Interleukin 10(IL-10) Inhibits Cytokine Synthesis by Human Monocytes: An Autoregulatory Role of IL-10 Produced by Monocytes

By René de Waal Malefyt, John Abrams,* Bruce Bennett, Carl G. Figdor,‡ and Jan E. de Vries

*From the *Departments of Human Immunology and Immunology, DNAX Research Institute, Palo Alto, California 94304; and the ‡Division of Immunology, the Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands*

Summary

In the present study we demonstrate that human monocytes activated by lipopolysaccharides (LPS) were able to produce high levels of interleukin 10 (IL-10), previously designated cytokine synthesis inhibitory factor (CSIF), in a dose dependent fashion. IL-10 was detectable 7 h after activation of the monocytes and maximal levels of IL-10 production were observed after 24–48 h. These kinetics indicated that the production of IL-10 by human monocytes was relatively late as compared to the production of IL-1α, IL-1β, IL-6, IL-8, tumor necrosis factor α(TNFα), and granulocyte colony-stimulating factor (G-CSF), which were all secreted at high levels 4–8 h after activation. The production of IL-10 by LPS activated monocytes was, similar to that of IL-1α, IL-1β, IL-6, IL-8, TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), and G-CSF, inhibited by IL-4. Furthermore we demonstrate here that IL-10, added to monocytes, activated by interferon γ(IFN-γ), LPS, or combinations of LPS and IFN-γ at the onset of the cultures, strongly inhibited the production of IL-1α, IL-1β, IL-6, IL-8, TNFα, GM-CSF, and G-CSF at the transcriptional level. Viral–IL-10, which has similar biological activities on human cells, also inhibited the production of TNFα and GM-CSF by monocytes following LPS activation. Activation of monocytes by LPS in the presence of neutralizing anti–IL-10 monoclonal antibodies resulted in the production of higher amounts of cytokines relative to LPS treatment alone, indicating that endogenously produced IL-10 inhibited the production of IL-1α, IL-1β, IL-6, IL-8, TNFα, GM-CSF, and G-CSF. In addition, IL-10 had autoregulatory effects since it strongly inhibited IL-10 mRNA synthesis in LPS activated monocytes. Furthermore, endogenously produced IL-10 was found to be responsible for the reduction in class II major histocompatibility complex (MHC) expression following activation of monocytes with LPS. Taken together our results indicate that IL-10 has important regulatory effects on immunological and inflammatory responses because of its capacity to downregulate class II MHC expression and to inhibit the production of proinflammatory cytokines by monocytes.

Murine–IL-10 was recently identified and its gene cloned based on its cytokine synthesis inhibitory (CSIF)[1] activity (1, 2). In murine systems, IL-10 was produced by the CD4⁺ Th2 subset and inhibits the cytokine production, particularly IFN-γ, by Th1 clones (1, 2). The inhibition of cytokine production by IL-10 was observed only when macrophages, but not when B cells were used as APCs (3). In addition to its CSIF activity, IL-10 was shown to be pleiotropic and to act on different cell types, including thymocytes (4),

cytotoxic T cells (5), mast cells (6), B cells (7), and macrophages (3).

Human IL-10 also exhibits CSIF activity (8). We have demonstrated that the production of IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) by PBMC activated by PHA or anti-CD3 mAbs was strongly inhibited by IL-10 and that this inhibition occurred at the transcriptional level (8). Both human and murine IL-10 have extensive sequence homology to a previously uncharacterized open reading frame in the Epstein Barr virus genome, BCRF-1 (2, 8). Expression of the open reading frame yielded an active protein, designated viral–IL-10 (v–IL-10), which shared most properties with human and murine IL-10, including CSIF activity on mouse and human T cells (9).

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[1] *Abbreviations used in this paper:* CSIF, cytokine synthesis inhibitory factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; v-IL-10, viral-IL-10.

Recently, we described that human IL-10 and v–IL-10 were able to inhibit antigen specific proliferative T cell responses by reducing the antigen presenting capacity of human monocytes via downregulation of class II MHC molecules (9a). Here we describe that human monocytes were able to produce high levels of IL-10 following activation with LPS and that this production was relatively late as compared to that of other monokines. In addition, it is reported here that IL-10 strongly inhibited the production of the proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, and TNFα and the haematopoietic growth factors GM-CSF and granulocyte colony-stimulating factor (G-CSF) by monocytes activated by LPS, IFN-γ, or LPS and IFN-γ. Endogenously produced IL-10 had not only autoregulatory effects on IL-1α, IL-1β, IL-6, IL-8, TNFα, GM-CSF, and G-CSF production by monocytes, but also downregulated its own production and class II MHC expression on monocytes in an autoregulatory fashion. These results indicate that IL-10 has important regulatory effects on immunological and inflammatory responses.

Materials and Methods

Isolation and Culture of Human Monocytes. Human PBMs were isolated from 500 ml blood of normal donors as described previously (10, 11). Mononuclear cells were isolated by density centrifugation in a blood component separator, followed by fractionation into lymphocytes and monocytes by centrifugal elutriation. The monocyte preparation was >95% pure, as judged by nonspecific esterase staining and contained more than 98% viable cells. Monocytes were cultured in Yssel's medium (12) containing HSA supplemented with 1% pooled heat inactivated human AB⁺ serum. This culture medium was endotoxin free as determined by the Limulus amebocyte lysate assay (<0.2 ng/ml of endotoxin). The monocytes were cultured at a concentration of 4 × 10⁶ cells/ml in teflon bags (Jansen MNL, St. Niklaas, Belgium), which prevented adhesion of these cells. After culture for the times indicated, monocytes were collected and analyzed for cell surface expression by indirect immunofluorescence or analyzed for lymphokine gene expression by Northern and PCR analysis. In addition, monocyte culture supernatants were collected for determination of IL-1α, IL-1β, IL-6, IL-8, IL-10, TNFα, GM-CSF, and G-CSF production following activation of these cells. The viability of the cells after culture always exceeded 95% as determined by trypan blue exclusion.

Reagents. Recombinant human IL-10 and v–IL-10 were expressed in *Escherichia coli* as glutathione-S-transferase fusion proteins, purified, and digested with thrombin to remove the N-terminal fusion part (13), resulting in active human and v–IL-10 as described previously (9a). Purified human rIL-4 and rIFN-γ were provided by Schering-Plough Research (Bloomfield, NJ). LPS (*E. coli* 0127:B8) was obtained from Difco Laboratories (Detroit, MI). The neutralizing anti–IL-10 mAb 19F1 was raised against v–IL-10 and efficiently neutralized both human and viral–IL-10 (Silver, J., and J. Abrams, manuscript in preparation).

Probes. The following probes were used for northern analysis: 600 bp Sma I fragment (nt 1299 - 1899) of pCD-hTGFβ (14), 1200 bp Pst I fragment of pAL (β-actin) (8), 567 bp BamHI-Xba I fragment (nt 1 - 567) of pCD-hIL-6 (14), 268 bp Hind III fragment (nt 29 - 297) of SP64-3-10c (IL-8) (15), 760 bp Bgl II - Hind III fragment (nt 159 - 919) of pCD-SRα-hIL-10 (8). The following oligonucleotides were used for Southern analysis of PCR products: IL-1α: 5'-CATGGGTGCTTATAAGTCATC-3' (nt 500-521) (16);

IL-1β: 5'-CGATCACTGAACTGCACGCTCCGGG-3' (nt 444 - 469) (16); IL-6: 5'GAGGTATACCTAGAGTACCTC-3' (nt 510 - 531) (17); IL-8: 5'-TAAAGACATACTCCAAACCTT-3' (nt 200 - 221) (15); IL-10: 5'-CAGGTGAAGAATGCCTTTAATAAGCTCCAA-GAGAAAGGCATCTACAAAGCCATGAGTGAGTTTGACATC-3' (nt 429 - 498) (8); TNFα: 5-GGCGTGGGAGCTGAGAGATAAC-3' (nt 500 - 521) (18); GM-CSF: 5'-CCGGCGTCTCCTGAACCT-3' (nt 150 - 168) (19); actin: 5'-CTGAACCCTAAGGCCAACCGTG-3' (nt 250 - 272) (20); and G-CSF: 5'-GCCCTGGAAGGGATC-TCCCCC-3' (nt 400 - 421) (21).

mRNA Isolation and Northern Analysis. Total RNA was isolated from 20 × 10⁶ monocytes by the guanidinium thiocyanate-CsCl procedure (22). For northern analysis, 10 μg total RNA per sample was separated according to size on 1% agarose gels containing 6.6% formaldehyde, transferred to Nytran nylon membranes (Schleicher & Schuell, Keene, NH) and hybridized with probes, labeled to high specific activity (>10⁸ cpm/μg) by the hexamer labeling technique (23). Filters were hybridized, washed under stringent conditions, and developed as previously described (24).

Polymerase Chain Reaction (PCR) Analysis. 1 μg of total RNA was reverse transcribed using oligo (dT) 12-18 as primer (Boehringer Mannheim, Indianapolis, IN) and AMV reverse transcriptase (Boehringer Mannheim) according to the procedure of Krug and Berger (25) in a 20 μl reaction. 2 μl of reverse transcript (equivalent to 100 ng of total RNA) was used directly for each amplification reaction. Conditions for PCR were as follows: in a 50 μl reaction, 25 nmol of each primer, 125 μM each of dGTP, dATP, dCTP and dTTP (Pharmacia, Uppsala, Sweden), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 1 mg/ml gelatin, 100 μg/ml nonacetylated BSA and 1 U Vent DNA polymerase (New England Biolabs, Beverly, MA). Primers used were as follows: IL-1α sense primer 5'-CATCGCCAATGACTCAGAGGAAG-3' (nt 302-325), IL-1α antisense primer 5'-TGCCAAGCACACCCAGTAGTCTTGCTT-3' (nt 770 - 743) (16), IL-1β sense primer 5'-CCAGCTACGAAT-CTCGGACCACC-3' (nt 230 - 253), IL-1β anti sense primer 5'-TTAGGAAGACACAAATTGCATGGTGAAGTCAGT-3' (nt 896 - 863) (16), IL-6 sense primer 5'-ATGAACTCCTTCTCCACA-AGC-3' (nt 1 - 21), IL-6 anti sense primer 5'-CTACATTTGCC-GAAGAGCCCTCAGGCTGGACTG-3' (nt 810 - 777) (17), IL-8 sense primer 5'-ATGACTTCCAAGCTGGCCGTG-3' (nt 1 - 21), Il-8 anti sense primer 5'-TTATGAATTCTCAGCCCTCTTCAA-AAACTTCTC-3' (nt 302 - 269) (15), IL-10 sense primer 5'-ATG-CCCCAAGCTGAGAACCAAGACCCA-3' (nt 323 - 349), IL-10 anti sense primer 5'-TCTCAAGGGGCTGGGTCAGCTATCC-CA-3' (nt 674 - 648) (8), TNFα sense primer 5'-AGAGGGA-AGAGTTCCCCAGGGAC-3' (nt 310 - 333), TNFα anti sense primer 5'-TGAGTCGGTCACCCTTCTCCAG-3' (nt 782 - 760) (18), GM-CSF sense primer 5'-GCATCTCTGCACCCGCCCG-CTCGCC-3' (nt 76-100), GM-CSF anti sense primer 5'-CCTGC-TTGTACAGCTCCAGGCGGGT-3' (nt 276 - 250) (19), G-CSF sense primer 5'-GAGTGTGCCACCTACAAGCTGTGCC-3' (nt 233 - 258), G-CSF anti sense primer 5'-CCTGGGTGGGCTG-CAGGGCAGGGGC-3' (nt 533 - 508) (21), β-actin sense primer 5'-GTGGGGCGCCCCAGGCACCA-3' (nt 1 - 20), β-actin anti sense primer 5'-GTCCTTAATGTCACGCACGATTTC-3' (nt 548 - 530) (20). Reactions were incubated in a Perkin-Elmer/Cetus DNA Thermal cycler for 20 cycles (denaturation 30 s 94°C, annealing 30 s 55°C, extension 60 s 72°C). Reactions were extracted with CHCl₃ and 40 μl per sample was loaded on 1% agarose gels in TAE buffer. Products were visualized by ethidium bromide staining. Subsequently, gels were denatured in 0.5 M NaOH, 1.5 M NaCl, neutralized in 10 M ammonium acetate, and transferred to Nytran nylon membranes. Membranes were pre-hybridized in 6 × SSC,
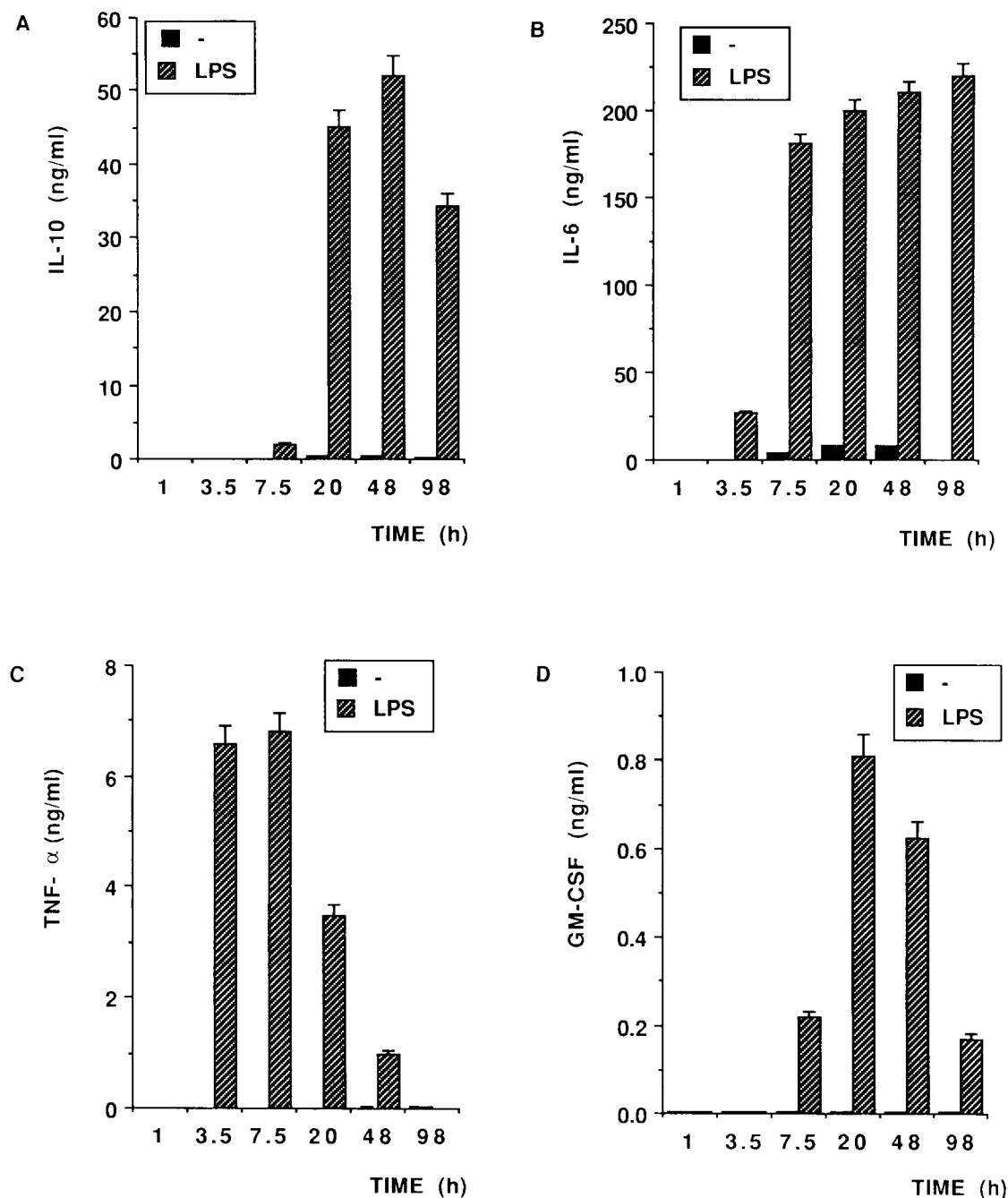
Figure 1. The kinetics of IL-10, IL-6, TNFα, and GM-CSF production by human monocytes, activated by LPS. Human monocytes, isolated by centrifugal elutriation were cultured in teflon bags (4 × 10⁶/ml) in the absence or presence of LPS (1 μg/ml) and production of (*A*) IL-10, (*B*) IL-6, (*C*) TNFα and (*D*) GM-CSF was determined in the culture supernatants harvested at the times indicated by cytokine specific ELISA's.

1% SDS, 10 × Denhardt's solution (0.2% Ficoll, 0.2% polyvinyl-pyrrolidone, 0.2% BSA, pentax fraction V) and 200 μg/ml *E. coli* tRNA (Boehringer, Mannheim, FRG) for 4 h at 55°C. Oligonucleotide probes (200 ng), specific for a sequence internal to the primers used in the amplification, were labeled at the 5' end by T4 polynucleotide kinase (New England Biolabs) and γ-³²P-ATP (Amersham, Arlington Heights, IL) as described (26). Probes were separated from nonincorporated nucleotides by passage over a Nick column (Pharmacia) and added to the hybridization mix. Following

hybridization for 12 h at 55°C, filters were washed in 0.1 × SSC (1 × SSC:150 mM NaCl, 15 mM Na-citrate pH = 7.0), 1% SDS at room temperature and exposed to Kodak XAR-5 films for 1-2 h.

Lymphokine Determinations. The production of IL-1α and TNFα by monocytes was measured by lymphokine specific ELISA's obtained from Endogen (Boston, MA). The lower detection limit of these ELISA's were 50 pg/ml and 10 pg/ml respectively. Production of IL-1β was determined by lymphokine specific ELISA obtained from Cistron (Pine Brook, NJ). The sensitivity of this ELISA

was 20 pg/ml. IL-6 levels were determined by lymphokine specific ELISA purchased from Genzyme (Boston, MA). The sensitivity of this assay was 0.313 ng/ml. IL-8 and G-CSF specific ELISA's were obtained from R&D Systems (Minneapolis, MN) and used to quantitate IL-8 and G-CSF production. The sensitivity of these ELISA's was 4.7 pg/ml and 7.2 pg/ml respectively. GM-CSF production was determined by lymphokine specific ELISA as described previously (27). The sensitivity of this ELISA was 50 pg/ml. IL-10 production was determined by a specific ELISA in which the anti-IL-10 mAb JES 9D7 was used as a coating antibody and the anti-IL-10 mAb JES3-12G8 as a tracer antibody (Silver, J., and J. Abrams, manuscript in preparation). The sensitivity of this ELISA was 50 pg/ml.

Immunofluorescence Analysis. Cells (10^5) were incubated in V bottom microtiter plates (Flow Laboratories, McLean, VA) with 10 μl of purified mAb (1 mg/ml) for 30 min at 4°C. After two washes with PBS containing 0.02 mM sodium azide and 1% BSA (Sigma Chemical Co., St. Louis, MO), the cells were incubated with 1/40 dilution of FITC labeled F(ab')$_2$ fragments of goat anti–mouse antibody (Tago, Inc., Burlingame, CA) for 30 min at 4°C. After three additional washes, the labeled cell samples were analyzed by flow microfluometry on a FACScan (Becton Dickinson and Co., Sunnyvale, CA). The anti–MHC class II mAbs PdV5.2 (HLA-DR/DP/DQ) (28), Q5/13 HLA-DR/DP (29), and L243 (HLA-DR) (30) were described previously.

Figure 2. The production of IL-10 by human monocytes in response to increasing concentrations of LPS. Human monocytes (4 × 10^6/ml) were cultured in teflon bags with increasing concentrations of LPS for 24 h and the production of IL-10 was determined by ELISA.

Results

IL-10 Is Produced by Human Monocytes. We have shown previously that IL-10 was produced by activated human T cell clones, activated peripheral blood T and B cells, EBV transformed B cell lines (8) and monocytes (Abrams, J., H. Yssel, and M. G. Roncarolo, manuscript in preparation). Here we further characterize IL-10 production by human monocytes. Highly-purified human monocytes, isolated by centrifugal elutriation, produced IL-10 following activation by LPS. In addition, it is shown that these human monocytes were able to produce high levels of IL-6, TNFα, and GM-CSF (Fig. 1). Kinetics of cytokine production by LPS activated monocytes indicated that IL-10 production by monocytes was relatively late. It was first detected in supernatants harvested at 7.5 h, but maximal production was observed 20–48 h after activation. In contrast, TNFα and IL-6 were
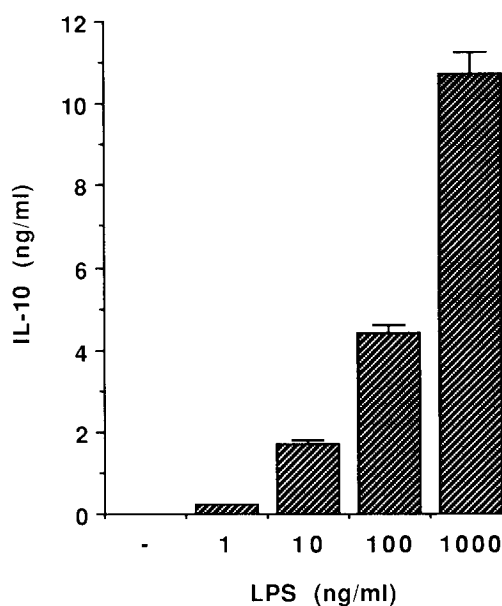
produced rapidly upon activated and reached maximal levels of production at 3.5 and 7.5 h following activation respectively (Fig. 1). However, GM-CSF production was also first detected 7.5 h after activation of monocytes by LPS, but in this case maximal production levels were reached at 20 h. Dose response studies indicated that activation of monocytes by LPS at 10 ng/ml already resulted in significant levels of IL-10 production, whereas the maximal IL-10 synthesis was observed at LPS concentrations of 1 μg/ml (Fig. 2).

IL-10 Inhibits Cytokine Production by Human Monocytes. IL-10 has been shown to inhibit IFN-γ and GM-CSF production by activated PBMC (8). To determine the effects of IL-10 on the production of cytokines by monocytes, highly-purified monocytes were activated for 24 h by LPS in the absence or presence of IL-10. In addition, monocytes were activated with

Table 1. *Effects of Exogenous IL-10, Endogenous IL-10, and IL-4 on Cytokine Production by Human Monocytes*

	IL-1α	IL-1β	IL-6	IL-8	IL-10	TNFα	GM-CSF	G-CSF
				ng/ml				
Medium 37°C	0	0	0	150	0	0	0	0
LPS	1.2	44.8	261.7	479	30.6	21.2	0.6	90
LPS + IL-4	0	9.7	135.7	418	11.9	5.1	0	17.5
LPS + IL-10	0	13.6	78	434	ND	2.6	0	21.1
LPS + αIL-10	2.7	50.6	323	672	ND	47.6	5.5	110

Human monocytes, isolated by centrifugal elutriation were cultured in teflon bags at a concentration of 4 × 10^6 cells/ml and activated by LPS (1 μg/ml) in the absence and presence of IL-10 (100 U/ml), IL-4 (100 U/ml) or anti–IL-10 mAb 19F1 (10 μg/ml) for 24 h and production of cytokines was determined in the supernatants by cytokine specific ELISA's. ND: not done.
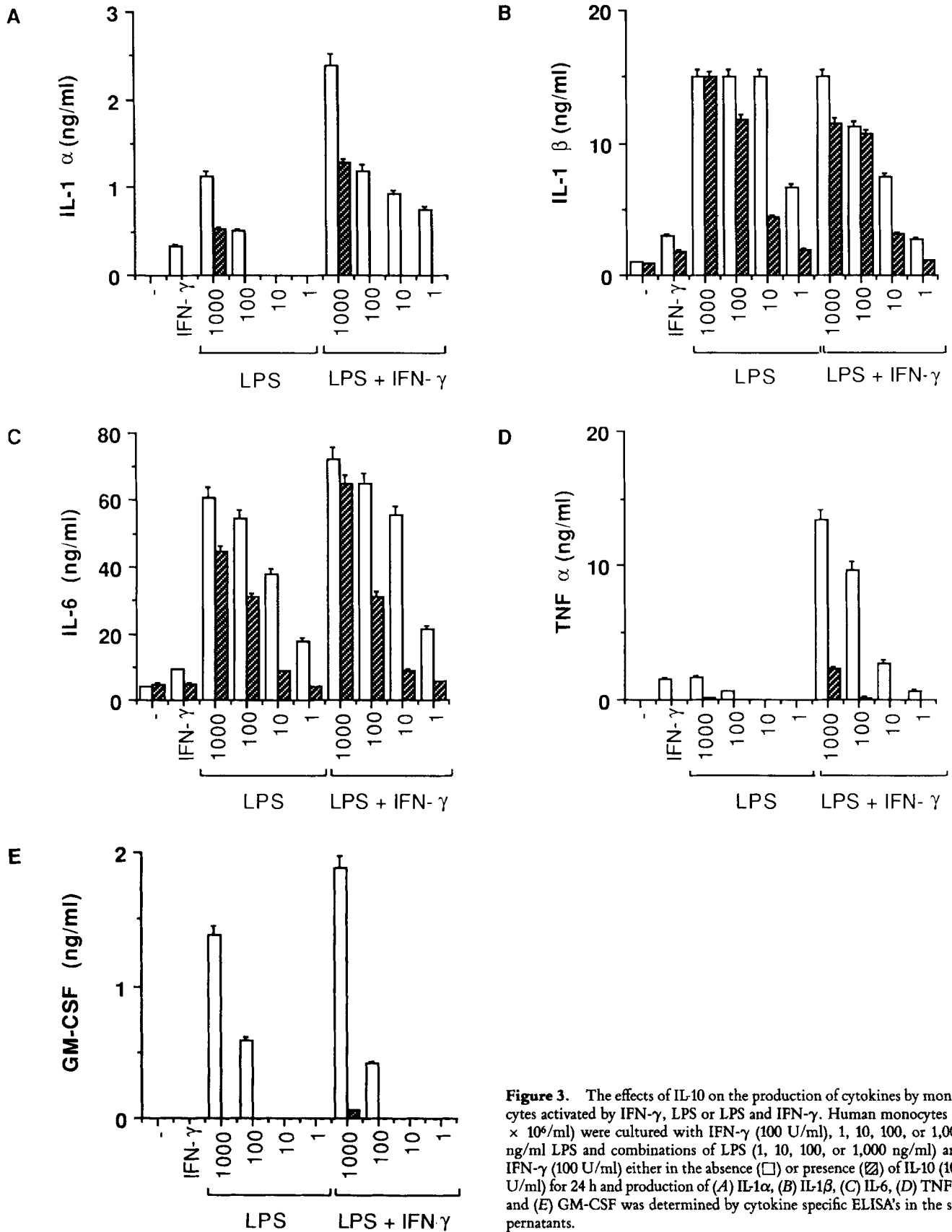
Figure 3. The effects of IL-10 on the production of cytokines by monocytes activated by IFN-γ, LPS or LPS and IFN-γ. Human monocytes (4 × 10⁶/ml) were cultured with IFN-γ (100 U/ml), 1, 10, 100, or 1,000 ng/ml LPS and combinations of LPS (1, 10, 100, or 1,000 ng/ml) and IFN-γ (100 U/ml) either in the absence (□) or presence (▨) of IL-10 (100 U/ml) for 24 h and production of (*A*) IL-1α, (*B*) IL-1β, (*C*) IL-6, (*D*) TNFα, and (*E*) GM-CSF was determined by cytokine specific ELISA's in the supernatants.

LPS for 24 h in the presence of IL-4 (100 U/ml) or neutralizing anti–IL-10 mAb 19F1, which was raised against v–IL-10 but efficiently neutralized both human IL-10 and v–IL-10 (Silver, J., and J. Abrams, manuscript in preparation) (The latter results are discussed below). Cytokine production was determined in the supernatants of these cultures, harvested 24 h after activation, by cytokine specific ELISA's. As shown in shown in Table 1, monocytes which were incubated in medium alone at 37°C did not produce IL-1α, IL-1β, IL-6, IL-10, TNFα, GM-CSF, and G-CSF. Under these conditions, only significant levels of IL-8 were synthesized. Activation of monocytes with LPS (1 μg/ml) resulted in production of high levels of IL-1α, IL-1β, IL-6, IL-8, IL-10, TNFα, GM-CSF, and G-CSF. Interestingly, IL-10 inhibited the production of IL-1α, IL-1β, IL-6, IL-8, TNFα, GM-CSF, and G-CSF to various extents (Table 1). The strongest inhibitory effects of IL-10 were observed on the production of IL-1α, TNFα, GM-CSF, and G-CSF, which were blocked by 80–100%. The inhibition of IL-1β and IL-6 production was less pronounced, whereas the synthesis of IL-8 was only slightly affected by IL-10.

IL-10 Also Inhibits Cytokine Production of Monocytes Activated by IFN-γ. IL-10 also inhibited cytokine production by monocytes activated by IFN-γ, or combinations of IFN-γ and LPS. In Fig. 3, it is shown that IFN-γ at optimal concentrations of 100 U/ml generally was a less potent inducer of cytokine secretion than was LPS at optimal concentrations of 1 μg/ml. Furthermore, it is demonstrated that the effects of combinations of IFN-γ and LPS on cytokine production by monocytes generally were additive. The strongest inhibitory effects of IL-10 were observed on IL-1α, TNFα and GM-CSF production. TNFα and GM-CSF secretion was suppressed by more than 90%, even following activation of the monocytes by optimal LPS and IFN-γ concentrations. Although considerable inhibitory effects on IL-1β and IL-6 secretion were observed at optimal stimulation conditions, their inhibition was more pronounced when the monocytes were stimulated by suboptimal concentrations of LPS, either in the absence or presence of optimal concentrations of IFN-γ.

Viral IL-10 Inhibits Cytokine Production by Monocytes. Viral IL-10 and human IL-10 have similar effects on human cells (9). In Fig. 4, it is shown that both IL-10 and v–IL-10 inhibited TNFα and GM-CSF production by monocytes in a similar fashion. IL-10 and v–IL-10, added at concentrations of 100 U/ml, had significant inhibitory effects on TNFα and GM-CSF production by monocytes following activation by LPS (1 μg/ml). These inhibitory effects of IL-10 and v–IL-10 on TNF-α and GM-CSF secretion were reversed when incubations were carried out in the presence of mAb 19F1 (Fig. 4), demonstrating the specificity of the inhibitory effects of v–IL-10. In fact, activation of monocytes by LPS in the presence of IL-10 or v–IL-10 and the neutralizing anti–IL-10 mAb resulted even in enhanced production of TNFα and GM-CSF, indicating that endogenously produced IL-10 suppressed the production of these cytokines.

Endogenously Produced IL-10 Inhibits Cytokine Production by Monocytes. The inhibitory effects of endogenously produced IL-10 on cytokine production by monocytes was further evalu-
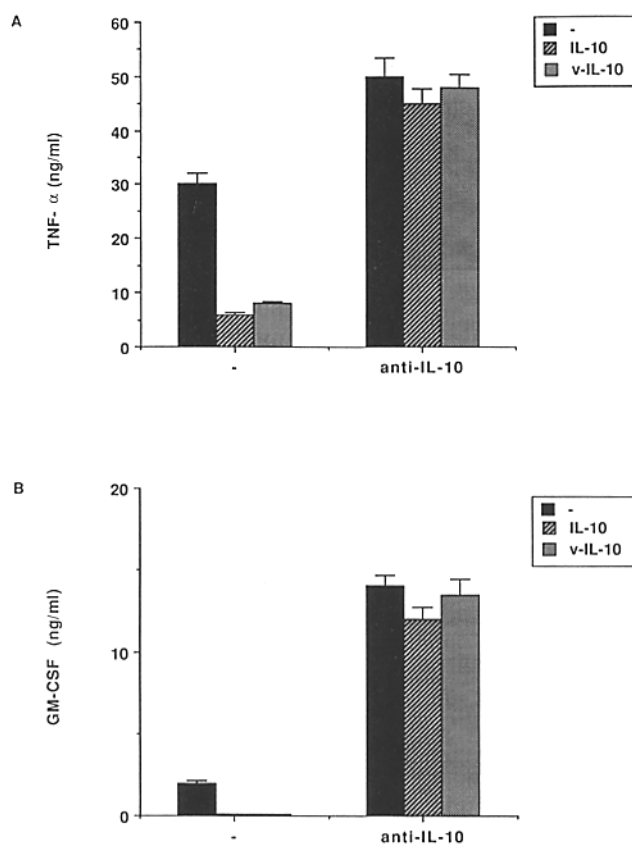
Figure 4. The effects of human IL-10 and viral–IL-10 on the production of TNFα and GM-CSF by LPS activated monocytes. Human monocytes (4 × 10⁶/ml) were activated by LPS (1 μg/ml) and cultured with human IL-10 (100 U/ml) or viral–IL-10 in the absence or in the presence of neutralizing anti–IL-10 mAb 19F1 (10 μg/ml) for 24 h and production of (*A*) TNFα and (*B*) GM-CSF was determined by cytokine specific ELISA's.

ated by quantifying cytokine levels produced by LPS activated monocytes in the presence of neutralizing anti–IL-10 mAb. In Table 1 it is shown that LPS plus anti–IL-10 treatment of monocytes resulted in higher levels of cytokine production as compared to activation by LPS alone, indicating that endogenously produced IL-10 in addition to its inhibitory effects on TNFα and GM-CSF production blocked the production of IL-1α, IL-1β, IL-6, IL8, and G-CSF. The most significant inhibitory effects were found on the production of IL-1α, GM-CSF, and TNFα, whereas the inhibitory effects on IL-1β, IL-6, IL-8, and G-CSF expression were considerable, but less pronounced. Taken together these results indicate that both exogenous IL-10 and endogenously produced IL-10 inhibit the production of IL-1α, IL-1β, IL-6, IL-8, TNFα, GM-CSF, and G-CSF by LPS activated monocytes.

IL-4 Inhibits IL-10 Production by Activated Monocytes. We demonstrated previously that IL-4 inhibited production of IL-1β, IL-6, and TNFα by LPS activated monocytes (31). To determine whether IL-4 also inhibited IL-10 production, monocytes were activated by LPS for 24 h and IL-10 secretion was measured. As shown in Table 1, IL-4 strongly inhibited IL-10 production by LPS activated monocytes. Furthermore, IL-4,
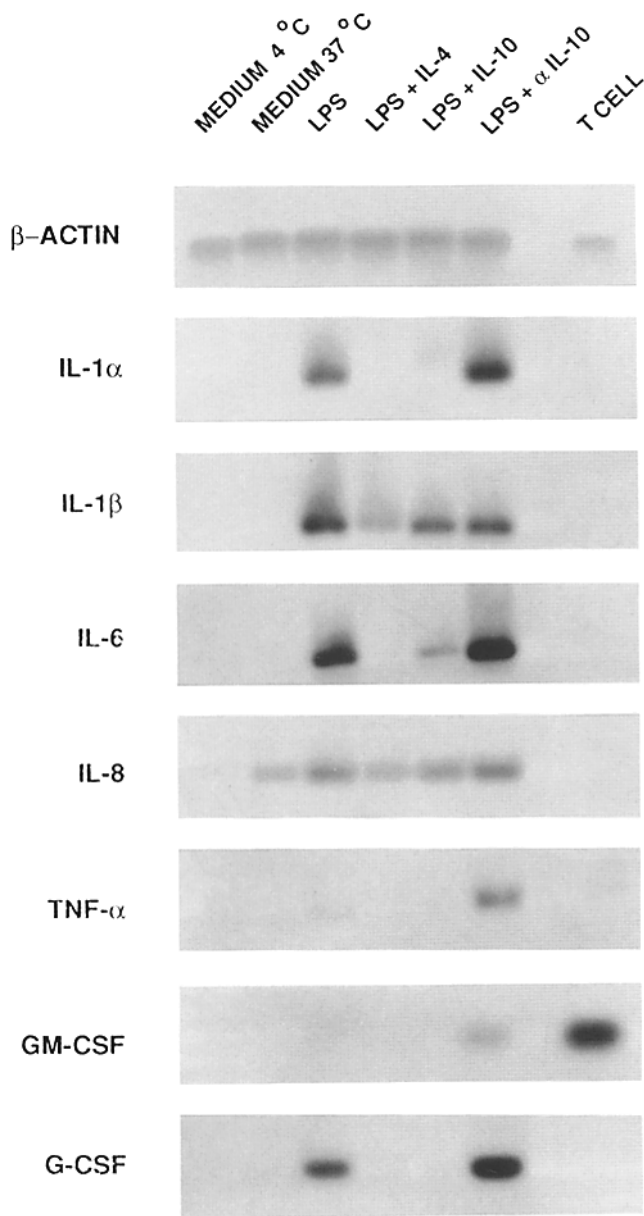
Figure 5. The effects of exogenous IL-10, endogenously produced IL-10 and IL-4 on the expression of cytokine specific mRNA levels by human monocytes activated by LPS. Human monocytes (4 × 10⁶/ml) were cultured in medium at 4°C and 37°C or activated by LPS (1 mg/ml) in the absence or presence of IL-4 (100 U/ml), IL-10 (100 U/ml) and neutralizing anti–IL-10 mAb 19F1 (10 μg/ml) and mRNA was isolated after 24 h. Expression of β-actin, IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF and G-CSF was determined on reverse transcribed mRNA by PCR analyses with cytokine specific primers followed by Southern blotting of the reaction products with internal probes. cDNA obtained from a CD4⁺ T cell clone was included as control for expression of TNFα and GM-CSF.

in addition to its inhibitory effects on IL-1β, IL-6, and TNFα secretion, efficiently blocked the production of GM-CSF and G-CSF. However, as observed for IL-10, the production of IL-8 was only slightly affected by IL-4. Collectively these data indicate that IL-4 and IL-10 have comparable inhibitory effects of cytokine production by activated monocytes.
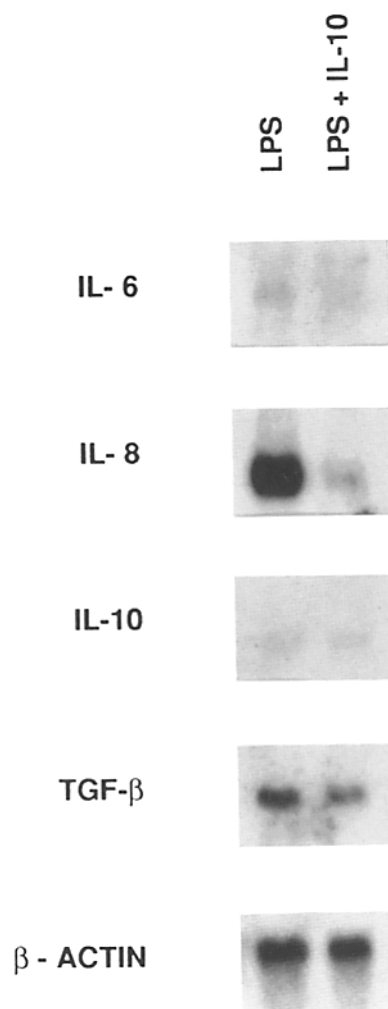

Figure 6. The effects of IL-10 on the expression of cytokine specific mRNA levels by human monocytes activated by LPS. mRNA was isolated from human monocytes (4 × 10⁶/ml), activated by LPS (1 μg/ml) in the absence or presence of IL-10 (100 U/ml) for 7 h and expression of IL-6, IL-8, IL-10, TGF-β, and β actin was determined by northern analyses.

Inhibition of Monokine Production Occurs at the Transcriptional Level. To determine at which level IL-10 inhibited the production of cytokines by monocytes, comparative PCR analyses were performed on RNA isolated from monocytes, activated by LPS in the absence or presence of IL-10, IL-4 or the neutralizing anti–IL-10 mAb for 24 h. The cytokine measurements of this experiment are shown in Table 1. mRNA isolated from these samples was reverse transcribed into cDNA and subsequently amplified with cytokine specific primers. A relatively small number of cycles was used for amplification to ensure that the amount of amplified DNA was proportional to the cycle number and correlated with the amount of specific mRNA in the original sample. It is shown in Fig. 5 that under these conditions equivalent amounts of β-actin specific cDNA were amplified. Monocytes incubated at 4°C in medium alone for 24 h expressed very low levels of IL-8 mRNA. Incubation of these cells at 37°C resulted in an in-
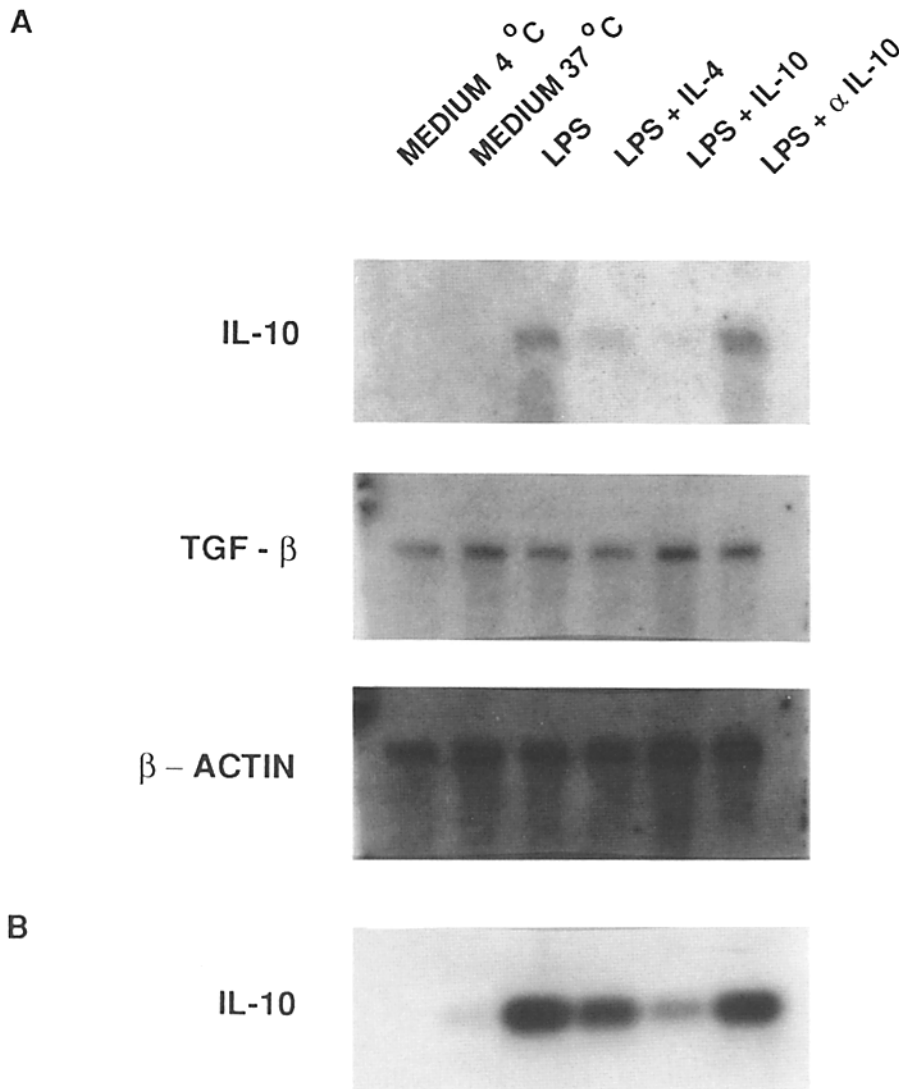
A

IL-10

TGF - β

β – ACTIN

B

IL-10

Figure 7. The effects of exogenous IL-10, endogenously produced IL-10 and IL-4 on the expression of IL-10 and TGFβ specific mRNA levels by human monocytes activated by LPS. Human monocytes (4 × 10⁶/ml) were cultured in medium at 4°C and 37°C or activated by LPS (1 mg/ml) in the absence or presence of IL-4 (100 U/ml), IL-10 (100 U/ml) and neutralizing anti–IL-10 mAb 19F1 (10 μg/ml) and mRNA was isolated after 24 h. Expression of (*A*) IL-10, TGFβ and β-actin was determined by northern analyses and (*B*) expression of IL-10 was determined on reverse transcribed mRNA by PCR analysis with cytokine specific primers followed by Southern blotting of the reaction products with internal probes.

creased expression of IL-8 mRNA, but did not induce expression of IL-1α, IL-1β, IL-6, IL-10, TNFα, GM-CSF, or G-CSF mRNA. LPS activation resulted in a strong expression of IL-1α, IL-1β, IL-6, IL-8, and G-CSF mRNA, whereas TNFα and GM-CSF mRNA were moderately induced. Furthermore, it is shown in Fig. 5 that IL-1α, IL-6, TNFα, GM-CSF, and G-CSF expression was strongly inhibited by IL-10 and IL-4 at the mRNA level (Fig. 5), whereas IL-1β and IL-8 expression were only slightly affected by IL-10. Activation of monocytes by LPS in the presence of the anti–IL-10 mAb resulted in a moderate enhancement in expression of IL-1α, IL-1β, IL-6, IL-8, and G-CSF mRNA and a strong increase in TNFα and GM-CSF mRNA synthesis. The levels of expression of cytokine specific mRNA and their modulation by exogenous and endogenous IL-10 or IL-4 correlated well with secretion of the corresponding proteins as shown in Table 1 and indicated that IL-10 and IL-4 inhibited cytokine expression by LPS activated monocytes at the transcriptional level.

IL-10 Regulates IL-10 mRNA but not TGFβ mRNA Expression in Activated Monocytes. Having demonstrated that

human monocytes produced IL-10 relatively late following activation by LPS, we determined whether IL-10 could affect endogenous IL-10 mRNA synthesis. Human monocytes were activated by LPS in the presence or absence of IL-10 for 7 h and mRNA expression was analyzed by northern blotting. In Fig. 6 it is shown that IL-10 mRNA was detected 7 h after activation by LPS and that IL-10 had no or only minimal inhibitory effects on IL-10 mRNA expression at this time point. In contrast, the expression of IL-6 and IL-8 mRNA was strongly inhibited by IL-10. However, in another series of experiments where monocytes were activated for 24 h by LPS, IL-10 strongly reduced the expression of IL-10 mRNA as shown by northern analysis in Fig. 7. Furthermore, it is shown in Fig. 7 that activation of monocytes with LPS in the presence of a neutralizing anti–IL-10 mAb resulted in an upregulation of IL-10 mRNA expression at 24 h. These results were confirmed by PCR analysis with IL-10 specific primers since the RNA used in this latter experiment was also used for the PCR analyses shown in Fig. 5. It is shown in Fig. 7 *B* that the quantitative differences observed in IL-10 mRNA
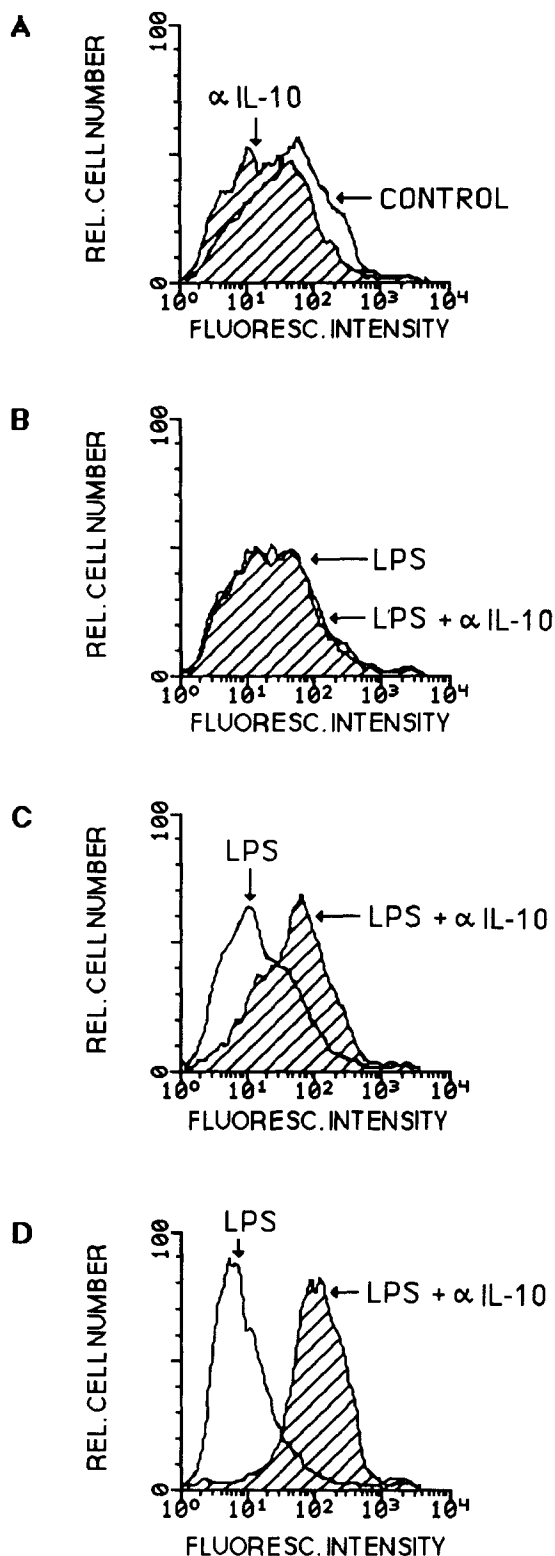
A

B

C

D

Figure 8. The effects of endogenously produced IL-10 on class II MHC expression by human monocytes, activated with LPS. Human monocytes were cultured with (A) 0 ng/ml LPS, (B) 0.1 ng/ml LPS, (C) 10 ng/ml LPS, or (D) 1,000 ng/ml LPS in the absence or presence of neutralizing anti–IL-10 mAb 19F1 (10 mg/ml) for 40 h and expression of HLA-DR/DP (Q5/13) was determined by indirect immunofluorescence.

expression by Northern analysis correlated with those observed by comparative PCR analysis. In addition, the more sensitive PCR analysis allowed the detection of low levels of IL-10 mRNA that were induced 24 h after culture of monocytes in medium alone at 37°C. These results indicate that IL-10 has autoregulatory effects on IL-10 mRNA synthesis and if mRNA levels accurately reflect IL-10 protein production, probably on IL-10 production by human monocytes as well. However, downregulation of IL-10 production occurred rather late in the activation process. TGFβ mRNA, which was expressed constitutively in freshly isolated non activated monocytes, was not enhanced by LPS activation for 7 or 24 h (Figs. 6 and 7). In addition, it is shown in Figs. 6 and 7 that the levels of TGFβ mRNA were not affected when activations were carried out in the presence of IL-4, IL-10, or neutralizing anti–IL-10 mAbs.

IL-10 Has Autoregulatory Effects on Class II MHC Expression by Monocytes. Recently, we demonstrated that IL-10 downregulated the expression of class II MHC molecules on the cell surface of human monocytes (9a). IL-10 was shown to downregulate constitutive, IL-4 or IFN-γ–induced MHC class II expression. Since monocytes produce high levels of IL-10 following activation by LPS, the possibility that endogenous IL-10 could inhibit class II MHC expression by LPS activated monocytes was investigated. Monocytes were activated by various concentrations of LPS in the presence or absence of the neutralizing anti–IL-10 mAb. In Fig. 8 it is shown that activation of monocytes with LPS reduced the constitutive HLA-DR/DP expression on these cells in a dose dependent manner. However, in the presence of the neutralizing anti–IL-10 mAb 19F1 strong induction of HLA-DR/DP expression was observed. Identical results were obtained with several HLA-DR or HLA-DR/DP specific mAb. These results indicate that endogenously produced IL-10, in an autoregulatory fashion, was responsible for the downregulation of class II MHC expression on human monocytes following LPS activation.

Discussion

In the present study we demonstrated that human IL-10 was produced in relatively large quantities by monocytes following activation. Kinetic studies showed that low levels of IL-10 could be detected 7 h after activation of the monocytes, and maximal IL-10 production occurred 24–48 h after activation. This was relatively late as compared to the production of IL-1α, IL-1β, IL-6, IL-8 and TNF-α, which were secreted at high levels between 4–8 h after activation (31–33). In addition, we showed that human IL-10 has strong inhibitory effects on cytokine production by monocytes following activation with IFN-γ, LPS or combinations of IFN-γ and LPS. These inhibitory effects are specific for IL-10 since they could be completely neutralized by mAb 19F1 which inhibits both IL-10 and v–IL-10 activity. IL-10 added at concentrations of 100 U/ml reduced IL-1α, TNF-α, GM-CSF, and G-CSF synthesis by more than 90% following optimal activation of the monocytes by combinations of IFN-γ (100 U/ml) and LPS (1 μg/ml). The inhibitory effects on IL-1β, IL-6, and IL-8

production were somewhat less pronounced, particularly when the monocytes were optimally activated by combinations of IFN-γ and LPS. The mechanism by which IL-10 inhibits cytokine production by monocytes is not clear. It remains to be determined whether these effects of IL-10 are direct or indirectly mediated via other factors. Since IL-1 can induce IL-6 production in fibroblasts, thymocytes, and monocytes (34–36), it is, for example, possible that the partial inhibition of IL-6 production is the result of reduced IL-1 production.

Viral–IL-10 which has been shown to possess biological activities, on human cells similar to human IL-10 (9), was less extensively tested. However, v–IL-10 inhibited TNFα and GM-CSF production by LPS activated monocytes to the same extent as did human IL-10. These inhibitory effects of v–IL-10 on TNFα and GM-CSF production were neutralized by mAb 19F1, illustrating the specificity of the v–IL-10 effects.

Inhibition of IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF, and G-CSF secretion occurred at the transcriptional level. IL-10 strongly inhibited cytokine specific mRNA synthesis induced by LPS, as determined by northern and PCR analyses. PCR analyses were performed under conditions that allowed a comparison of the reaction products of individual samples in a semiquantitative manner. This was validated by the fact that amplification of cDNA's with primers specific for β-actin resulted in equivalent amounts of reaction products between the samples and quantitatively comparable results were obtained when IL-10 mRNA expression was determined in the same samples by both northern and PCR analysis. In addition, cytokine mRNA expression levels correlated with the protein levels in the supernatants of these cultures. IL-10 failed to affect TGF-β mRNA expression in activated monocytes. However, it has to be noted here that TGF-β was constitutively expressed in nonactivated monocytes and that activation of the monocytes by LPS did not affect TGF-β mRNA levels. Assoian et al. (37) have demonstrated that monocytes constitutively express TGF-β mRNA and that TGF-β secretion requires monocyte activation. However, it remains to be determined whether IL-10 has an effect on the conversion of TGF-β from its latent to its active form.

The production of IL-10 was shown to be inhibited by IL-4 at the transcriptional level. Although the inhibitory effects of IL-4 on IL-10 production were considerable, IL-4 was not able to block the production of IL-10 completely. IL-4 inhibited the production of IL-10 only up to 70%, even when high IL-4 concentrations (400 U/ml), which were sufficient to completely inhibit the production of IL-1, IL-6, and TNFα, were used (results not shown). We (31) and others (38–40) have demonstrated previously that IL-4 was able to inhibit the production of IL-1α, IL-1β, IL-6, IL-8 (41), and TNFα by human monocytes. These findings were confirmed here and extended by demonstrating that IL-4 also inhibited the production of IL-8, GM-CSF, and G-CSF by LPS activated human monocytes. This inhibition occurred at the transcriptional level. The data furthermore illustrate that IL-4 and IL-10 have similar effects on cytokine expression by human monocytes, which underlines the pleiotropic effects of cytokines and redundancy in the immune system.

Interestingly, IL-10 is an autoregulatory cytokine, since it strongly inhibited IL-10 mRNA synthesis in monocytes activated for 24 h. In addition, activation of monocytes by LPS in the presence of neutralizing anti–IL-10 mAbs resulted in an increased expression of IL-10 mRNA at 24 h, indicating that endogenously produced IL-10 also inhibited IL-10 mRNA synthesis. The fact that IL-10 is able to downregulate its own production by human monocytes makes this the first cytokine that is regulated by a negative feedback mechanism. Autoregulatory effects of endogenously produced IL-10 were also observed on the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF, and G-CSF by LPS activated monocytes. However, the inhibitory effects of endogenous IL-10 on the production of these cytokines were less pronounced than those of exogenous IL-10 added at the onset of the culture. This is related to the fact that IL-1α, IL-1β, IL-6, IL-8, TNF-α, and G-CSF are already produced at high levels 4–8 h after activation, whereas maximal endogenous IL-10 production occurs much later at 24–48 h after activation. This notion is supported by the observation that the strongest inhibitory effects of endogenously produced IL-10 were found on GM-CSF secretion which was shown to be produced late following activation of the monocytes. Assuming that IL-10 mRNA synthesis reflects and precedes IL-10 protein secretion, and that IL-10 can only interact with its receptor on the cell surface, these results suggest that the inhibitory effects of endogenously produced IL-10 occur relatively late and may therefore be of particular importance in the later phases of an immune response.

IL-10 was first described in the mouse as a CSIF produced by Th2 cells, which inhibited cytokine production (predominately IFN-γ by Th1 cells (1). This inhibitory effect of mouse–IL-10 on cytokine production by Th1 cells required the presence of macrophages (3). Recently we demonstrated that human IL-10 and v–IL-10 strongly inhibited antigen specific T cell proliferation when monocytes were used as APC (9a). In addition, it was shown that reduction in the antigen specific proliferative T cell responses were for a major part due to the reduced antigen presenting capacity of the monocytes caused by strong downregulatory effects of IL-10 on class II MHC antigen expression on these cells. These data, together with the present finding that IL-10 is produced late by monocytes and has autoregulatory effects on IL-10 secretion by these cells, indicate that IL-10 may have strong downregulatory effects on ongoing antigen specific T cell responses. Therefore IL-10 may be a cytokine that plays a major role in dampening antigen driven proliferative T cell responses. The notion, that IL-10 produced by monocytes may also have strong autoregulatory feedback activity on T cell activation, is supported by the observation that IL-10 produced by monocytes following activation by LPS is responsible for downregulation of class II MHC antigens on these cells, since class II MHC expression was not reduced and even strongly enhanced when LPS stimulations were carried out in the presence of the neutralizing anti–IL-10 mAb.

The finding that IL-10 has strong downregulatory effects on the secretion of the proinflammatory cytokines IL-1α, IL-

1β, IL-6, IL-8, and TNF-α which are all involved in acute and chronic inflammatory processes, many autoimmune diseases, including rheumatoid arthritis and not only modulate the activation and function of cells of the immune system but also those of endothelial cells, keratinocytes and hepatocytes (42–44), suggests that IL-10 is a powerful inhibitor of inflammation. Based on its properties described thus far, which include inhibition of antigen specific T cell proliferation by reducing the APC capacity of monocytes through downregulation of class II MHC antigens on these cells and inhibitory effects on proinflammatory cytokine secretion by monocytes, it seems that IL-10 plays a major role as a suppressor of immune and inflammatory responses.

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