INTERLEUKIN-10 (IL-10) is a noncovalent homodimeric cytokine of 35 kDa that is predominantly produced by monocytes, B cells, and T cells (for review, see Moore et al.1). Although it was originally defined as a product of murine Th2 subsets by its function of inhibiting cytokine synthesis of Th1 clones, human IL-10 was found to be produced by all T-helper cell subsets and CD8\(^+\) T cells.2 The pleiotropic actions of IL-10 on macrophages and T cells include inhibition of production of proinflammatory cytokines,3,4 inhibition of T-cell proliferation,5 and ability to block several accessory cell functions such as antigen presentation and expression of major histocompatibility complex class II expression.6 Therefore, IL-10 has been regarded as an important suppressor of immune functions mediated by macrophages, T cells, and natural killer cells. In animal models, administration of IL-10 was shown to be protective against lethal endotoxemia, suggesting a role for IL-10 as a potent antiinflammatory therapeutic agent.7

In addition to direct proinflammatory actions, interferon-\(\alpha\) (IFN-\(\alpha\)) has recently been found to exhibit activities on cytokine production by various cell types, indicating an immunosuppressive function of IFN-\(\alpha\) as well. Expression of prototypic proinflammatory cytokines such as IL-1,8,9,10 IL-6,11,12 and granulocyte-macrophage colony-stimulating factor (GM-CSF),12 is inhibited by IFN-\(\alpha\), whereas production of the immunosuppressive IL-1 receptor antagonist (IL-1RA) is stimulated in vitro13 and in vivo.14 The present experiments were designed to investigate the role of IFN-\(\alpha\) in the regulation of IL-10 production by human monocytes and purified CD4\(^+\) T cells.

MATERIALS AND METHODS

Cytokines and antibodies. Recombinant human (rhu) IFN-\(\alpha\)2b with a specific activity of \(1.8 \times 10^{9}\) U/mg was obtained from Essex Pharma (Münchener, Germany), and rhuIFN-\(\gamma\) from Reentscher (Laupheim, Germany). rhU-IL-4 was kindly provided by Schering Plough (Kenilworth, NJ), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) by Knoll (Ludwigshafen, Germany). VIT3, a CD3 monoclonal antibody (MoAb) of IgM isotype,14 was a generous gift from O. Majdic (Institute of Immunology, Vienna, Austria). CD28 was obtained from Jansen (Brüggen Bracht Germany), CD8, CD14, CD19, and CD56 MoAbs from Coulter (Krefeld, Germany), and CD16 from Becton Dickinson (San Jose, CA). A neutralizing anti-IFN-\(\alpha\) antiserum was purchased from Sigma Biociences (Deisenhofen, Germany).

Separation procedures. Peripheral blood mononuclear cells (PBMCN) were isolated from heparinized blood samples of normal volunteers by centrifugation over Ficoll-Hypaque. Erosette-negative (E-), and -positive (E\(^+\)) cells were purified by rosetting with neuraminidase (Sigma)-treated sheep red blood cells using standard procedures. Highly purified CD4\(^+\) cells were obtained by negative selection of cells expressing CD8, CD14, CD16, CD19, or CD56. E\(^+\) cells were incubated with a cocktail of these MoAbs followed by depletion using sheep anti-mouse IgG-coated magnetic beads (Dynabeads M450; Dynal, Oslo, Norway) according to the manufacturer’s instructions. This separation procedure was repeated once and resulted in a purity of CD4\(^+\) cells of more than 97%. Contamination with monocytes and B cells was less than 1%. Monocytes were isolated from E\(^+\) cells by negative selection using Dynabeads M450 Pan T (CD2) and M450 Pan B (CD19) to a purity of more than 95%.

Stimulation of cytokine synthesis. For analysis of cytokine synthesis, purified CD4\(^+\) cells were cultured in 24-well flat-bottomed
plates (Greiner, Nuringen, Germany) at 2.5 × 10⁵ cells/mL in a total volume of 1.5 mL RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum (Biochrom) and supplements as previously described.¹⁰ T cells were stimulated with a combination of soluble CD28 and CD3 (VIT-3), which was immobilized by coating the culture plate as recently described.¹³ For coating, the wells were incubated with 1.0 μg/well VIT-3 at 4°C overnight and subsequently washed three times with phosphate-buffered saline (PBS). For RNA analysis, CD4⁺ cells were incubated at 1.0 × 10⁶ cells/mL in the presence of physiohemagglutinin (PHA) and phorbol myristate acetate (PMA). Monocytes were cultured with supplemented medium containing 10% fetal calf serum in 24-well plates and stimulated with factors as indicated in the Results. Total PBShovine serum albumin (lo%), the standards and supernatants were applied and incubated overnight at 4°C. After extensive washes, the wells were incubated with 1.0 μg/well VIT-3 at 4°C overnight.

**Northern blot analysis.** Total cytoplasmic RNA was purified from PBMCs, purified monocytes, or CD4⁺ T cells and subjected to Northern blot analysis previously described.¹⁰ A cDNA probe for huIL-10 was constructed by reverse transcriptase–polymerase chain reaction (RT-PCR) using total RNA obtained from normal PBMCs according to standard procedures.¹⁰ Primer sequences were as follows: sense primer corresponding to cdNA position 320 to 351, 5’ AAGCTGAGAACCAAGACCCAGACATCAAGGCG 3’; and anti-sense primer corresponding to cdNA position 617 to 647, 5’ AGC-TATCCCCAGGCCCCAGATCGATTGG 3’. The 528-bp IL-10 PCR fragment was purified by agarose gel electrophoresis and cloned in TA-cloning vector (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. An EcoRI fragment (350 bp) excised from this plasmid was used for Northern blot analysis.

**Enzyme-linked immunosorbent assay.** huIL-10, anti-IL-10 MoAb clone JES3-9D7 (capture AB), and biotin-anti-11-10 clone JES3-12G8 were obtained from Dianova (Hamburg, Germany) and used for IL-10 enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottomed 96-well plates (Maxi Sorb; Nunc, Wiesbaden, Germany) were coated with 2 pg/well capture antibody overnight. After extensive washing, 100 ng biotinylated anti-IL-10 antibody was added to each well and incubated at room temperature for 45 minutes. Wells were washed again and incubated with avidin-peroxidase 25 μg/mL (Sigma) for 30 minutes at room temperature. After washing, a mixture (1,000:1) of substrate solution (0.03% in 0.1 m, sodium citrate) and 30% H₂O₂ was added, and the reaction was stopped with 1 mol L sulfuric acid after 1 hour. Optical density was measured at 450 nm and corrected for adsorption at 540 nm in an Anthos ELISA reader (Labtec Instruments, Salzburg, Austria). IL-4 protein level was measured in an ELISA using antibodies kindly provided by Sandoz Research Institute (Vienna) as recently described.¹⁵

**Clinical specimens.** Five healthy volunteers and five patients with chronic hepatitis C infection treated with IFN-α2b (Aesda-Schering-Plough Corp, Vienna, Austria) were studied. Details of this study protocol were recently published.¹⁵ Study participants received increasing doses of IFN-α (1, 3, and 5 × 10⁸ U) subcutaneously in a single dose at weekly intervals. EDTA plasma samples were prepared before and 2, 12, 48, and 72 hours after injection, and circulating IL-10 levels were measured by ELISA.

**RESULTS**

**Regulation of IL-10 mRNA expression by IFN-α.** The effect of IFN-α on the expression of IL-10 mRNA was first investigated in PBMCs. PBMCs were incubated with IFN-α alone or in combination with lipopolysaccharide (LPS) or TNF-α, and RNA extracted from such stimulated cells was examined for IL-10 expression in Northern blot analysis (Fig 1A). In the experiment depicted in Fig 1A, a low expression of IL-10 mRNA was detected even in unstimulated cultures, which was clearly enhanced in the presence of IFN-α. Co-stimulation of IFN-α with TNF-α or LPS caused a further increase of IL-10 expression. The induction of IL-10 mRNA by IFN-α was also detected when de novo protein synthesis was blocked by cycloheximide (Fig 1B), strongly suggesting a direct effect of IFN-α.

**Monocytic cells and T cells represent major sources of IL-10.** Therefore, we examined the influence of IFN-α on the expression of IL-10 mRNA in these cell populations. Purified unstimulated PBMC failed to express IL-10 mRNA (Fig 2). In the presence of IFN-α, a faint message of IL-10 was observed. The strong induction of IL-10 mRNA by LPS was not further enhanced by IFN-α. In purified CD4⁺ T cells, induction of IL-10 expression by IFN-α was not clearly detected (Fig 3) over the background of ribosomal RNA. However, IL-10 expression induced by PHA/PMA stimulation was markedly enhanced in the presence of IFN-α.

**Regulation of IL-10 secretion by IFN-α in monocytes.** The production of IL-10 was studied in culture supernatants of monocytes after 24, 48, and 96 hours of incubation with various stimuli (Fig 4A). Detectable levels of IL-10 were only observed upon stimulation with LPS. Although IFN-α induced the expression of IL-10 mRNA in PBMCs (Fig 1), the cytokine as a single stimulant failed to promote significant production of IL-10 protein. However, IFN-α potently enhanced the production of IL-10 in the presence of LPS, after prolonged periods of incubation in particular. In contrast, IFN-γ slightly reduced the production of IL-10 stimulated by LPS after 24 hours, whereas in supernatants obtained after prolonged incubation, no difference was observed with or without IFN-γ added. The induction of IL-10 secretion was only observed at high concentrations of IFN-α. As shown in dose-titration experiments with IFN-α (Fig 4B), the maximum effect on IL-10 secretion by monocytes was only observed at a concentration of 1,000 U/mL IFN-α. Whereas in purified monocytes, expression and secretion of IL-10 and its enhancement by IFN-α was strictly dependent on stimulation with LPS, in unseparated PBMC, IL-10 was constitutively expressed and further enhanced by IFN-α alone. To further investigate an interaction between monocytes and T cells in the production of IL-10, we mixed purified monocytes and T cells in cell cultures with and without IFN-α and measured IL-10 secretion in these culture supernatants. However, IL-10 was always below the detection limit of our ELISA (data not shown), suggesting that a more complex interaction of cells contained in the MNC population might be responsible for the spontaneous IL-10 expression that is further induced by IFN-α.

**Regulation of IL-10 secretion by IFN-α in CD4⁺ T cells.** Freshly isolated CD4⁺ T cells were stimulated via the TCR/CD3 complex using immobilized CD3 MoAb in combination with CD28. This combination has been found to provide optimal conditions for cytokine synthesis of T-helper cells.¹⁵ Maximal secretion of IL-10 was observed after 96 hours
INDUCTION OF IL-10 BY IFN-α

A B

Fig 1. Effect of IFN-α on IL-10 mRNA expression in PBMNC. (A) Expression of IL-10 mRNA after incubation of PBMNC with medium alone, TNF-α (500 U/mL), or LPS (10 μg/mL) with and without 1,000 U/mL IFN-α for 8 hours. 18S and 28S rRNA from ethidium bromide-stained gel were used as loading control. (B) Induction of IL-10 mRNA in the presence of cycloheximide (CHX). PBMNC were incubated for 3 hours with medium alone or 10 μg/mL CHX with and without 1,000 U/mL IFN-α.

(IFig 5A). IFN-α enhanced the secretion of IL-10 in CD4⁺ T cells, and, comparable to our findings in monocytes, this effect was most pronounced after longer incubation periods. In dose-titration experiments, we observed an upregulation of IL-10 secretion in CD4⁺ T cells at lower doses, as seen in monocytes (Fig 5B). At IFN-α 100 U/mL, the amount of IL-10 measured in the supernatant was twice as high as the level at baseline stimulation and further increased to more than fourfold at 1,000 U/mL. The specificity of the IFN-α–mediated effect was examined using neutralizing anti–IFN-α antisera. The induction of IL-10 secretion by IFN-α was completely blocked in the presence of anti–IFN-α antibody (Table 1). The effect of IFN-α on IL-10 secretion was compared with effects of other immunoregulatory cytokines (Fig 6). In contrast to IFN-α, IL-4 and IFN-γ failed to regulate the synthesis of IL-10 in CD3/CD28-stimulated CD4⁺ T cells (Fig 6A). In the same culture supernatants, IL-4 protein levels were measured after 96 hours of incubation (Fig 6B). Whereas IL-10 was differentially regulated by type I and type II IFNs, IFN-α and IFN-γ both inhibited the production of IL-4 in CD4⁺ T cells (Fig 6).

![Diagram](image-url)

Fig 2. Effect of IFN-α on IL-10 mRNA expression in purified monocytes. Highly purified monocytes were incubated for 8 hours in medium alone or LPS with or without IFN-α. For concentrations of stimulants, see Fig 1.

![Diagram](image-url)

Fig 3. Effect of IFN-α on IL-10 mRNA expression in CD4⁺ T cells. Highly purified CD4⁺ T cells were incubated for 24 hours in medium or with the stimulants as indicated: PHA 10 μg/mL, PMA 5 ng/mL, and IFN-α 1,000 U/mL.
Influence of therapeutic administration of IFN-α on circulating IL-10 levels. IL-10 plasma levels were measured in blood samples collected from patients and normal individuals after subcutaneous administration of various doses of IFN-α. An increase of circulating IL-10 levels was not observed in the clinical specimens upon IFN-α therapy (data not shown), whereas in the same samples an increase of IL-1RA was detected, as recently described.13

DISCUSSION

In the present report, we describe the induction of a prototypic immunosuppressive cytokine, IL-10, by IFN-α, which by itself exerts proinflammatory activities. Our results, suggesting a role of IFN-α in limiting inflammatory processes, are functionally in keeping with earlier observations demonstrating the inhibition of mediators of inflammation, including IL-8,9,10 IL-1,11,12 and GM-CSF,12 and the induction of IL-1RA.12,13 However, in contrast to its role in the regulation of IL-1RA, IFN-α alone is not sufficient to stimulate production of IL-10. Another proinflammatory signal, such as endotoxin, is required for IL-10 induction. In LPS-stimulated monocytes, induction of IL-10 was observed to be a late effect that follows the secretion of proinflammatory cytokines such as TNF and IL-1,13 suggesting that an inflammatory signal finally limits its own biologic action via a negative-feedback mechanism. IFN-α appears to primarily enhance this late negative-feedback mechanism, whereas the early inflammatory response to LPS is not counteracted by increased IL-10 secretion. Functionally, the late enhancement of IL-10 secretion could indicate that IFN-α mediates this effect indirectly by interacting with cytokine cascades in response to LPS. However, the experiments involving cycloheximide as an inhibitor of protein neosynthesis clearly prove direct action of IFN-α.

The present results clearly demonstrate that type I and type II IFNs exert opposite effects on the regulation of cytokine expression, including IL-10. Inhibition of LPS-induced IL-10 by IFN-γ has been reported recently18 and was confirmed.
in our experiments, which showed slightly reduced levels of IL-10 in culture supernatants after 24 hours of incubation. Similarly, the influence of IFN-α on IL-1RA synthesis is more pronounced, as found with IFN-γ, and the production of GM-CSF is stimulated by IFN-γ and inhibited by IFN-α.

The enhancement of IL-10 production by IFN-α appears to be tightly regulated. In unseparated MNC, the constitutive expression of IL-10 was further enhanced by IFN-α. However, purified T cells fail to express IL-10 with or without IFN-α, unless these cells are activated with appropriate stimuli. In purified monocytes, a faint induction of IL-10 mRNA expression was seen with IFN-α alone, that appeared to be of no biologic significance, since it did not translate into measurable protein levels in culture supernatants. In accordance with these in vitro results, induction of IL-10 was not detected in plasma samples of IFN-α-treated individuals, whereas induction of IL-1RA was clearly detected in response to therapy with IFN-α in these samples.

Maximal effects on cytokine production are seen at relatively high doses of IFN-α only, suggesting that biologic action in vivo might be locally restricted to the inflammatory process without inducing systemic effects.

The influence of IFN-α on IL-10 synthesis appears to be more effective in activated CD4+ T cells. A remarkable increase of IL-10 was observed at lower doses of IFN-α, as seen in monocytes. However, comparable to our findings in monocytes, this effect was more pronounced after longer incubation periods and required preactivation of the target cell population, again suggesting that IFN-α might enhance negative-feedback mechanisms related to IL-10 secretion. Activities of IFN-α in T cells reported thus far point toward a shift in the differentiation of T-cell subpopulations. Recently, it has been suggested that IFN-α promotes development of the Th1 subtype by increasing the number of T cells that preferentially produce IFN-γ. In fact, our observation that IFN-α significantly downregulated the production of IL-4 in T-cell supernatants is in keeping with a preferential stimulation of the Th1 subtype. However, in human T cells, IL-10 has been shown to be produced by all subpopulations of T-helper cells. Since our experiments were performed in freshly isolated T cells, we currently cannot determine whether the IFN-mediated induction of IL-10 is restricted to certain subpopulations of T-helper cells.

The biologic role of this novel activity of IFN-α in the regulation of inflammation remains to be determined. IFN-α enhances the late induction of IL-10 in vitro, that occurs physiologically upon stimulation of monocytes and T cells with bacterial products, cytokines, or antigen. This effect might enhance the negative-feedback mechanism ascribed to IL-10 that limits an inflammatory reaction. However, the biologic functions of IL-10 are not solely restricted to suppression of the immune system. IL-10 potently enhances proliferation and Ig production by B cells, particularly in the context of stimulation via the CD40 pathway. Prevention of apoptosis by IL-10 has been described for germinal-center B cells and T cells starved of IL-2. Even in monocytes, stimulation of Fc gamma receptor I (CD64) by IL-10 has been shown to occur in an enhanced cytotoxic activity. Thus, the biologic consequence of an upregulation of IL-10 production by IFN-α might not be only an enhancement of the inhibitory effects—it is conceivable that IFN-α might also indirectly enhance the IL-10–mediated positive effects. In particular, by an enhancement of B-cell function, IFN-α might contribute to an improvement of the humoral immune response to microbial invasion.

### Table 1. Influence of Anti–IFN-α Antibodies on Induction of IL-10 by IFN-α in CD4+ T Cells Stimulated by CD3/CD28

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>48 Hours (pg/mL)</th>
<th>96 Hours (pg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Anti–IFN-α</td>
<td>387 ± 23</td>
<td>397 ± 17</td>
</tr>
<tr>
<td>IFN-α (1,000 U/mL)</td>
<td>2,106 ± 67</td>
<td>6,258 ± 96</td>
</tr>
<tr>
<td>IFN-α + anti–IFN-α</td>
<td>336 ± 29</td>
<td>210 ± 21</td>
</tr>
</tbody>
</table>

![Graph A](image1.png)

**Graph A:** Regulation of IL-10 and IL-4 secretion by IFN-α, IFN-γ, and IL-4. CD4+ T cells were incubated with CD28 and immobilized CD3 MoAb alone or in combination with IFN-α (1,000 U/mL), IFN-γ (1,000 U/mL), or IL-4 (500 U/mL). After 96 hours of incubation, concentrations of IL-10 (A) and IL-4 (B) were determined. Data are represented as single values and as the median of 7 different experiments. For calculating statistical significance of the influence of IFN-α on cytokine secretion, the Wilcoxon matched-pairs signed-rank test was used.
In conclusion, we demonstrate a potent effect of IFN-α on the production of IL-10 by activated monocytes and T-helper cells. These findings might contribute to our understanding of the complex interaction of stimulatory and inhibitory cytokines in the regulation of inflammatory immune responses.

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Interferon-alpha stimulates production of interleukin-10 in activated CD4+ T cells and monocytes

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