walkersville, MD), 50 μg/ml gentamicin sulfate, 0.1 mM nonessential amino acids, and 2 mM l-glutamine. For activation, purified NK cells were incubated with various concentrations of IL-2 and/or IL-12 for the indicated time periods as described in Results and figure legends. For proliferation assays, cells were cultured at 10⁶ cells/ml in 0.2 ml/well in 96-well microtiter plates with different cytokines and/or Abs to cytokines for 1 to 6 days. Wells were pulsed with 1 μCi of [3H]TdR (DuPont-New England Nuclear, Boston, MA) for 6 to 7 h and harvested onto glass-fiber filters (Cambridge, MA) for 6 to 7 h and harvested onto glass-fiber filters (Wallac, Inc., Gaithersburg, MD). Proliferation was assessed by measuring [3H]TdR incorporation and was expressed as counts per minute. YTN10 and YTN17, two subclones of YT, an NK cell-like cell line obtained from Dr. Junji Yodoi, Kyoto University (Kyoto, Japan) (31), was maintained in RPMI 1640 medium containing 10% FCS.

**Intracytoplasmic staining**

Freshly purified NK cells were activated for 24 h in the presence of IL-2 and IL-12. Monensin was added at a final concentration of 2 μM, 3 h before termination of the culture. Cells were stained for intracellular cytokines and for cell surface Ags using a modified method of Prussin and Metcalf (32). Briefly, cells were washed in FACS buffer (1% FCS and 0.1% saponin, and 0.1% sodium azide) and resuspended in permeabilization buffer (1% FCS, 0.1% saponin, and 0.1% sodium azide) for 2 to 3 h at 4°C, followed by incubation with goat anti-mouse IgG-coated magnetic beads (PerSeptive Diagnostics, Inc., Cambridge, MA) and exposure to a magnet to remove residual T cells, B cells, and monocytes. Unbound cells were treated with a second cycle of goat anti-mouse IgG-coated Dynal magnetic beads at a 1:1 ratio (Dynal) and exposed to a magnetic field. In replicate experiments, the purity of NK cells (CD16⁺ and/or CD56⁺) was approximately 90 to 95%, with <1% monocytes, 1 to 3% T cells (often positive for CD56 Ag), and 1 to 2% B cells as analyzed by FACScan (Becton Dickinson).

**Extraction of mRNA and RT-PCR**

Total cellular RNA was isolated from activated cells by acid guanidinium thiocyanate/phenol chloroform extraction as previously described (33). Levels of cytokine mRNA were assessed by a semiquantitative RT-PCR (34). RNA (2 μg) was reverse transcribed, using reverse transcriptase and oligo(dT) primers (Superscript, Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s protocol. The cDNA was diluted with water to a final volume of 100 μl. Each sample was subjected to an initial amplification using primers specific for glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Table 1). Primers designed for human IL-10 do not amplify murine IL-10. PCR was performed in a reaction mixture containing 5 μl of cDNA, 200 μl of each dNTP, 0.4 μmol/l of each primer, 1.5 mmol/l MgCl₂, 2.5 U of Taq DNA polymerase (Life Technologies), and 1 μCi of [3H]dCTP (3000 Ci/mmol; DuPont-New England Nuclear) in reaction buffer supplied by the manufacturer. cDNAs were amplified in a thermocycler (Robocycler, Stratagene, CA) as follows. Samples were initially denatured at 95°C for 3 min, then at 95°C for 1 min, at 61°C for 1 min, and at 72°C for 1 min (20 cycles for G3PDH; 30 cycles for IL-10), with a final extension at 72°C for 7 min. Samples were analyzed by electrophoresis through a 6% acrylamide (Long Ranger, AT Biochem, Malvern, PA) Tris-borate-EDTA gel, followed by autoradiography and quantitation by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

**Results**

Combined effects of IL-2 and IL-12 on NK cell proliferation

The activation of NK cells by IL-2 is accompanied by an increase in proliferation and cytolytic function. IL-12 has previously been shown to induce the proliferation of preactivated PBMC (17, 18, 35) and preactivated NK cells (35–37). However, IL-12 alone has been observed to induce little or no proliferation of fresh NK cells (38, 39) and certainly less proliferation than IL-12–40. We examined the ability of IL-12 together with IL-2 to induce the proliferation of fresh NK cells. NK cells were activated in the presence of different concentrations of IL-2 and IL-12 for 1, 2, 3, or 6 days (Fig. 1). IL-2 alone induced NK cell proliferation in a time- and dose-dependent fashion, and the combination of IL-12 (1 or 10 U/ml) with a suboptimal concentration of IL-2 (0.6 ng/ml) consistently produced an additive or greater than additive (depending on donor) effect. In contrast, when IL-12 was tested in combination with high concentrations of IL-2 (6.0 ng/ml), proliferation was markedly inhibited after 2, 3, or 6 days of culture. This inhibition was IL-12 dose and time dependent. A 30 to 35% decrease in thymidine incorporation was seen on day 2, a 50% decrease on day 3, and approximately a 70% inhibition on day 6 (to ensure sufficient nutrient levels, 6-day cultures had been replenished with fresh medium and cytokines on day 3; Fig. 1).

Analysis of IL-10 mRNA expression by RT-PCR

It has been shown that human T cells activated with mitogens such as Con A proliferate and produce a high level of IL-10 (24), a cytokine with known inhibitory effects on T cells (41, 42). Recently, IL-12 has been shown to induce IL-10 production by T cells (27, 28, 43). Activated NK cells are known to produce a number of cytokines, including IFN-γ, TNF-α, and GM-CSF, in vitro (2, 13), but IL-10 production by NK cells has not been reported. IL-10 has previously been shown either to augment IL-2-induced NK activity or proliferation (44) or to have no effect on IL-2-induced NK activity or proliferation (45, 46). Because of conflicting effects of IL-10 on NK proliferation, we hypothesized that the IL-12-induced inhibitory effect on NK proliferation may be due to endogenous IL-10 production. We first determined whether IL-10 mRNA was expressed in freshly purified NK cells in response to IL-2 and IL-12. Activation of NK cells with IL-2 (6 ng/ml) for 16 to 96 h resulted in the induction of IL-10 gene expression. IL-10 mRNA levels were detectable as early as 16 h, reached peak levels at 24 h (10-fold), and later declined (Fig. 2A). Very low levels of IL-10 mRNA were consistently detected in control unstimulated cells. Cells that were stimulated with different concentrations of IL-2 (0–60 ng/ml) for 24 h showed an increase

Table I. PCR primers and product size

<table>
<thead>
<tr>
<th>RNA Detected</th>
<th>GenBank Accession No</th>
<th>Primer Pair (upper strand; lower strand)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3PDH</td>
<td>M32599</td>
<td>GCCACCCAGAGACCTGTTGATGCCC</td>
<td>446</td>
</tr>
<tr>
<td>hIL-10</td>
<td>U16720</td>
<td>CTCGGAGATCTCCSGAGGCTCCTTC</td>
<td>312</td>
</tr>
</tbody>
</table>

**Cytokine ELISA**

Human NK cells were stimulated with cytokines as indicated in the figure legends. The levels of IL-10 protein in NK cell culture supernatants were measured using an ELISA kit obtained from BioSource International (Camarillo, CA). A human IFN-γ ELISA kit was purchased from Inctar Inc. (Stillwater, MN). Assays were performed according to the manufacturer’s instructions. The lower limit of IL-10 detection was 5 pg/ml. All samples were assayed in duplicate. Results are expressed as the mean levels of IL-10 in picograms per milliliter.
in IL-10 mRNA (Fig. 2B). After normalizing IL-10 mRNA expression levels to levels of mRNA encoding for G3PDH, a housekeeping gene, the ratio demonstrated that IL-10 mRNA expression was induced after stimulation with IL-2 in a dose-dependent manner (Fig. 2C).

We next evaluated the effects of IL-12 alone or in combination with IL-2 on the induction of IL-10 mRNA. Although IL-12 alone (1–100 U/ml) did not increase IL-10 mRNA beyond the baseline (data not shown), it synergized with IL-2 in augmenting the expression of IL-10 mRNA (2.5-fold; Fig. 3). IL-10 mRNA expression levels (quantified by phosphoimage analysis) induced by IL-2 and IL-12 together were compared with the sum of the IL-10 mRNA (quantified) levels produced by each cytokine alone. The ratio was 2.11 ± 0.36 for three experiments (p, 0.02, by one-sample t test on logarithms of the ratio). Thus, a 2.2-fold increase in IL-10 mRNA was observed when IL-2 and IL-12 were used together over what would be expected if the effect were merely additive.

To confirm that IL-10 was produced by NK cells, we investigated whether IL-10 was expressed in an NK-like cell line, YT. A high level of constitutive expression of human IL-10 message was observed in two subclones of YT (YTN10 and YTN17; Fig. 3 and data not shown). Consistent with detection of IL-10 mRNA, YTN10 or YTN17 cells constitutively produced IL-10 as measured by ELISA (1286 or 634 pg/ml). Neither expression nor production of IL-10 by YT cells was altered by IL-2 treatment.

**Synergistic effects of IL-2 with IL-12 on IL-10 secretion**

To determine whether secretion of IL-10 was associated with IL-10 mRNA expression, highly purified fresh NK cells were stimulated with different concentrations of IL-2 and IL-12. Two days later, supernatants were collected and assayed for IL-10 by ELISA. NK cells stimulated with IL-12 produced no detectable IL-10, but cells stimulated with IL-2 alone (0.6 or 6.0 ng/ml) produced low levels of IL-10 protein (10 and 30 pg/ml, respectively, after 2 days of culture; Fig. 4A). However, when NK cells were activated with IL-2 plus IL-12, IL-10 production was synergistically enhanced with as little as 1 U/ml of IL-12 and 0.6 ng/ml of IL-2. The combined effect of IL-2 and IL-12 increased with the duration of culture; after 3 days the synergistic effect on IL-10 secretion was even more pronounced, especially with higher doses of IL-2 (6 ng/ml; Fig. 4B). Figure 4C shows a direct comparison between NK cells from a single donor stimulated with IL-2 and/or IL-12 for 3 and 6 days. As cells were refed on day 3, IL-10 levels on days 3 and 6 each represent cumulative IL-10 accumulated over 3-day periods (0–3 and 3–6 days, respectively). Under these conditions, the IL-10 production in response to IL-2 plus IL-12 increased dramatically with time (150 pg/ml for days 0–3 and 400 pg/ml for days
show that most CD16+ and CD56+ NK cells contain cytoplasmic IL-10. The specificity of the IL-10 staining was demonstrated in two ways. First, addition of a molar excess of rIL-10 to the anti-IL-10-PE Ab before staining completely blocked the intracellular IL-10 staining. Second, preincubation of cells with unlabeled Ab blocked intracellular IL-10 staining. Contaminating T cells, monocytes, and B cells, identified by CD3, CD14, and CD22 cell surface markers, respectively, exhibited low or no staining with anti-IL-10-PE (data not shown). Intracellular IL-10 staining was further confirmed in YT cells using the same protocol. All YT cells stained positive for both CD56 and IL-10 (Fig. 5).

**Role of endogenous IL-10 in the inhibition of NK cell proliferation by IL-12**

Previously, it has been shown that IL-10 can directly inhibit T cell proliferation by a monocyte-independent pathway (47). Because its production is synergistically enhanced by IL-2 and IL-12 treatment of NK cells, we investigated whether endogenously produced IL-10 is responsible for the inhibition of proliferation of NK cells by IL-12. NK cells were activated with IL-2 and/or IL-12 for 3 to 6 days. Neutralizing anti-IL-10 Abs (clone JES3-9D7) from two different commercial sources or normal rat IgG were added at the beginning of culture. The ability of the anti-human IL-10 mAbs at 2 μg/ml to neutralize the IL-10 activity was previously confirmed by their ability to neutralize the endogenously produced IL-10 in cultures of LPS-stimulated human monocytes (48). Moreover, these anti-IL-10 Abs prevented the measurement of IL-10 protein by ELISA in the supernatants from NK cells treated with IL-2 and IL-12 (data not shown). Nevertheless, the addition of neutralizing Abs to the culture did not alter the inhibition of IL-2-induced proliferation by IL-12 (Fig. 6). These data suggest that endogenous IL-10 is probably not responsible for IL-12-induced inhibition of IL-2-enhanced proliferation.

**Discussion**

In this study we demonstrate that purified preparations of human peripheral blood NK cells express IL-10 mRNA and secrete IL-10 protein upon IL-2 stimulation. Although IL-12 alone had no effect on IL-10 synthesis at the concentrations tested, it synergized with IL-2 for production of IL-10 mRNA and protein. IL-10 was first described as a T cell product (24, 41), but subsequent studies have shown that it is produced by various other cells, including B cells (49), mast cells (50), and monocytes (51). Previous reports have shown that NK cells isolated from 10-day coculture with the lymphoblastoid cell line RPMI-8866 and PBMC expressed IFN-γ, TNF-α, GM-CSF (13), and CSF-1 (14) when stimulated with IL-2.
and CD16 ligand. Similarly, IL-2-activated NK cells produced TNF-α when stimulated with anti-CD94 Ab (52). Recently, Warren et al. (15) have shown that either fresh NK cells isolated from peripheral blood or NK cells obtained from 17-day cultures, when cocultured with gamma-irradiated MM-170 or JY stimulator cells in the presence of IL-2, produced IL-5. The results from the present study extend the list of cytokines that NK cells produce to include IL-10.

Earlier studies have been unable to demonstrate IL-10 production by NK cells (44, 45). Spagnoli et al. (45) studied IL-10 expression in PBMC stimulated with IL-2. Carson et al. (44) characterized IL-10R expression and its potential functional role on human NK cells. They reported that neither resting nor activated NK cells produced human IL-10, although data were not shown. In contrast, we observed that IL-2-stimulated NK cells expressed IL-10 mRNA and protein in a dose-dependent manner. However, the sensitivity of ELISA used by Carson et al. (44) was 40 pg/ml, eightfold less sensitive than that used in the current study, and they did not examine mRNA expression. It is thus possible that the level of IL-10 secreted may not have been detectable with the low sensitivity ELISA.

Our time-course studies showed that expression of IL-10 mRNA expression occurs relatively late, peaking at 24 h, with a subsequent decline. This is consistent with findings in T cells (24, 27) and monocytes (53), in which IL-10 is produced late and inhibits the synthesis of other cytokines produced earlier (42, 53, 54). The production of IL-10 protein increased in a time-dependent manner, was detectable as early as 48 h following IL-2 stimulation, increased at 72 h, and further increased on days 3 to 6 after NK cells were replenished with fresh medium and cytokines. Similarly, Warren et al. (15) have shown that restimulation of NK cells with IL-2 resulted in much higher levels of IL-5 compared with those after the initial stimulation.

IL-10 production by activated NK cells was further confirmed by intracytoplasmic staining using flow cytometry with fluorescence-conjugated Abs to IL-10 and NK cell surface markers (CD16 and CD56). No fundamental differences in the proportion of IL-10-positive cells among CD16+ (89%) or CD56+ (86%) NK cells was observed. Specific blocking with either a molar excess of rIL-10 or the unconjugated anti-IL-10 Ab with conjugated anti-IL-10 Ab before staining supported the conclusion that IL-10 is present in NK cells. Finally, IL-10 production by NK cells was confirmed in the NK-like cell line, YT (N10 and N17 clones), which constitutively expresses IL-10 mRNA and protein under standard culture conditions.

The expression and production of IL-10 in IL-2-activated NK cells are synergistically enhanced by IL-12. These results suggest that regulation of IL-10 production in NK cells may be similar to that reported in T cells (43). In addition to augmenting IL-10 production, the combination of IL-2 and IL-12 augmented IFN-γ production in NK cells, but IFN-γ production occurs much earlier than that of IL-10. Recently, Windhagen et al. (55) have reported that stimulation of an Ag-specific T cell clone with anti-CD3 Ab

![Graph A: IL-10 production by IL-2-stimulated NK cells.](http://www.jimmunol.org/)

![Graph B: IL-10 production by IL-2-stimulated NK cells.](http://www.jimmunol.org/)

![Graph C: IL-10 production by IL-2-stimulated NK cells.](http://www.jimmunol.org/)

![Graph D: IL-10 production by IL-2-stimulated NK cells.](http://www.jimmunol.org/)

**FIGURE 4.** IL-12 synergistically enhances IL-10 production by IL-2-stimulated NK cells. Purified cells were stimulated with IL-2 (0–6 ng/ml) and/or IL-12 (0–10 U/ml) for 2 days (A) and 3 days (B); IL-10 and IFN-γ productions were compared on days 3 and 6 (C and D, respectively). Cells were stimulated with IL-2 (6 ng/ml) and/or IL-12 (10 U/ml). Six-day cultures had medium removed on day 3 and were replenished with fresh medium and cytokines. Supernatants were collected, and IL-10 was quantified by ELISA. Results are expressed in picograms per milliliter, and values represent one of three experiments. The SEM fell within a range of 5 to 10% of the mean.
induced the secretion of both IL-10 and IFN-\(\gamma\), and the effect was enhanced by the addition of IL-12. The present observations extend the previous reports of synergistic interactions of IL-2 with IL-12 in inducing IFN-\(\gamma\) (19, 56–58) or IL-10 (43) production by PBL and T cells.

Of the known cytokines, IL-2 (59, 60), IL-7 (61), and IL-15 to some extent (62) can induce significant proliferation of resting NK cells. Our results support and extend the finding that IL-12 in combination with low dose IL-2 can invariably enhance the proliferation of NK cells (38) in an additive or synergistic manner, but with higher IL-2 concentrations, it substantially inhibited NK cell proliferation. This is consistent with previous findings in T cells (63), an effect that has been reported to be mediated in part by TNF-\(\alpha\) (35, 64). However, the reversal of the effect by blocking with anti-TNF-\(\alpha\) Ab was never complete. Therefore, Perussia et al. (35) speculated that IL-2 together with IL-12 may induce the production of other soluble factors that may be involved in the inhibition of proliferation. Recently, Jeannin et al. (43) reported that IL-12 directly, independently of APC, can induce IL-10 production by human T cells. IL-10 has been shown to suppress both cytokine production (41, 65, 66) and Ag-specific proliferation (51) of Th1-type clones in an accessory cell-dependent (47) or independent (67) manner. Previously, conflicting reports have appeared showing that IL-10 augments IL-2-induced NK proliferation (44) or has no effect on it (45, 46). Therefore, we investigated whether the inhibitory effect of IL-12 on IL-2-induced NK proliferation was in part due to endogenously produced IL-10. However, neutralizing Abs against IL-10 failed to block IL-12-induced growth inhibition, suggesting that IL-10 production, augmented in response to IL-12, does not contribute to the inhibition by IL-12 of IL-2-induced proliferation of NK cells.

Previous studies have demonstrated that certain viral infections activate NK cells to produce IFN-\(\gamma\) (68, 69), which has been shown to be a consequence of virus-induced IL-12 production (70), resulting in increased antiviral activity of NK cells. However, inappropriately up-regulated levels of IL-12 may have detrimental effects on the host (71–73). It can synergize with endogenously produced IL-2 and can down-modulate CD8\(^+\) T cells responses (72, 73). However, overexpression of IL-12 is controlled through a negative feedback mechanism involving IL-10 production (54).

**FIGURE 5.** Intracellular staining for IL-10. NK cells were stimulated with 6 ng/ml of IL-2 plus 10 U/ml of IL-12 for 24 h in the presence of 2 \(\mu\)M monensin for the last 3 h, surface stained using CD16-FITC and CD56-Cy5 Abs, fixed, and preincubated with no addition (A), rat IgG control (B), no addition (C), or unlabeled anti-IL-10 Ab at 100 \(\mu\)g/ml (D). Samples B and D were then stained with anti-IL-10-PE Ab, while samples A were stained with control IgG-PE, and samples C were stained with anti-IL-10-PE Ab that had been preincubated with a molar excess (1–2 \(\mu\)g) of human rIL-10. YTN17 cells were stained using protocol described above. The experiment was repeated at least four times. The results were analyzed on a Macintosh Quadra 650 (Apple Computers, Sunnyvale, CA), using B-D software program CellQuest (Becton Dickinson, Mountain View, CA). Live/dead separation of cells was based on side scatter on the \(x\)-axis and forward scatter on the \(y\)-axis. A gate was drawn to compass the live cells.
Although the amounts of IL-10 produced by NK cells are small compared with those produced by T cells or monocytes, it is possible that in certain viral infections in vivo, IL-10 produced by NK cells in response to IL-2 and IL-12 may have a feedback inhibitory effect on cytokines produced within the microenvironment of NK cells. In summary, our studies suggest that human NK cells can produce IL-10 and IFN-γ upon IL-2 stimulation, and the production can be enhanced synergistically by costimulation with IL-12.

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