Interleukin 10 (IL-10) Inhibits Human Lymphocyte Interferon γ-Production by Suppressing Natural Killer Cell Stimulatory Factor/IL-12 Synthesis in Accessory Cells

By Annalisa D'Andrea, Miguel Aste-Amezaga, Nicholas M. Valiante, Xiaojing Ma, Marek Kubin, and Giorgio Trinchieri

From The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Summary

Natural killer cell stimulatory factor or interleukin 12 (NKSF/IL-12) is a heterodimeric cytokine produced by monocytes/macrophages, B cells, and possibly other accessory cell types primarily in response to bacteria or bacterial products. NKSF/IL-12 mediates pleiomorphic biological activity on T and NK cells and, alone or in synergy with other inducers, is a powerful stimulator of interferon γ (IFN-γ) production. IL-10 is a potent inhibitor of monocyte-macrophage activation, that inhibits production of tumor necrosis factor α (TNF-α), IL-1 and also IFN-γ from lymphocytes acting at the level of accessory cells. Because TNF-α and IL-1 are not efficient inducers of IFN-γ, the mechanism by which IL-10 inhibits IFN-γ production is not clear. In this paper, we show that IL-10 is a potent inhibitor of NKSF/IL-12 production from human peripheral blood mononuclear cells activated with *Staphylococcus aureus* or lipopolysaccharide (LPS). Both the production of the free NKSF/IL-12 p40 chain and the biologically active p70 heterodimer are blocked by IL-10. NKSF/IL-12 p40 chain mRNA accumulation is strongly induced by *S. aureus* or LPS and downregulated by IL-10, whereas the p35 mRNA is constitutively expressed and only minimally regulated by *S. aureus*, LPS, or IL-10. Although IL-10 is able to block the production of NKSF/IL-12, a powerful inducer of IFN-γ both in vitro and in vivo, the mechanism of inhibition of IFN-γ by IL-10 cannot be explained only on the basis of inhibition of NKSF/IL-12 because IL-10 can partially inhibit IFN-γ production induced by NKSF/IL-12, and also, the IFN-γ production in response to various stimuli in the presence of neutralizing antibodies to NKSF/IL-12. Our findings that antibodies against NKSF/IL-12, TNF-α, or IL-1β can significantly inhibit IFN-γ production in response to various stimuli and that NKSF/IL-12 and IL-1β can overcome the IL-10–mediated inhibition of IFN-γ, suggest that IL-10 inhibition of IFN-γ production is primarily due to its blocking production from accessory cells of the IFN-γ-inducer NKSF/IL-12, as well as the costimulating molecule IL-1β.

Natural killer cell stimulatory factor or interleukin 12 (NKSF/IL-12)[1] is a heterodimeric cytokine produced by monocyte/macrophages, B cells, and possibly other accessory cell types primarily in response to bacteria or bacterial products (1–4). NKSF/IL-12 is active on T and NK cells, inducing cytokine production, proliferation, and enhanced cytotoxic activity (5–10). NKSF/IL-12 is particularly efficient in inducing IFN-γ production by T and NK cells, and in this effect, it acts synergistically with other IFN-γ-inducers such as IL-2 and phorbol diesters in both NK and T cells; antigens, alloantigens, mitogens, and anti-CD3 Abs in T cells; and target cells, immune complexes, and anti-CD16 Abs in NK cells (5, 11). Neutralizing anti-NKSF/IL-12 Abs significantly and often completely inhibit IFN-γ production from human PBMC induced by several stimuli (e.g., *Staphylococcus aureus*, anti-CD3 Abs, PHA, and IL-2), suggesting that NKSF/IL-12, either induced or constitutively produced, is required for optimal IFN-γ production by resting human lymphocytes (4). However, production of IFN-γ by activated NK or T cells, in the absence of NKSF/IL-12–producing cells, is not inhibited by anti-NKSF/IL-12 Abs (4). IL-12 also induces production of other cytokines by NK and T cells, including TNF-α, although in this case, a synergistic effect with IL-2 is not observed (6).

By a mechanism that is at least in part independent from IFN-γ production, IL-12 has been shown to induce a Th

[1] *Abbreviations used in this paper:* NKSF, natural killer cell stimulatory factor; TPA, tetradecanoylphorbol acetate.

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type 1 (Th-1) response and to inhibit the differentiation of IL-4–producing Th-2 cells, both in the response of human PBLs to allergens or bacterial antigens (12), and in the response of anti-OVA TCR transgenic mice to OVA (13). Importantly, in both systems (12, 13), anti-IL-12 Abs prevented Th-1 differentiation, suggesting that IL-12 is a necessary factor for Th-1 cell differentiation.

IL-10 is a product of various cell types including monocytes and B and T cells, that, unlike IL-12, is associated with differentiation of Th-2 cells (14, 15). IL-10 inhibits lymphocyte cytokine production, particularly IFN-γ, by T and NK cells, and also inhibits proliferation of T cells, acting primarily at the level of monocyte/macrophage accessory cells (16–18). IL-10 is a potent inhibitor of monocyte/macrophage functions, including oxidative burst, nitric oxide production, cytotoxicity, and production of cytokines, such as TNF-α and IL-1 (19–24). TNF-α has been shown to be an important cofactor for the induction of IFN-γ production by murine T and NK cells, and the ability of IL-10 to prevent TNF production by monocyte/macrophages has been proposed to be one of the mechanisms by which IL-10 inhibits IFN-γ production (25). However, TNF-α by itself is unable to induce IFN-γ production, and cells other than monocytes and macrophages, including T and NK cells, also produce TNF-α, making it unlikely that inhibition of TNF-α production by IL-10 may fully account for its effect on IFN-γ production (25).

In this paper, we demonstrate that IL-10 efficiently inhibits, in addition to TNF-α and IL-1β, IL-12 production by human PBMC. Abs against IL-12, TNF-α, and IL-1β and, more effectively, their combinations, inhibit IFN-γ production from human PBMC in response to several different inducers. IL-12 and IL-1β, but not TNF-α, are able to partially restore IFN-γ production in the presence of IL-10. These results suggest that IL-10 inhibits IFN-γ production by preventing production by accessory cells of the IFN-γ–inducing cytokine NKSF/IL-12 and, in part, the costimulatory cytokines IL-1β and possibly TNF-α.

Materials and Methods

Cytokines and Reagents. CHO cell–activated rNKSF/IL-12 (5×10^6 U/mg) was a gift from Dr. S. Wolf (Genetics Institute, Boston, MA); rIL-1β (3.8×10^7 U/ml) was provided by the Division of Cancer Treatment (National Cancer Institute, Bethesda, MD); rIL-2 (10^7 U/mg) by Dr. T. Taguchi (Osaka University, Osaka, Japan); CHO cell–derived hIL-10 (1.5×10^7 U/mg) by Dr. K. Moore (DNAX, Palo Alto, CA), and TNF-α (5×10^7 U/mg) by Dr. H.M. Shepard (Genentech, South San Francisco, CA). The following reagents were purchased from commercial sources: PHA-M (Wellcome Diagnostics, Dartford, England); fixed *S. aureus* Cowan strain 1 (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA); LPS (from *Escherichia coli*, serotype 0127:B8; Sigma Chemical Co., St. Louis, MO); and 12-0-tetradecanoylphorbol 13-acetate (TPA; Sigma Chemical Co.).

Antibodies. mAbs B154.2 (anti-TNF-α, IgG1) and C8.6.2 (anti-NKSF/IL-12 p40, IgG1) were produced and characterized in our laboratory as previously described (4, 26). Neutralizing anti-IL-10 mAb 19F1 was kindly provided by Dr. K. Moore and anti-IL-1β F18.609 and F18.206 were kindly provided by Dr. M.A. Cousin (Roussel-Uclaf, Romainville, France) (27).

Cytokine Assays. RIAs for human IFN-γ and human TNF-α were performed as previously described (26, 28) using mAbs B133.1/B133.5 and B154.9/B154.7, respectively. NKSF/IL-12 p40 was measured in cell-free supernatants by RIA as described (4) using the mAb pairs C11.79/C8.6. RIA for IL-1β was performed using F18.609/F18.206 mAbs (27).

Quantitation of Biologically Active NKSF/IL-12 Heterodimer by an Ab Capture Bioassay. The biological activity of NKSF/IL-12 was determined using a modification of the capture bioassay previously described (29). Briefly, the anti-NKSF/IL-12 p40 Ab C11.5 (4) was absorbed (15 μg/ml, 100 μl/well, in 0.1 M carbonate buffer, pH 9.5) for 24 h or longer at 4°C to bacteriological 96-well plates (Flow Laboratories, McLean, VA). The plates were washed three times with PBS and PBS/FCS 5% was added to plates (200 μl, 1 h, 37°C). After three washes, dilutions of rNKSF/IL-12 standard or supernatant fluids to be tested were added (100 μl/well, 3 h, room temperature), the plates were again washed (five times) and PBMC (5×10^5/well) together with 100 U/ml or rIL-2 were added to the plates. After an 18-h incubation (37°C, 5% CO_2), the supernatants were collected and tested for IFN-γ production by RIA. The assay detects $\geq$ 1 pg/ml of biologically active NKSF/IL-12, is specific for NKSF/IL-12, and at least in the concentration range used in the present experiments, is not affected by the presence of an excess of free p40 chains.

Preparation of Human PBMC. Peripheral blood obtained from healthy donors was anticoagulated with heparin. PBMC were separated on Ficoll-Hypaque (Lymphoprep; Nyegard and Co., Oslo, Norway) density gradient, and PBL were obtained after adherence of PBMC to plastic flasks (1 h, 37°C). PHA blasts (>98% activated T cells) were obtained after 5-d culture of PBL in the presence of 1% PHA-M (5, 11). Pure preparations (>98%) of activated NK cells were purified from 8-d coculture of PBL with irradiated RPMI-8866 B lymphoblastoid cell line, as described (30, 31). Cultures were performed in 250 μl RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Irvine Scientific, Santa Ana, CA) in 96-well round-bottomed plates (Flow Laboratories) with 10^6 cells/well. Reagents to be tested were added at the onset of the culture and supernatants were harvested after 18 h, and p40, TNF-α, IL-1β, or IFN-γ release were determined using specific RIAs. In some experiments, neutralizing anti-NKSF/IL-12 p40 mAb (C8.6), anti-TNF-α mAb (B154.2), anti-IL-1-β (F18.609), at the concentration of 10 μg/ml each, were added to PBL during the 18-h culture assays before harvesting the supernatants for the specific RIAs.

Northern Blot Hybridization. Northern blots were performed as already described (32). Briefly, total RNA was extracted from induced and uninduced PBMC by the guanidine isothiocyanate method. Equal amounts of RNA (15 μg/lane) were fractionated in a 1% agarose-formaldehyde gel. The genes were detected by sequential hybridizations of nylon membranes (Schleicher & Schuell, Keene, NH) to ^{32}P-cDNA probes for NKSF/IL-12 p40, p35, TNF-α, IL-1β, and β-actin. The filters were then exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) between double intensifying screens (DuPont Co., Wilmington, DE).

RNAse Protection Assay. The entire coding regions of p40- and p35-NKSF/IL-12 were amplified by PCR and subcloned into the pCR™1000 pCR™II vectors, respectively, according to the suggestions of the manufacturer (Invitrogen Co., San Diego, CA). The vectors were linearized with appropriate restriction enzymes and transcribed using ^{32}P-UTP (NEN; DuPont Co.) and the riboprobe kit (Promega Biotec, Madison, WI) into complementary RNA (antisense) riboprobes containing regions of 266 and

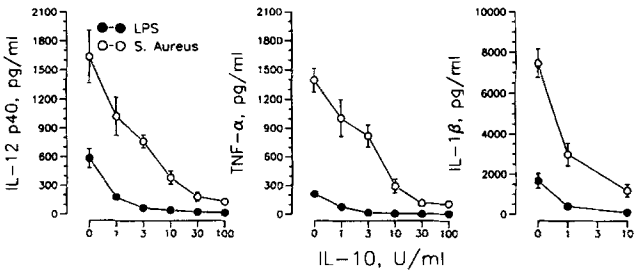

Figure 1. Inhibition of cytokine production by human rIL-10. PBMC were preincubated for 4 h in the presence of the indicated concentrations of human rIL-10 and then stimulated in the presence of rIL-10 with: (●—●) LPS (1 μg/ml) or (○—○) *S. aureus* (1:10,000 wt/vol). After an 18-h incubation at 37°C, supernatant fluids were collected and tested for the presence of IL-12 p40, TNF-α, and IL-1β by RIA. All the experiments were performed in endotoxin-free conditions. Results are mean ± SE, number of donors, $n = 4$.

240 bp complementary to sequences in the p40 and p35 NKSF/IL-12, respectively. RNA samples (5–20 μg in 20 μl) were hybridized in solution with an excess of riboprobes (8–10 × 10^4 cpm, sp act 10^9 cpm/μg, 90°C for 5 min, 42°C for > 10 h). 200 μl of RNase solution was added for 30 min at 37°C and the instructions of the Ribonuclease Protection Assay RPA II Kit (Ambion Inc., Austin, TX) were followed. The protected fragments were fractionated on 5% polyacrylamide/urea sequencing gels and detected by autoradiography.

Results

Inhibition of IL-12 Production by IL-10. In endotoxin-free conditions, insignificant levels of NKSF/IL-12 p40 are spontaneously released by human PBMC in vitro (data not shown), whereas LPS or *S. aureus* are powerful inducers of NKSF/IL-12 production (Fig. 1). Human rIL-10 induced a dose-dependent inhibition of NKSF/IL-12 production in response to either inducer which was almost complete at IL-10 concentrations of 10–30 U/ml (Fig. 1). Murine rIL-10, inactive on human cells, did not inhibit NKSF/IL-12 production at concentrations up to 100 U/ml (data not shown). When PBMC were separated into adherent (>95% monocytes) and monocyte-depleted, nonadherent (<0.5% monocytes) populations, IL-10 inhibited IL-12 production in both preparations (data not shown). In the nonadherent cell preparation, IL-12 is produced in part by B cells, although the major nonadherent producer cells are HLA-DR$^+$, non-B, nonmonocytic cells (4). At concentrations inhibitory for IL-12 production, IL-10 also inhibited LPS- and *S. aureus*-induced TNF-α and IL-1β production, confirming and extending the results of other investigators (21).

The RIA used to measure NKSF/IL-12 in Fig. 1 detects the p40 chain either in free form or associated with the p35 chain in the biologically active p70 heterodimer (4). Because PBMC always produce an excess of the free p40 chain over the p70 heterodimer (4), we tested whether IL-10 also inhibits the production of the p70 heterodimer using an antibody-capture biological assay based on the ability of IL-12 captured by anti-IL-12 p40 coated on plastic to induce IFN-γ production from PBL in the presence of IL-2 (29).

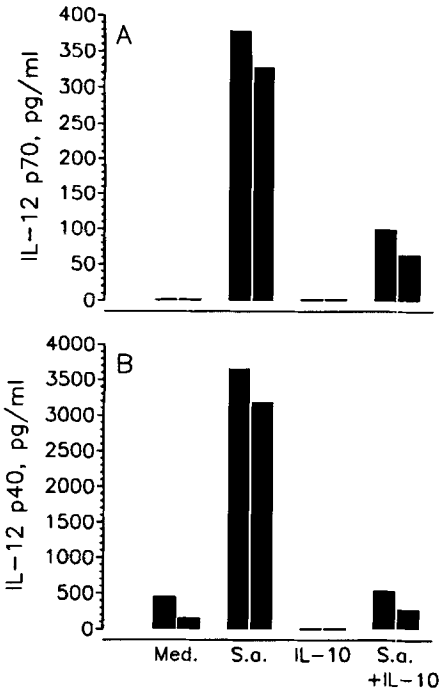

Figure 2. Inhibition of NKSF/IL-12 production by rIL-10. Human PBMC from two donors were preincubated for 4 h in the presence of 10 U/ml of rIL-10 and then stimulated for 18 h with *S. aureus* (1:10,000 wt/vol). Supernatant fluids were collected and tested for the presence of IL-12 p70 biologically active heterodimer, Ab capture assay (*A*), and IL-12 p40 double determinant RIA (*B*) as described in Materials and Methods. Each of the paired bars represents the results obtained from an individual donor.

As shown in Fig. 2, IL-10 was found to inhibit production of both the free p40 chain and the p70 heterodimer.

We analyzed by Northern blotting the ability of IL-10 to inhibit *S. aureus*- (Fig. 3 *A*) and LPS-induced (data not shown) accumulation of NKSF/IL-12 p40 and TNF-α mRNA. *S. aureus* induced maximal accumulation of p40 mRNA at 3–5 h, whereas accumulation of TNF-α mRNA was maximal at 1–3 h. At all time points analyzed, 50 U/ml IL-10 almost completely blocked *S. aureus* and LPS-induced accumulation of both p40 and TNF-α mRNA. Because p35 mRNA is at very low abundance, RNase protection assay was used for its detection (Fig. 3 *B*). Unlike p40 mRNA, that was strongly induced by SAC stimulation and inhibited by IL-10, p35 mRNA accumulation was only minimally stimulated by SAC at 4–6 h and also minimally downregulated by IL-10. These results confirm previous data (4) that p35 expression is constitutive in PBMC and only minimally regulated.

Role of NKSF/IL-12, TNF-α, and IL-1β Production by Accessory Cells in the Induction of IFN-γ. As shown in Fig. 4, IL-10 efficiently suppressed IFN-γ production by resting PBMC in response to IL-2, PHA, *S. aureus*, and, to a lesser extent, NKSF/IL-12. Abs to NKSF/IL-12 also significantly inhibited IFN-γ production in the same experimental conditions. However, the ability of IL-10 to still inhibit IFN-γ production in the presence of anti-NKSF/IL-12 Abs (Fig. 4),

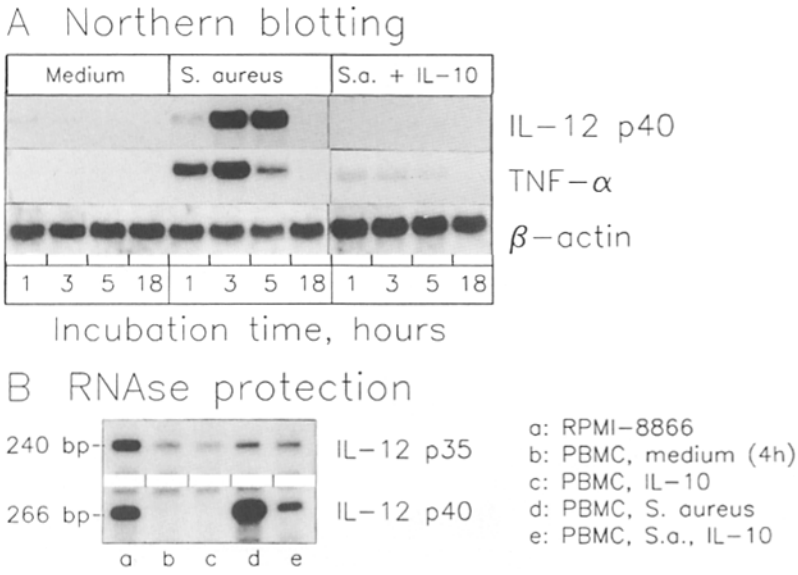

Figure 3. Effect of rIL-10 on the *S. aureus* induced accumulation of IL-12 p40, p35, and TNF-α mRNA in human PBMC. Total RNA was extracted from PBMC incubated for the indicated times in tissue culture medium or in the presence of *S. aureus* (1:10,000 wt/vol) with or without 50 U/ml human rIL-10. (*A*) Cells stimulated in the presence of IL-10 were pretreated for 4 h with the same concentrations of IL-10. The RNA (15 μg/lane) was then hybridized in a Northern blotting with ^{32}P-labeled IL-12 p40, TNF-α, or β-actin cDNA. Representative experiment out of four with similar results. (*B*) Total RNA from the RPMI-8866 cell line, constitutively expressing high levels of NKSF/IL-12 (1) and from PBMC incubated for 4 h in medium, IL-10 50 U/ml, *S. aureus* (1:10,000 wt/vol), or *S. aureus* and IL-10, was hybridized with ^{32}P-riboprobes transcribed from NKSF/IL-12 p40 and p35 cDNA. 20 μg RNA were used for hybridization with the p35 probe and 5 μg with the p40 probe. Equivalency of the different RNA preparations was determined by optical density and by hybridization with a β-actin probe in a Northern blotting (data not shown). mRNA accumulation was quantitated by RNase protection and autoradiograms showing the protected RNA fragment (240 bp for p35 and 266 bp for p40) are presented. Representative experiment out of three with similar results. All the experiments shown in this figure were performed in endotoxin-free conditions.

suggested that IL-10–mediated inhibition of IFN-γ production was not solely due to suppression of NKSF/IL-12 production. We therefore investigated the role in IFN-γ production of two other cytokines inhibited by IL-10, IL-1β, and TNF-α. Neutralizing Abs against NKSF/IL-12, TNF-α, and IL-1β at least partially inhibited IFN-γ production individually (Fig. 5). IL-2, IL-12, and TPA-induced IFN-γ production also on purified preparations of cultured NK and T cells, whereas *S. aureus*, which presumably acts indirectly by stimulating accessory cells to produce NKSF/IL-12 (4), was unable to induce IFN-γ by the purified cell preparations. The IFN-γ production by purified activated NK and T cells in response to IL-2, IL-12, and TPA was not inhibited by IL-10, unlike the significant inhibition observed in the same conditions with resting PBMC (Fig. 6). The inability of IL-10 to suppress IFN-γ in the purified activated cell preparations was likely due to the absence of accessory cells which are required for IFN-γ production by resting but not by activated lymphocytes (5). Addition of cultured monocyte–derived macrophages to the purified NK and T cells in most experiments reconstituted the ability of the cells to produce IFN-γ in response to *S. aureus* and enhanced the production in response to IL-2 or IL-12. The IFN-γ production in response to *S. aureus* or the enhanced production of IFN-γ in response to IL-2 or IL-12 in the presence of macrophages was inhibited by IL-10 (data not shown). Purified NK and T cell preparations were unable to produce NKSF/IL-12 (4) and anti-NKSF/IL-12 Abs failed to inhibit IL-2 or TPA-induced IFN-γ production in purified NK cells (Fig. 7) and T cells (data not shown), unlike what was observed with PBMC (Fig. 4). The enhanced IFN-γ production in response to *S. aureus* obtained by adding monocyte-derived macrophages to NK or T cells, was, however, inhibited by anti-NSKF/IL-12 Abs. Anti-TNF-α Abs, unlike anti-NKSF/IL-12, significantly inhibited IFN-γ production in response to IL-2, NKSF/IL-12, and TPA in purified NK (Fig. 7) and T cells (data not shown), indicating that TNF-α, known to be produced by NK and T cells in response to these stimuli (6, 26, 31), participates in IFN-γ production not only by resting, but also by activated NK and T cells.

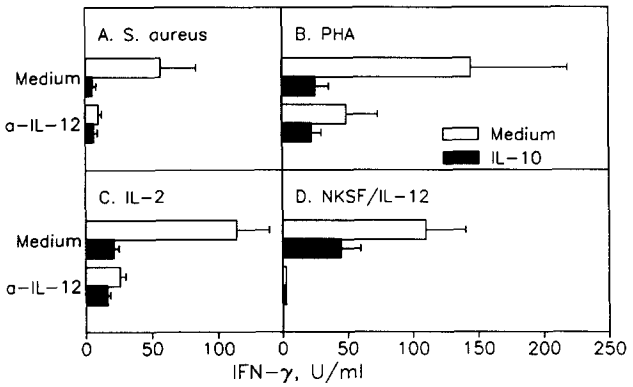

Figure 4. Effect of rIL-10 and neutralizing Abs anti-NKSF/IL-12 on IFN-γ production by human PBMC in response to various inducers. Human PBMC were incubated (5 × 10^6/ml, 18 h, 37°C) with or without human rIL-10 (PBMC stimulated in the presence of IL-10 were also preincubated for 4 h in the presence of the same IL-10 concentration) and with or without anti-NKSF/IL-12 (C8.6, 10 μg/ml). The cell-free supernatant fluid was then assayed for IFN-γ content by RIA. Results are mean ± SE, n = 6.

NKSF/IL-12 and IL-1β Reconstitute IFN-γ Production in the Presence of IL-10. The observation that neutralizing Abs against NKSF/IL-12, TNF-α, and IL-1β inhibited IFN-γ production by PBMC in response to various stimuli, prompted us to test the ability of these three cytokines to stimulate IFN-γ production and to prevent IL-10 inhibition. To de-

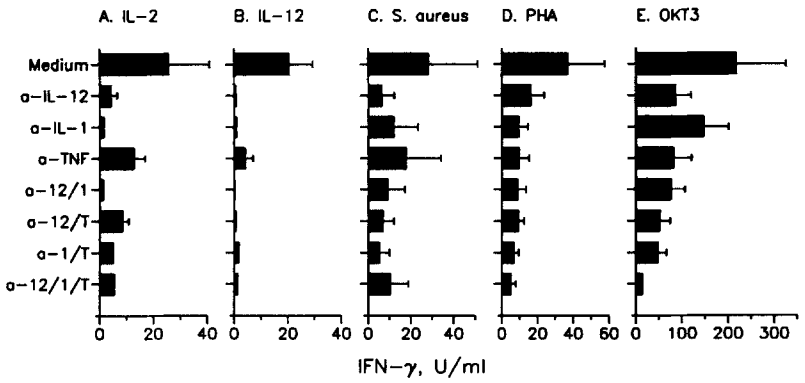

Figure 5. Effect of neutralizing Abs anti-NKSF/IL-12, anti-TNF-α, and anti-IL-1β on IFN-γ production by human PBMC in response to various inducers. Human PBMC were incubated (5×10^6/ml, 18 h, 37°C) with various IFN-γ inducers (IL-2, 100 U/ml; IL-12, 1 ng/ml; *S. aureus*, 1:10,000 vol/vol; PHA, 1%; anti-CD3 and mAb OKT3, and the indicated combinations of anti-NKSF/IL-12 (C8.6, 10 μg/ml) anti-TNF-α (B154.2, 10 μg/ml) or anti-IL-1β (F18.609, 10 μg/ml) Abs. The cell-free supernatant fluid was then assayed for IFN-γ content by RIA. Results are mean ± SE, n = 4.

crease the constitutive production of these cytokines, their effect was tested in endotoxin-free conditions. In these conditions, IFN-γ production was found to be consistently lower than that observed when nonendotoxin tested lots of PBS and FCS were used, suggesting that activation of accessory cells by contaminant endotoxin has an enhancing role on IFN-γ production by PBMC. To obtain consistently measurable levels of IFN-γ in these experiments, 100 U/ml of rIL-2 were added. In these conditions, NKSF/IL-12, but not TNF-α or IL-1β induced IFN-γ production (Fig. 8). IL-1β but not TNF-α (tested in a range of concentrations from 1 to 30 U/ml), slightly enhanced IFN-γ production induced by NKSF/IL-12 and IL-2. IL-10 (10 U/ml) inhibited IFN-γ production induced by NKSF/IL-12 and IL-2, and this inhibition was largely reversed by the addition of 10 ng/ml IL-1β, whereas addition of TNF-α had no effect (Fig. 8).

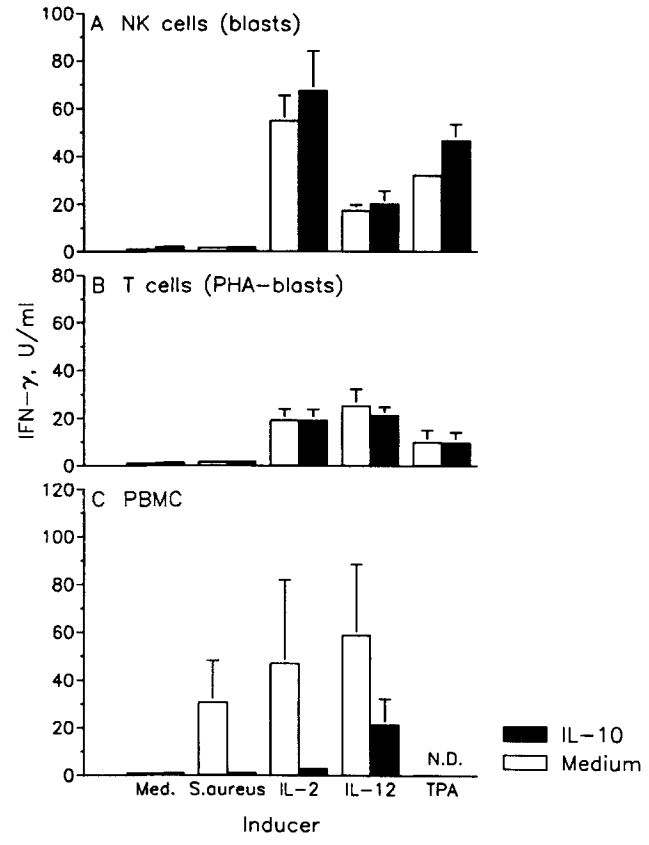

Figure 6. Effect of rIL-10 on IFN-γ production by purified preparations of activated NK cells (*A*), T cells (*B*), and resting PBMC (*C*), in response to various stimuli. Purified NK cells from 8-d cocultures of PBL stimulated with irradiated RPMI-8866 cells, purified T cells from 5-d PHA-stimulated cultures of PBMC, and total resting PBMC were incubated (18 h, 5×10^6 cells/ml, 37°C) in medium or in the presence of *S. aureus* (1:10,000 wt/vol), rIL-2 (100 U/ml), rNKSF/IL-12 (1 ng/ml), or TPA (10^{-8} M), and in the presence (during the incubation and a 4-h pretreatment) or absence of 50 U/ml human rIL-10. Cell-free supernatant fluids were then collected and assayed for IFN-γ content by RIA. Results are expressed as mean ± SE, n = 7 for NK and T cells, n = 4 for PBMC.

Discussion

IL-10 was shown to inhibit macrophage activation and production of cytokines such as TNF-α and IL-1 (19–24). IL-10 can also inhibit T cell lymphokine production and proliferation by suppressing the function of accessory and APCs (16). IL-10 inhibits the accessory function of macrophages, but not of other APCs such as dendritic or B cells, by suppressing both secretion of costimulatory cytokines and

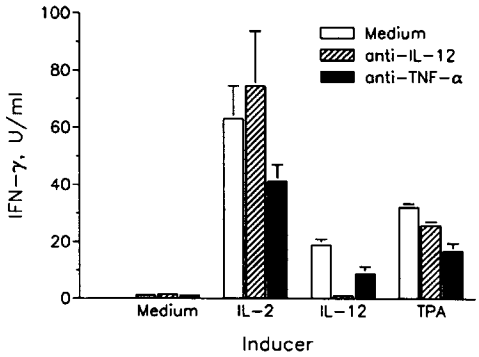

Figure 7. Anti-TNF-α, but not anti-NKSF/IL-12 Abs inhibit IFN-γ production by purified cultured NK cells in response to various inducers. NK cells were purified from 8-d cocultures of PBMC with irradiated RPMI-8866 B cells and stimulated for 18 h with rIL-2 (100 U/ml), rNKSF/IL-12 (1 ng/ml), or TPA (10^{-8} M), in the presence or absence of anti-NKSF/IL-12 (C8.6, 10 μg/ml) or anti-TNF-α (B154.2, 10 μg/ml) Abs. Cell-free supernatant fluids were collected and assayed for IFN-γ content by RIA. Results are expressed as mean ± SE, n = 5.

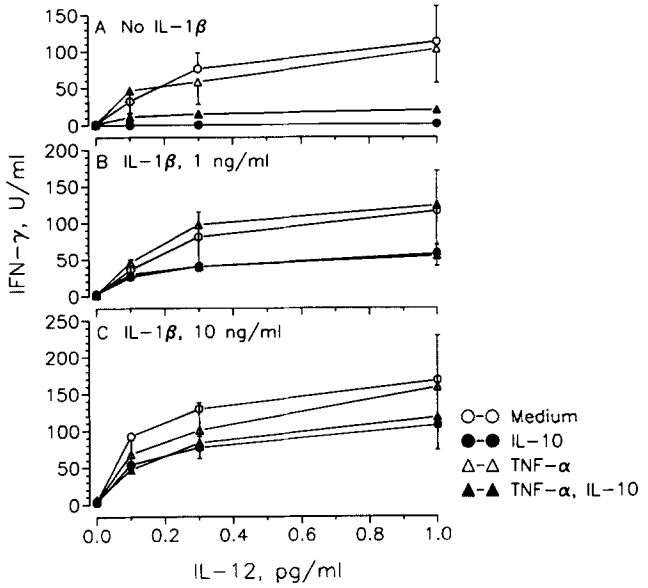

Figure 8. Effect of rIL-1β and rTNF-α on the inhibitory effect of IL-10 on the IFN-γ production by PBMC in response to various concentrations of rNKSF/IL-12. PBMC were purified and incubated (5×10^6/ml, 18 h, 37°C, 100 U/ml rIL-2) in endotoxin-free conditions at the indicated concentrations of rNKSF/IL-12 and in the absence (*A*) or in the presence of 1 ng/ml (*B*) or 10 ng/ml (*C*) rIL-1β, in medium (O—O), or in medium containing 10 U/ml human rIL-10 (including a 4-h pretreatment with IL-10) (●—●), 30 U/ml rTNF-α (Δ—Δ); 30 U/ml rTNF-α, and 10 U/ml rIL-10 (▲—▲). Cell-free supernatant fluids were collected and assayed for IFN-γ content by RIA. Results are expressed as mean ± SE, $n = 3$.

expression of costimulatory molecules (e.g., B7) on the macrophage surface (33). The ability of IL-10 to inhibit IFN-γ production by T and NK cells is not easily explained by its inhibition of cytokines such as TNF-α and IL-1, which have costimulatory or enhancing effects on IFN-γ production, but which by themselves are not able to induce IFN-γ production (25).

We (1, 4, 5, 11) have reported that NKSF/IL-12, a product of monocytes/macrophages and other accessory cells, is a powerful inducer of IFN-γ production by NK and T cells, acting alone or in synergy with IL-2 or other IFN-γ inducing stimuli. NKSF/IL-12 has a major role in IFN-γ production by human PBL in vitro. When IFN-γ production is induced by stimuli able to induce monocytes to produce NKSF/IL-12, such as *S. aureus* or other bacteria or bacterial products, anti-NKSF/IL-12 Abs almost completely abolish IFN-γ production (4). However, a significant inhibition of IFN-γ production by anti-NKSF/IL-12 Abs is also observed when IFN-γ inducers are used such as IL-2 or PHA, that are not able to significantly induce NKSF/IL-12 production. These results, therefore, suggest that NKSF/IL-12, constitutively produced or possibly induced by contaminant endotoxin, plays a major role in IFN-γ production. Similarly, in the murine system, it was shown that NKSF/IL-12 is required for Toxoplasma-induced IFN-γ production in vitro (34) and we (Wysocka, M., and G. Trinchieri, unpublished observations) have observed that anti-NKSF/IL-12 mAbs block LPS-induced IFN-γ production in vivo in mice. Because of this central role of NKSF/IL-12 in IFN-γ production, we investigated the effect of IL-10 on the ability of monocytes to produce NKSF/IL-12. Similarly to what was previously reported for TNF-α, IL-1, and other monokines (21–23), IL-10 very efficiently suppressed in PBMC IL-12 p40 mRNA accumulation and secretion of IL-12 p40 free chain and p70 heterodimers induced by LPS or *S. aureus*. Production of NKSF/IL-12 from both monocytes and nonadherent mononuclear cells was suppressed by IL-10. Because B cells apparently are responsible for only a fraction of NKSF/IL-12 production by nonadherent mononuclear cells and the nature of the NKSF/IL-12 producer HLA-DR$^+$ nonadherent cells remains to be determined (4), it is not possible to conclude yet on which cells, in addition to monocytes, IL-10 is active in suppressing NKSF/IL-12 production.

Although NKSF/IL-12 plays a major role both in in vitro and in vivo induction of IFN-γ production in response to various stimuli, IL-10–mediated inhibition of IFN-γ cannot be explained only by inhibition of NKSF/IL-12 production by accessory cells, because of the ability of IL-10 to at least partially suppress IFN-γ production by PBMC in response to NKSF/IL-12. We therefore analyzed the possibility that inhibition of other cytokines, such as TNF-α and IL-1β, play a role in the effect of IL-10 on IFN-γ production. Although TNF-α and IL-1β, unlike NKSF/IL-12, did not directly stimulate IFN-γ production, neutralizing Abs against these cytokines significantly suppressed IFN-γ production, suggesting that they are required costimulatory factors for IFN-γ production. Only IL-1β and not TNF-α was shown to be able to prevent the ability of IL-10 to inhibit IFN-γ production in response to NKSF/IL-12. We interpret these data to indicate that IL-10 inhibits IFN-γ production in PBMC by suppressing both NKSF/IL-12 and IL-1 production by monocytes and other accessory cells. Although TNF-α is also an important cofactor for IFN-γ production and IL-10 inhibits its production by monocytes, it appears not to play a primary role in the effect of IL-10 on IFN-γ production, probably because upon induction with IL-2, NKSF/IL-12 or other stimuli, TNF-α is produced not only by monocytes but also by NK and T cells, not affected by IL-10. This conclusion is supported by the observation that IFN-γ production by purified preparations of NK and T cells is partially inhibited by anti-TNF-α Abs, but is unaffected by IL-10 or by anti-NKSF/IL-12 Abs. Another possible mechanism of action of IL-10, not investigated in this study, could be mediated by its effect on costimulatory molecules on the membrane of accessory cells (33). For example, we have observed that costimulation of T cells with anti-CD28 Abs or B7 antigen on accessory cells synergizes with NKSF/IL-12 in inducing IFN-γ production (Kubin, M., and G. Trinchieri, unpublished observations).

The ability of IL-10 to inhibit NKSF/IL-12 production has particular interest in light of the opposite effects of these two cytokines on Th-1–Th-2 cell differentiation (12, 13). Because NKSF/IL-12 may be an obligatory factor for efficient Th-1 responses (12, 13), IL-10 production by Th-2 cells or by monocytes may represent a feedback mechanism preventing differentiation of Th-1 clones and favoring the maintenance

of a Th-2 response. The understanding of the reciprocal regulation of these cytokines in vitro and in vivo will shed light on the regulation of cellular and humoral immune responses and will possibly provide means of therapeutic intervention for modulating them.

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Address correspondence to Dr. Giorgio Trinchieri, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104.

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References

1. Kobayashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170:827.
2. Wolf, S.F., P.A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R.M. Hewick, et al. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.* 146:3074.
3. Gubler, U., A.O. Chua, D.S. Schoenhaut, C.M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A.G. Wolitzky, P.M. Quinn, P.C. Familletti, and M.K. Gately. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA.* 88:4143.
4. D'Andrea, A., M. Rengaraju, N.M. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg, et al. 1992. Production of natural killer cell stimulatory factor (NKSF/IL-12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176:1387.
5. Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Pospísil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark, and G. Trinchieri. 1991. Induction of IFN-γ production by NK cell stimulatory factor (NKSF): characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 173:869.
6. Perussia, B., S. Chan, A. D'Andrea, K. Tsuji, D. Santoli, M. Pospísil, D. Young, S. Wolf, and G. Trinchieri. 1992. Natural killer cell stimulatory factor or IL-12 has differential effects on the proliferation of TCR$\alpha\beta^+$, TCR$\gamma\delta^+$ T lymphocytes and NK cells. *J. Immunol.* 149:3495.
7. Gately, M.K., B.B. Desai, A.G. Wolitzky, P.M. Quinn, C.M. Dwyer, F.J. Podlaski, P.C. Familletti, F. Sinigaglia, R. Chizzonite, U. Gubler, and A.S. Stern. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J. Immunol.* 147:874.
8. Chehimi, J., S. Starr, I. Frank, M. Rengaraju, S.J. Jackson, C. Llanes, M. Kobayashi, B. Perussia, D. Young, E. Nickbarg, et al. 1992. Natural killer cell stimulatory factor (NKSF) increases the cytotoxic activity of NK cells from both healthy donors and HIV-infected patients. *J. Exp. Med.* 175:789.
9. Gately, M.K., A.G. Wolitzky, P.M. Quinn, and R. Chizzonite. 1992. Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cell. Immunol.* 143:127.
10. Desai, B.B., P.M. Quinn, A.G. Wolitzky, P.K.A. Mongini, R. Chizzonite, and M.K. Gately. 1992. The IL-12 receptor. II. Distribution and regulation of receptor expression. *J. Immunol.* 148:3125.
11. Chan, S.H., M. Kobayashi, D. Santoli, B. Perussia, and G. Trinchieri. 1992. Mechanisms of IFN-γ induction by natural killer cell stimulatory factor (NKSF/IL-12): role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J. Immunol.* 148:92.
12. Manetti, R., P. Parronchi, M.G. Giudizi, M.-P. Piccini, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (NKSF/IL-12) induces Th1-type specific immune responses and inhibits the development of IL-4 producing Th cells. *J. Exp. Med.* 177:1199.
13. Hsieh, C., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Listeria-induced Th1 development in $\alpha\beta$-TCR transgenic CD4$^+$ T cells occurs through macrophage production of IL-12. *Science (Wash. DC).* 260:547.
14. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann. 1989. Two types of mouse helper T cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170:2081.
15. Moore, K.W., P. Vieira, D.F. Fiorentino, M.L. Trounstine, T.A. Khan, and T.R. Mosmann. 1988. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRF1. *Science (Wash. DC).* 248:1230.
16. Fiorentino, D.F., A. Zlotnik, P. Vieira, T.R. Mosmann, M. Howard, K.W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* 146:3444.
17. Hsu, D.H., K.W. Moore, and H. Spits. 1992. Differential effects of IL-4 and IL-10 on IL-2–induced IFN-gamma synthesis and lymphokine-activated killer activity. *Int. Immunol.* 4:563.
18. Taga, K., and G. Tosato. 1992. IL-10 inhibits human T cell proliferation and IL-2 production. *J. Immunol.* 148:1143.
19. Bogdan, C., Y. Vodovotz, and C. Nathan. 1991. Macrophage deactivation by interleukin 10. *J. Exp. Med.* 174:1549.
20. Gazzinelli, R.T., I.P. Oswald, S.L. James, and A. Sher. 1992. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-γ–activated macrophages. *J. Immunol.* 148:1792.
21. de Waal Malefyt, R., J. Abrams, B. Bennett, C.G. Figdor, and J.E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174:1209.
22. Ralph, P., I. Nakoinz, A. Sampson Johannes, S. Fong, D. Lowe, H.Y. Min, and L. Lin. 1992. IL-10, T lymphocyte inhibitor

of human blood cell production of IL-1 and tumor necrosis factor. *J. Immunol.* 148:808.
23. Fiorentino, D.F., A. Zlotnik, T.R. Mosmann, M.H. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* 147:3815.
24. Oswald, I.P., R.T. Gazzinelli, A. Sher, and S.L. James. 1992. IL-10 synergizes with IL-4 and transforming growth factor-β to inhibit macrophage cytotoxic activity. *J. Immunol.* 148:3578.
25. Wherry, J.C., R.D. Schreiber, and E.R. Unanue. 1991. Regulation of gamma interferon production by natural killer cells in scid mice: role of tumor necrosis factor and bacterial stimuli. *Infect. Immun.* 59:1709.
26. Cuturi, M.C., M. Murphy, M.P. Costa-Giomi, R. Weinmann, B. Perussia, and G. Trinchieri. 1987. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. *J. Exp. Med.* 165:1581.
27. Fontaine, S., C. Damais, D. Lando, and M.A. Cousin. 1989. Monoclonal antibodies to human interleukin-1 beta and their use in a sensitive two-site enzyme linked immunosorbent assay. *Lymphokine Res.* 8:129.
28. Murphy, M., R. Loudon, M. Kobayashi, and G. Trinchieri. 1986. Gamma interferon and lymphotoxin, released by activated T cells, synergize to inhibit granulocyte-monocyte colony formation. *J. Exp. Med.* 164:263.
29. Gately, M.K., and R. Chizzonite. 1992. Measurement of human and mouse interleukin 12. *In* Current Protocols in Immunology, Volume 1. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, Editors. John Wiley & Sons, New York. 6.16.1–8.
30. Perussia, B., C. Ramoni, I. Anegón, M.C. Cuturi, J. Faust, and G. Trinchieri. 1987. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. *Nat. Immun. Cell Growth Regul.* 6:171.
31. Anegón, I., M.C. Cuturi, G. Trinchieri, and B. Perussia. 1988. Interaction of Fcγ receptor (CD16) with ligands induces transcription of IL-2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. *J. Exp. Med.* 167:452.
32. Cassatella, M.A., L. Hartman, B. Perussia, and G. Trinchieri. 1989. Tumor necrosis factor and immune interferon synergistically induce cytochrome b_{-245} heavy chain gene expression and NADPH oxidase in human leukemic myeloid cells. *J. Clin. Invest.* 83:1570.
33. Ding, L., and E.M. Shevach. 1992. IL-10 inhibit mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. *J. Immunol.* 148:3133.
34. Sher, A., J. Oswald, S. Hieny, and R.T. Gazzinelli. 1993. *Toxoplasma gondii* induces a T-independent IFN-γ response in NK cells which requires both adherent accessory cells and TNF-α. *J. Immunol.* 150:3982.