Interleukin-10 (IL-10) is a potent monocyte regulatory cytokine that inhibits gene expression of proinflammatory mediators. In this study, we investigated the mechanism by which IL-10 downregulates expression of intercellular adhesion molecule-1 (ICAM-1) on the cell surface of normal human monocytes activated with interferon-γ (IFN-γ). IL-10 inhibition of IFN-γ-induced ICAM-1 expression was apparent as early as 3 hours and was blocked by an anti–IL-10 antibody but not by an isotype-matched control antibody. Northern blot analysis showed that IL-10 reduced the accumulation of ICAM-1 mRNA in IFN-γ–stimulated monocytes. IL-10 inhibition of IFN-γ–induced steady-state mRNA was detected at 3 hours and remained at 24 hours. Nuclear run-on transcription assays showed that IL-10 inhibited the rate of IFN-γ–induced transcription of the ICAM-1 gene, and mRNA stability studies showed that IL-10 did not alter the half-life of IFN-γ–induced ICAM-1 message. Thus, IL-10 inhibits IFN-γ–induced ICAM-1 expression in monocytes primarily at the level of gene transcription. Activation of IFN-γ–responsive genes requires tyrosine phosphorylation of the transcription factor STAT-1α (signal transducer and activator of transcription-1α). However, IL-10 did not affect IFN-γ–induced tyrosine phosphorylation of STAT-1α or alter STAT-1α binding to the IFN-γ response element (IRE) in the ICAM-1 promoter. Instead, IL-10 prevented IFN-γ–induced binding activity at the NF-κB site of the tumor necrosis factor α (TNF-α)–responsive NF-κB/C/EBP composite element in the ICAM-1 promoter. These data indicate that IL-10 inhibits IFN-γ–induced transcription of the ICAM-1 gene by a regulatory mechanism that may involve NF-κB.

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media contained RPMI 1640 (GIBCO Laboratories, Grand Island, NY), 10% heat-inactivated fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA), 2 mM/L-glutamine, penicillin-streptomycin (100 μg/mL), and 10 mM/L HEPES (Sigma Chemical Co) (complete media). Anti–DR, anti–ICAM-1, and isotype control were purchased from Shift assays (EMSAs) were performed as we have previously described. FCS. Cell preparations yielded greater than 85% CD14+ cells. Monocytes were incubated with phycoerythrin (PE)- and FITC-24 to 36 hours with 10^7 cpm labeled RNA. Filters were washed with PstI digestion double-stranded synthetic deoxyoligonucleotide probes for 30 min at 0°C. Adherent monocytes were harvested by removing the cell pellet by adherence to 150-mm tissue culture dishes (Corning Inc, Corning, NY) for 1.5 hours at 37°C in complete media. Nonadherent cells were collected by vigorous washing in cold RPMI 1640 in 3% FCS. Cell preparations yielded greater than 85% CD14+ monocytes as determined by flow cytometry.

**FACS analysis.** Peripheral blood mononuclear cells (2 × 10^9) were cultured in 24-well Falcon culture dishes (Becton Dickinson Co, Lincoln Park, NJ) for 1.5 hours, and then nonadherent cells were removed by vigorous washing with cold RPMI 1640 with 3% FCS. Monocytes were stimulated in the presence or absence of IFN-γ, IL-10, or both in combination in complete media. Adherent cells were harvested using a cell scraper and then washed with buffer (phosphate-buffered saline, 3% bovine serum albumin, and 0.01% NaN3). Cells were incubated with phycoerythrin (PE)- and FITC-conjugated monoclonal antibodies for 30 minutes on ice, washed twice in buffer, and fixed with 1% paraformaldehyde. Monocytes were identified as CD14+ using PE-conjugated anti-CD14 monoclonal antibody, and electronically gated CD14+ cells were analyzed for intensity of green fluorescence with FITC-conjugated anti–ICAM-1 and FITC-conjugated anti–HLA-DR monoclonal antibody. PE-conjugated anti-CD14 monoclonal antibody, FITC-conjugated anti-DR, anti–ICAM-1, and isotype control were purchased from Coulter (Hialeah, FL).

**Northern blot analysis.** Total cellular RNA was isolated using RNAzol (Tel-test ‘B’ Inc, Friendswood, TX) from adherent monocytes (1 × 10^9) stimulated with cytokines in complete media for the times and cytokine concentrations indicated in the figure. Total RNA samples (5 to 10 μg) were loaded onto a 1.2% agarose gel containing formaldehyde, and then blotted onto BA-S NC nitrocellulose membranes (Midwest Scientific, St Louis, MO). The membrane was baked for 2 hours at 80°C and then probed in a 50% formamide hybridization solution overnight at 42°C. The probes, 1,400-basepair (bp) ICAM-1 (SalI-BglII digestion fragment) and 1,400-bp gyaleraldehyde-3-phosphate dehydrogenase (GAPDH) Pro1-Pro3 digestion fragment were labeled by random priming (Stratagene, La Jolla, CA) with 50 μCi [α-32P]dCTP (3,000 Ci/mmol; Amersham, Arlington Heights, IL). The membrane was washed and autoradiographed at −70°C for 1 to 3 days. Filters were stripped and rehybridized with [32P]-labeled GAPDH under the same conditions. Levels of ICAM-1 mRNA are expressed as the ratio of ICAM-1 to GAPDH mRNA determined by laser densitometry (Personal Densitometer SI, Molecular Dynamics Inc, Sunnyvale, CA).

**ICAM-1 mRNA stability analysis.** Monocytes were stimulated for 16 hours with IFN-γ (100 U/mL) in the presence or absence of IL-10 (100 U/mL) in complete media. Actinomycin D (5 μg/mL) was added to the cells, and RNA was extracted at 0, 1, 2, and 3 hours. Northern blot analysis was performed as described earlier. The maximum levels of ICAM-1 mRNA determined by densitometry for IFN-γ and IFN-γ plus IL-10 were set at 100% and plotted against the percent reduction in the amount of mRNA.

**Nuclear run-on analysis.** Monocytes were unstimulated or stimulated with IFN-γ (100 U/mL), IL-10 (100 U/mL), or a combination of both for 3 hours at 37°C in complete media. Cells were washed, scraped from the culture dishes, centrifuged, resuspended in Nonidet P-40 (NP-40) lysis buffer (10 mM/L Tris hydrochloride, pH 7.4, 10 mM/L NaCl, 3 mM/L MgCl₂, and 0.5% NP-40) for 5 minutes at 4°C, and pelleted to isolate the nuclei. Nuclei were resuspended in 200 μL glycerol buffer (0.1 mM/L EDTA, 40% glycerol, 5 mM/L MgCl₂, and 50 mM/L Tris hydrochloride, pH 8.3) and flash-frozen in liquid nitrogen for storage at −80°C. Intact nuclei (200 μL) were thawed, and 200 μL transcription buffer (10 mM/L Tris hydrochloride, Tris, 5 mM/L MgCl₂, 0.3 μM/L KCl, 1 mM/L unlabeled ATP, CTP, and UTP ribonucleotides, and 5 mM/L DTT), 250 mCi [α-32P]GTP (3,000 Ci/mmol; Amerham Corp), and 500 U RNAse (Promega, Madison, WI) were incubated at 30°C for 30 minutes. For RNA extraction, nuclei were mixed with 30 μL (1 mg/mL) RNase-free DNase I (Promega) and 600 μL HSB buffer (0.5 mol/L NaCl, 50 mM/L MgCl₂, 2 mM/L CaCl₂, and 10 mM/L Tris hydrochloride, pH 7.4) and vigorously pipetted and incubated for 5 minutes at 30°C. For RNA purification, 200 μL sodium dodecyl sulfate (SDS)/Tris buffer (5% SDS, 0.5 mM/L Tris hydrochloride, pH 7.4, and 0.125 mol/L EDTA) and 5 μL (50 mg/mL) proteinase K (Promega) were incubated at 30°C for 30 minutes at 42°C. RNA was extracted with an equal volume of phenol/chloroform/isooamyl alcohol (25:24:1). Nonribonucleosides were solated with 5 μg linearized ICAM-1 cDNA and GAPDH cDNA, washed six times with SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), dried, and baked for 2 hours at 80°C. Hybridization was performed at 42°C for 24 to 36 hours with 10 6 cpm labeled RNA. Filters were washed with 0.2× SSC/0.1% SDS for 15 minutes at room temperature, followed by 0.2× SSC/0.5% SDS for 10 minutes at 42°C, and with 0.2× SSC/0.1% SDS for 10 minutes at 42°C. Membranes were autoradiographed at −70°C for 4 to 7 days. Levels of ICAM-1 mRNA were normalized by expressing the ratio of ICAM-1 RNA to GAPDH RNA determined by laser densitometry.

**Electrophoretic mobility shift analysis.** Electrophoretic mobility shift assays (EMSAs) were performed as we have previously described.11 Nuclear extracts were prepared by treating the cell pellet with cold buffer (10 mM/L HEPES, pH 7.9, 10 mM/L KCl, 0.1 mM/L EDTA, 0.1 mM/L EGTA, 1 mM/L DTT, and 0.5 mM/L phenylmethylsulfonyl fluoride [PMSF]) for 15 minutes to allow cells to swell, and then 25 μL 10% NP-40 was added and the nuclei were pelleted. The nuclear pellet was resuspended in buffer (20 mM/L HEPES, pH 7.9, 0.4 mol/L NaCl, 1 mM/L EDTA, 1 mM/L EGTA, 1 mM/L DTT, and 1 mM/L PMSF) for 15 minutes at 4°C. Debris was pelleted, and supernatants were frozen at −70°C. Nuclear extracts (5 to 7 μg protein) prepared from cytokine-activated monocytes were incubated with 50,000 cpm (0.5 ng) [32P]-end-labeled double-stranded synthetic deoxyoligonucleotide probes for 30 minutes at room temperature in a 20-μL reaction volume containing 12% glycerol, 12 mM/L HEPES-NaOH (pH 7.9), 60 mM/L KCl, 5 mM/L MgCl₂, 4 mM/L Tris hydrochloride (pH 7.9), 0.6 mM/L EDTA (pH 7.9), 0.6 mM/L DTT, and 1 μg poly(dI:dC). Protein-DNA complexes were resolved in 5% native polyacrylamide gels. Gels were dried and exposed overnight to x-ray film (Eastman Kodak Co, Rochester, NY) with an intensifying screen at ~70°C. For supershift, nuclear extracts (2 to 5 μg protein) were incubated with 2 μg anti–STAT-1α or anti–STAT3 antibodies for 30 minutes before incubation with labeled probe. The oligonucleotides used in these studies were as follows: ICAM-1 IRF, 5'-GAGGTTTCCCGGGGAAA-GCAGC-3'; c-fos SIE, 5'-GTGACATTTCGGATAACTTGGTCTACA-3'; ICAM-1 Sp-1, 5'-ACCGCGCCCGCGCGCG-3'; and ICAM-1 proximal NF-κB, 5'-GCTCGGGAACTTCCAGC-3'.

**Immunoprecipitation of STAT-1α.** Adherent monocytes were stimulated in the presence or absence of cytokines for 5 minutes at 37°C in complete media. Cells were lysed with lysis buffer (50 mM/L Tris hydrochloride, 1 mM/L EDTA, 0.15 mol/L NaCl, 1%
IL-10 inhibits ICAM-1 gene transcription. IL-10 inhibition of ICAM-1 mRNA may result from either a reduction

Table 1. IL-10 Inhibits Cell Surface Expression of ICAM-1

<table>
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<th>Donor</th>
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<th>HLA-DR</th>
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<td>+</td>
<td>597</td>
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Values in parentheses indicate ICAM-1 and MHC-DR expression on freshly isolated cells given as fluorescence values.
in transcription of the ICAM-1 gene and/or an increase in degradation of ICAM-1 mRNA. We performed nuclear run-on assays to determine if IL-10 inhibited ICAM-1 gene transcription. Nuclei isolated from monocytes activated with IFN-γ in the presence or absence of IL-10 for 3 hours were monitored for ICAM-1 transcription. IFN-γ increased the rate of ICAM-1 gene transcription in monocytes, and IL-10 prevented this increased transcription (Fig 2). These data demonstrate that IL-10 regulation of ICAM-1 in monocytes is mediated at least in part by transcription.

It has been reported that IL-10, in addition to inhibiting cytokine gene transcription, in some instances enhanced mRNA degradation. To determine if IL-10 alters the half-life \( t_{1/2} \) of ICAM-1 mRNA, we used Actinomycin D to block transcription in activated monocytes and determined the rate of mRNA degradation by Northern blot analysis. Monocytes were stimulated with IFN-γ alone or IFN-γ plus IL-10 for 16 hours. Actinomycin D (5 μg/ml) was then added to prevent further mRNA synthesis. RNA was isolated at different time points, and ICAM-1 mRNA was analyzed by Northern blot. There was a steady reduction in ICAM-1 mRNA over the 3-hour period, with a \( t_{1/2} \) of about 1 hour (Fig 3). However, IL-10 did not enhance the rate of ICAM-1 mRNA degradation, suggesting that IL-10 does not alter ICAM-1 mRNA stability. Taken together, these data demonstrate that IL-10 inhibits IFN-γ–induced ICAM-1 expression primarily at the level of gene transcription.

Effect of IL-10 on STAT-1α binding activity. IFN-γ has been shown to activate the transcription factor STAT-1α. Look et al described an IRE in the ICAM-1 promoter between nucleotide positions −116 and −106 that is required for ICAM-1 promoter induction by IFN-γ. To determine whether the IRE motif could bind IFN-γ–induced or IL-10–induced DNA binding proteins in monocytes, we synthesized a 20-bp oligonucleotide encompassing the IRE, prepared nuclear extracts from IFN-γ–treated monocytes in the presence or absence of IL-10 for 0.5 and 1 hour, and assessed DNA binding activity by EMSA. IFN-γ induced a complex that was evident as early as 30 minutes (Fig 4). In contrast, IL-10 induced two weak complexes: one comigrated with the IFN-γ–induced complex, and the second migrated more slowly. When IFN-γ and IL-10 were combined to stimulate monocytes, we detected a dominant complex corresponding to the IFN-γ–induced complex and a much weaker complex corresponding to the slower-migrating IL-10 complex. These data show that IL-10 does not inhibit the IFN-γ–induced complex.

Competitive-inhibition studies demonstrated the specificity of the IFN-γ–activated IRE binding complex. We used the c-sis inducible element (SIE) from the c-fos promoter shown previously to bind STAT-1α, to assess whether the IRE is also recognized by STAT-1α. Nuclear extracts from monocytes stimulated with IFN-γ plus IL-10 were incubated with labeled IRE and a 100-fold molar excess of IRE or SIE. Both the IRE and the SIE competed for proteins binding to the IRE, whereas no competition was observed for an unrelated ICAM-1 Sp1 binding sequence (Fig 5). These data demonstrate that IFN-γ and IL-10 induced specific gel shift complexes on the ICAM-1 IRE that may contain STAT-1α.

To directly demonstrate the presence of STAT-1α, we incubated STAT-1α antibody with nuclear extracts and assessed the effects on binding activity by EMSA. Anti–STAT-1α antibody supershifted the IFN-γ–induced IRE binding complex, whereas the anti–c-fos B antibody had no effect on the binding complex (Fig 6A). The combination of IFN-γ and IL-10 also induced an IRE binding complex that was supershifted by the anti–STAT-1α antibody. These data demonstrate that IL-10 does not alter STAT-1α binding to the ICAM-1 IRE. To determine if STAT-3 was present in the ICAM-1 IRE binding complexes, antibody to STAT-3...
IL-10 BLOCKS IFN-γ-INDUCED ICAM-TRANSCRIPTION

IL-10 inhibits IFN-γ–induced ICAM-1 gene transcription. (A) Nuclei were isolated from monocytes stimulated with IFN-γ (100 U/mL) in the presence or absence of IL-10 (100 U/mL) for 3 hours. Transcription occurred in the presence of labeled ribonucleotides. Isolated RNA was hybridized to ICAM-1 cDNA and GAPDH cDNA that was slot blotted onto nitrocellulose. (B) Normalized absorption values were obtained by densitometry scanning of the ICAM-1. From the ratio of ICAM-1 to GAPDH, the fold increase over untreated control cells was calculated. Data are representative of 2 experiments.

Effect of IL-10 on STAT-1α phosphorylation. STAT-1α is rapidly activated by tyrosine phosphorylation, which allows STAT-1α dimers to bind IRE sequences. Because we were unable to identify a difference in STAT-1α binding in the presence of IL-10, it appears that IL-10 does not inhibit tyrosine phosphorylation of STAT-1α. To directly demonstrate this, we immunoprecipitated STAT-1α from IFN-γ–activated monocytes in the presence or absence of IL-10. Adherent monocytes were treated with cytokines for 5 minutes, solubilized, and immunoprecipitated with anti–STAT-1α antibody. The immunocomplexes were washed, resolved on 8% SDS-PAGE, transferred to nitrocellulose, and probed with antiphosphotyrosine antibody 4G10. In monocytes treated with IFN-γ and IL-10 (Fig 7, lane 4), there was approximately the same level of STAT-1α phosphorylation versus IFN-γ alone (lane 2). Normalization of phosphorylated STAT-1α to the level of STAT-1α protein by laser densitometry showed that an equivalent amount of phosphorylated STAT-1α was detected from IFN-γ and IFN-γ plus IL-10 precipitates. These data in combination with the DNA binding experiments indicate that IL-10 does not inhibit the activity of IFN-γ by altering the binding activity of tyrosine phosphorylated STAT-1α protein.

IL-10 inhibits binding of NF-κB to a proximal site in the ICAM-1 promoter. ICAM-1 promoter also contains binding sites for AP-1, AP-2, AP-3, NF-κB, and Sp1. In EMSAs, we were unable to detect binding of nuclear proteins was incubated with nuclear extracts and assessed by EMSA. Anti–STAT-3 antibody did not supershift the IFN-γ–induced binding complex (lane 4), consistent with previous reports that IFN-γ specifically activates STAT-1α (Fig 6B). However, STAT-3 and STAT-1α were identified in the IRE binding complexes in IL-10–activated monocytes (lanes 7 and 8).

IL-10 does not enhance mRNA degradation. (A) Monocytes were stimulated for 24 hours with IFN-γ (100 U/mL) in the presence or absence of IL-10 (100 U/mL). Actinomycin D (5 μg/mL) was added to the cells, and RNA was extracted at 0, 1, 2, and 3 hours. Northern blot analysis was performed. (B) Induced levels of ICAM-1 mRNA at time zero were determined by densitometry for IFN-γ and IFN-γ plus IL-10. The values were set at 100% and plotted against the percent reduction in the amount of mRNA. The RNA half-life measured the time at which mRNA declined by 50%. Data are representative of 3 experiments.
We show that regulation of ICAM-1 by IL-10 occurs at the level of gene transcription and not at the level of mRNA stability. These observations suggest that IL-10 targets transcription factors interacting with the ICAM-1 promoter elements. Feldman et al. reported that IgG complexes inhibit FcγRI gene transcription in monocytes by inhibiting phosphorylation and binding activity of STAT-1α. However, we were unable to detect any effect of IL-10 on IFN-γ induction of STAT-1α phosphorylation or binding to the ICAM-1 IRE. These findings suggest that IL-10 inhibition of ICAM-1 may involve a novel mechanism distinct from that used to downregulate the FcγRI gene.

It is interesting that ICAM-1 expression in microglial cells, macrophage-like cells in the central nervous system, is induced by IFN-γ stimulation and inhibited by IL-10. However, unlike the effect in human monocytes, IL-10 does not inhibit ICAM-1 mRNA accumulation. In microglial cells, IL-10 affects ICAM-1 expression at the translational and/or posttranslational level, whereas in human monocytes it affects transcription. There are several possible differences between these two cell populations. Microglial cells are a differentiated macrophage-like cell, and transcriptional activation of genes in monocytes is developmentally regulated. There are a number of possible mechanisms that may be responsible for inhibition of IFN-γ-induced ICAM-1 transcription in monocytes. Several observations indicate that not only tyrosine phosphorylation but also serine phosphorylation is required for transcriptional activity of STAT proteins. Eilers et al. showed that a reduction in STAT-1α

**DISCUSSION**

In this study, we investigated the mechanism by which IL-10 inhibits ICAM-1 expression in human monocytes activated with IFN-γ. We show that regulation of ICAM-1 by IL-10 occurs at the level of gene transcription and not at the level of mRNA stability. These observations suggest that IL-10 targets transcription factors interacting with the ICAM-1 promoter elements. Feldman et al. reported that IgG complexes inhibit FcγRI gene transcription in monocytes by inhibiting phosphorylation and binding activity of STAT-1α. However, we were unable to detect any effect of IL-10 on IFN-γ induction of STAT-1α phosphorylation or binding to the ICAM-1 IRE. These findings suggest that IL-10 inhibition of ICAM-1 may involve a novel mechanism distinct from that used to downregulate the FcγRI gene.

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There are a number of possible mechanisms that may be responsible for inhibition of IFN-γ-induced ICAM-1 transcription in monocytes. Several observations indicate that not only tyrosine phosphorylation but also serine phosphorylation is required for transcriptional activity of STAT proteins. Eilers et al. showed that a reduction in STAT-1α
IL-10 BLOCKS IFN-γ–INDUCED ICAM-TRANSCRIPTION

Fig 7. IL-10 does not inhibit tyrosine phosphorylation of STAT-1α.
Monocytes were stimulated with IFN-γ (100 U/mL) in the presence or absence of IL-10 (100 U/mL) for 5 minutes. Cells were lysed, and STAT-1α was immunoprecipitated with Sepharose-bound anti-STAT-1 antibody. Immunoprecipitates were resolved on SDS-PAGE, blotted to nitrocellulose, probed with an antiphosphotyrosine antibody (4G10), and developed by enhanced chemiluminescence. In the lower panel, the blot was stripped and reprobed with anti-STAT-1 antibody and developed. Data are representative of 2 separate experiments.

One of the main outcomes of the inhibitory activity of IL-10 is its ability to alter the composition of dimers formed on the IRE. In monocytes stimulated with IFN-γ, STAT-1α would form homodimers capable of activating the ICAM-1 promoter, whereas in IL-10–activated monocytes, there would be mostly STAT-3 homodimers, which cannot activate the ICAM-1 promoter. Consequently, the combination of IFN-γ/IL-10 could produce heterodimers of STAT-1α and STAT-3 that may function as an inhibitory complex, thereby reducing ICAM-1 transcription.
IL-10 in monocytes is the reduction in cytokine synthesis. Although IL-10 controls cytokine production at the level of transcription, little is known about the mechanism. Experiments from Geng et al.\(^{15}\) showed that LPS induces the early tyrosine kinase p56 lyn in monocytes and that IL-10 inhibits p56 lyn activity. Since tyrosine kinase activity is necessary for LPS activation of IL-1, IL-6, and TNF-\(\alpha\), this may be the mechanism by which IL-10 inhibits cytokine production. Wang et al.\(^{41}\) also reported that LPS- and TNF-\(\alpha\)-induced NF-\(\kappa\)B activity is inhibited by IL-10. They postulated that since NF-\(\kappa\)B activity is required for transcription of many cytokine genes, this may be a common mechanism by which IL-10 inhibits transcription. Consistent with a role for NF-\(\kappa\)B in IL-10 inhibition, we showed that IL-10 inhibited binding of IFN-\(\gamma\)-induced nuclear factors to the proximal ICAM-1 NF-\(\kappa\)B binding site. Taken together, these observations suggest that IL-10 inhibition of IFN-\(\gamma\)-induced ICAM-1 transcription is complex and may involve the interaction of multiple transcription factor binding sites.

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