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Molecular Mechanisms of the Induction of IL-12 and Its Inhibition by IL-10[1]

Miguel Aste-Amezaga, Xiaojing Ma, Alexandrina Sartori,[2] and Giorgio Trinchieri[3]

Exogenously added IL-10 rapidly inhibited *Staphylococcus aureus*- or LPS-induced cytokine mRNA expression in human PBMCs and monocytes, with a maximal effect observed when IL-10 was added from 20 h before until 1 h after the addition of the inducers. Nuclear run-on assays revealed that the inhibition of IL-12 p40, IL-12 p35, and TNF-α was at the gene transcriptional level and that the addition of IL-10 to *S. aureus*- or LPS-treated PBMCs did not affect mRNA stability. The inhibitory activity of IL-10 was abrogated by cycloheximide (CHX), suggesting the involvement of a newly synthesized protein(s). The addition of CHX at 2 h before *S. aureus* or LPS also inhibited the accumulation of IL-12 p40 mRNA, but did not inhibit IL-12 p35 and TNF-α mRNA. This finding suggests that p40 transcription is regulated through a de novo synthesized protein factor(s), whereas the addition of CHX at 2 h after *S. aureus* activation caused superinduction of the *IL-12 p40*, *IL-12 p35*, and *TNF-α* genes. These results indicate that in human monocytes, the mechanism(s) of IL-10 suppression of both *IL-12 p40* and *IL-12 p35* genes is primarily seen at the transcriptional level, and that the induction of the *IL-12 p40* and *p35* genes have different requirements for de novo protein synthesis. ***The Journal of Immunology,*** **1998, 160: 5936–5944.**

The immune response to infectious agents involves a complex interaction of different cell types, which are often regulated in their function by a network of soluble factors or cytokines. Early in the inflammatory response, phagocytic cells produce IL-12, a cytokine that provides an important functional bridge between innate resistance and the adaptive immune response (1). IL-12 is a heterodimeric molecule composed of two covalently linked proteins of 40 kDa (p40) and 35 kDa (p35) (2). Human PBMCs produce high levels of IL-12 after stimulation with bacteria or bacterial products (3). The principal IL-12-producing cell types within the PBMC population are monocytes, but B cells and other accessory cells also produce IL-12 (3). The production of the IL-12 heterodimer requires the coordinated expression of both p40 and p35 chains (4). The expression of the *p40* gene is restricted to those cells that are able to produce the IL-12 heterodimer; in contrast, the *p35* gene is constitutively expressed in most cell types analyzed, although at low levels (3) and although secretion of the isolated p35 protein has not been detected (3). The cells that produce the biologically active IL-12 heterodimer secrete the isolated p40 chain at an excess of several-fold to 1000-fold above the heterodimer.

IL-12 mediates several biologic activities on T and NK cells, including the induction of IFN-γ production, the enhancement of cell-mediated cytotoxicity, and the costimulation of mitogenesis (5–8). In addition, the IL-12 that is produced by accessory cells during early antigenic stimulation is required for the induction of Th1 responses (9); this IL-12 is regulated by a positive feedback mechanism mediated by Th1 cells through IFN-γ or by negative feedback through Th2 cells secreting IL-10 (10). IL-10 is a potent inhibitor of many of the functions of monocyte/macrophages, including oxidative burst, nitric oxide production, phagocytosis, and the production of proinflammatory cytokines, i.e., IL-12, TNF-α, IL-6, and IL-8 (4, 11, 12). Previous studies of human monocytes demonstrated that IL-10 inhibits the LPS-stimulated production of inflammatory cytokines by blocking gene transcription (13, 14). In contrast with these reports, studies of murine macrophages regarding their activation of TNF-α and IL-1 by LPS show that the down-regulation of mRNA gene expression by IL-10 is posttranscriptional (15). However, in LPS-activated monocytes, IL-10 was shown to inhibit granulocyte CSF and granulocyte-macrophage CSF production by destabilizing their mRNA (16). Recently, the mechanism of IL-10 inhibition of class II MHC expression was shown to be dependent upon the inhibition of the transport of the class II molecules to the cell membrane (17). In the present study, we examined the effect of IL-10 on IL-12 gene regulation in human PBMCs and purified monocytes stimulated by *Staphylococcus aureus* or LPS. Our results demonstrate that IL-10 exerts its suppressive effect on *IL-12 p40* and *p35* as well as *TNF-α* gene expression mainly at the transcriptional level by a mechanism that requires de novo protein synthesis. Furthermore, we observe that the LPS- and *S. aureus*-induced expression of the *IL-12 p40* gene, but not that of *IL-12 p35* or *TNF* genes, requires de novo protein synthesis, suggesting that the *IL-12 p40* gene is regulated differently from the *p35* gene and those genes encoding other proinflammatory cytokines.

Materials and Methods

Cytokines and reagents

Chinese hamster ovary cell-derived human rIL-12 (rhIL-12)[4] was provided by Dr. S. Wolf (Genetics Institute, Cambridge, MA); Chinese hamster ovary cell-derived rhIL-10 and murine rIL-10 (rmIL-10) was provided by Dr. K. Moore (DNAX, Palo Alto, CA); and human rTNF-α and human

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[4] Abbreviations used in this paper: hIL, human IL; mIL, murine IL; Act D, actinomycin D; kb, kilobase; UTP, uridine triphosphate; CHX, cycloheximide; RIA, radioimmunoassay; GAPD, glyceraldehyde phosphate dehydrogenase; iNOS, inducible nitric oxide synthase.

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rIFN-γ were provided by Dr. H.M. Shepard (Genentech, South San Francisco, CA). The following reagents were purchased from commercial sources: fixed *S. aureus* Cowan strain I (Calbiochem-Behring, La Jolla, CA), LPS (from *Escherichia coli*, serotype 0127:B8, Sigma, St. Louis, MO), actinomycin D (Act D) (Calbiochem-Behring), cycloheximide (CHX) (Sigma), and DMSO (Sigma).

Abs and cytokine assays

The radioimmunoassays (RIAs) for human TNF-α and human IFN-γ were performed as described previously (18, 19) using mAb pairs B154.9/B154.7 and B133.1/B133.5, respectively. IL-12 p70 and IL-12 p40 were measured in cell-free supernatants by RIA as described previously (3) using the mAb pairs 12H4/C8.6 and C11.79/C8.6, respectively. The RIA for IL-10 was performed using JES3–9D7/JES-12G8 mAbs, which were kindly provided by Dr. A. O'Garra (DNAX).

Cell preparations

Peripheral blood obtained from healthy donors was anticoagulated with heparin. PBMCs were separated on a Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway) density gradient. Monocytes were purified as described previously (20). Briefly, gelatin-coated 175-cm^2 tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ) were incubated with autologous plasma for 1 h at 37°C; plasma was aspirated, and monocytes were obtained after a 1-h adherence of the PBMCs to the flasks at 37°C using 10 mM EDTA to remove the cells after carefully rinsing away nonadherent cells. *Mycoplasma*-free THP-1 human monocytic leukemia cells (21) and murine RAW 264.7 cells (American Type Culture Collection (ATCC), Manassas, VA) were grown in RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 20% heat-inactivated FBS (Irvine Scientific, Santa Ana, CA). All tissue culture media and supplements were endotoxin-free. Cells were cultured in RPMI 1640 medium supplemented with 10% (20% for THP-1 cells) heat-inactivated FBS in T25 tissue culture flasks (Becton Dickinson Labware) for cytokine and RNA analysis (5 ml, 25×10^6 PBMCs or monocytes/flask; 1×10^7 cell lines/flask), and in T75 cm^2 (10 ml, 1×10^8 PBMCs/flask) or T25 flasks (5 ml, 1×10^7 monocytes/flask) for nuclear run-on experiments. Cells were incubated (37°C, 5% CO_2) in the presence of the indicated inducers and/or inhibitors following the protocols outlined in the respective figure legends. Cell lines were cultured under the same conditions as the PBMCs and monocytes, except that the cell lines were pretreated with 1.2% DMSO 24 h before the addition of the inducer (22).

Northern blot hybridization

Northern blots were performed as described previously (23). Briefly, total RNA was extracted from induced and uninduced cells by the guanidine isothiocyanate method, loaded onto a 1% agarose-formaldehyde gel (15 μg/lane), and fractionated. IL-12 p40, TNF-α, IL-10, β-actin, and glyceraldehyde phosphate dehydrogenase (GAPD) mRNA were detected by sequential hybridization on nylon membranes (Schleicher & Schuell, Keene, NH) to the respective [^{32}P]-labeled (Random Primed Kit, Boehringer, Mannheim, Germany) cDNA fragments. Filters were analyzed on a PhosphorImager 445S1 (Molecular Dynamics, Sunnyvale, CA); signal intensities relative to the β-actin GAPD control were determined using ImageQuant software (Molecular Dynamics).

RNase protection assay

RNase protection was performed as described previously (4). Briefly, the vector, containing the entire coding region of IL-12 p35, was linearized with the appropriate restriction enzymes and transcribed using [^{32}P]uridine triphosphate (UTP) (800 Ci/mmol; Dupont, Boston, MA) and the riboprobe kit (Promega, Madison, WI) into a complementary RNA (antisense) riboprobe containing a 266-base pair region which was complementary to the sequence in the IL-12 p35. RNA samples (20 μg) were hybridized in solution with an excess of riboprobes (3×10^5 cpm, specific activity 10^9 cpm/μg, 90°C for 5 min, 42°C for >10 h). A total of 200 μl of RNase solution was added for 30 min at 37°C and the instructions provided for the Ribonuclease Protection Assay RPA II Kit (Ambion, Austin, TX) were followed. The protected fragments were fractionated on 5% polyacrilamide/urea gel and detected using a PhosphorImager 445S1 (Molecular Dynamics).

Nuclear transcription analysis (run-on assay)

The isolation of nuclei and in vitro transcription in the presence of [^{32}P]UTP (3000 Ci/mmol, Dupont) were performed essentially as described previously (24, 25). Nuclear RNA was then isolated after DNase I (Boehringer) and proteinase K (Boehringer) treatment, followed by four phenol/chloroform/isoamyl alcohol extractions and ethanol precipitation at −70°C for 2 h. Unincorporated [^{32}P]UTP was removed using Sephadex G-50 columns (Boehringer). RNA was partially degraded by treatment with 0.2 N NaOH for 10 min at 4°C. [^{32}P]-labeled nuclear RNA was hybridized for 2 days at 60°C to prehybridized nylon filters (Schleicher & Schuell) on which 500 ng of denatured PCR-amplified cDNA corresponding to the coding regions of the *IL-12 p40* and *p35*, *TNF-α*, and *β-actin* genes had been immobilized using a slot-blot apparatus (Hoeffer Scientific, San Francisco, CA). After hybridization, filters were washed at room temperature with 2× SSC, and ssRNA was digested with the same solution containing 10 μg/ml RNase A at 37°C for 30 min. Filters were then washed twice in 2× SSC plus 0.1% SDS for 15 min at 50°C and once in 0.1× SSC plus 0.1% SDS for 30 min at 50°C. The extent of hybridization was quantitated using ImageQuant software on a PhosphorImager 445S1 (Molecular Dynamics).

Generation of hIL-12 p40 promoter-luciferase stable transfectants

RAW 264.7 cells (ATCC) were electroporated using a Genepulser (Bio-Rad, Hercules, CA) at 350 V and 960 μFD with the 3.3-kilobase (kb) hIL-12 p40 promoter-luciferase construct (26) along with a CMV-neomycin expression vector in a molar ratio of 5:1. At 24 h after electroporation, the cells were plated in 96-well plates at 5000 live cells/well in the presence of 800 fg/ml of G418 (Life Technologies, Grand Island, NY). After a 2-wk period, G418-resistant cells were replated in 96-well plates by limiting dilution to generate single-cell clones. The integration of the construct was verified by PCR with genomic DNA isolated from the clones.

Luciferase assay

Luciferase activity was determined in cell extracts prepared according to the Luciferase Assay Kit (Promega).

Results

Kinetics of IL-12 p40, IL-12p70, TNF-α, IL-10, and IFN-γ secretion in S. aureus*-stimulated PBMCs*

S. aureus stimulation induces the expression of multiple cytokines in PBMCs, including IL-12 (p40 and p70), TNF-α, IL-1β, and IL-10 (4). To determine the temporal appearance of IL-12 relative to that of the other induced cytokines, cell-free supernatant fluids were collected after 4, 8, 12, and 20 h of *S. aureus* stimulation and assessed for cytokine production. *S. aureus*-treated PBMCs secreted IL-12 p40, IL-12 p70, TNF-α, IFN-γ, and IL-10 in a time-dependent manner (Fig. 1); TNF-α production was already maximal as early as 4 h after *S. aureus* addition, while IL-12 p40, IL-12 p70, and IL-10 production reached near maximal levels at 8 h poststimulation. Induced levels of IFN-γ were detectable starting at 8 h and increased up to 20 h poststimulation. At 20 h, the levels of IL-12 p40 were 100-fold higher than those of IL-12 p70, in agreement with previous reports (27).

Kinetics of the inhibitory effect of IL-10 on the steady-state mRNA accumulation of IL-12 p40, IL-12 p35, TNF-α, and IL-10 in S. aureus*- or LPS-stimulated PBMCs or monocytes*

To determine the optimal time of cytokine gene expression, mRNA for IL-12 p40, IL-12 p35, TNF-α, and IL-10 were analyzed by either Northern blot or RNase protection assays at different times after *S. aureus* ($1/10^4$ wt/v) or LPS (1 μg/ml) treatment. In PBMCs and monocytes, steady-state levels of IL-12 p40 and p35 showed similar kinetics of expression and were maximal at ~4 h after *S. aureus* stimulation (Fig. 2, *A* and *B*). TNF-α mRNA peaked at 4 h after *S. aureus* induction and at 1 h after LPS stimulation, followed by a rapid decline (Fig. 2). The induction of IL-10 mRNA was delayed and more gradual than that of other genes, peaking between 6 and 12 h after *S. aureus* or LPS stimulation (data not shown and Fig. 2*B*). As previously described (4), when monocytes or PBMCs were stimulated with *S. aureus* or LPS in the presence of added IL-10 (50 U/ml), the cytokine release (data not shown) and mRNA steady-state levels of IL-12 p40,

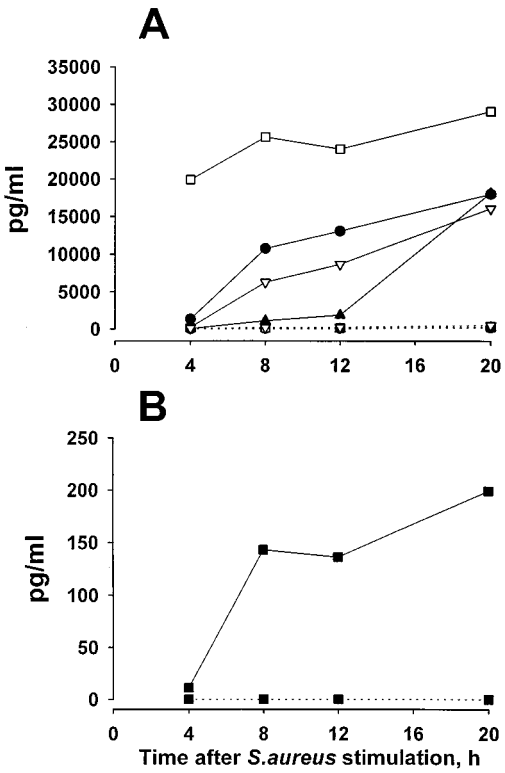

FIGURE 1. Kinetics of IL-12 p40, IL-12 p70, TNF-α, IL-10, and IFN-γ secretion in *S. aureus*-stimulated PBMCs. PBMCs (5×10^6 cells/ml) were stimulated with (solid lines) or without (dashed lines) *S. aureus* ($1/10^4$ w/v) at 37°C, and cell-free supernatant fluids were collected at the time periods indicated and tested by RIA for the presence of: *A*, IL-12 p40 (●), TNF-α (□), IL-10 (▽), IFN-γ (▲); and *B*, IL-12 p70 (■). Data from one of four representative experiments performed with similar results are shown, in which similar kinetics of production of the cytokines were observed, although the absolute concentrations of the cytokines produced showed interdonor variability.

IL-12 p35, and TNF-α (Fig. 2) were inhibited at all time points, with inhibition ranging from 68 to 90% (Table I). The *S. aureus*-induced accumulation of IL-10 mRNA in the presence of IL-10 was not inhibited up to 6 h poststimulation but was completely blocked by 20 h (Fig. 2*B*). Studies performed in the presence of anti-IL-10 demonstrated that endogenous IL-10 inhibited the induction of IL-10 and IL-12 p40 mRNA accumulation after *S. aureus* treatment (data not shown), indicating an autocrine negative feedback control of IL-10. By contrast, LPS-induced IL-10 mRNA was inhibited by the addition of IL-10 throughout the time course.

Time-dependent suppression of S. aureus*-induced IL-12 p40 mRNA by IL-10*

In a series of experiments, IL-10 was added to the PBMC culture at different times relative to *S. aureus*. At 4 h after *S. aureus* stimulation, total RNA was extracted and analyzed by Northern blot hybridization. As shown in Figure 3, *S. aureus*-induced IL-12 p40 and TNF-α mRNA steady-state levels were significantly inhibited when IL-10 was added over a wide time course. Maximal suppression occurred when IL-10 was added at 20 h before *S. aureus* stimulation. The simultaneous addition of *S. aureus* and IL-10 (Fig. 2) resulted in a significant inhibition of both IL-12 p40 mRNA and TNF-α mRNA accumulation. The extent of inhibition remained the same when the cells were pretreated with IL-10 from 3 h before until 1 h after the addition of *S. aureus* and was still detectable when IL-10 was added after 3 h (Fig. 3 and data not shown). Because a significant inhibition was observed when IL-10 was added at 1 h before stimulation, this time point was selected for additional experiments on the mechanism of inhibition; although a 20-h treatment resulted in a more complete inhibition, this time point was not selected, because it was often associated with a loss of viability in the cultures.

Effect of IL-10 on transcription rates of IL-12 p40, IL-12 p35, *and* TNF-α *genes in monocytes and PBMCs*

To determine whether IL-10 inhibits *S. aureus*- or LPS-induced gene transcription, nuclear run-on assays were performed using nuclei isolated from PBMCs or monocytes that had been pretreated with IL-10 for 1 h followed by *S. aureus* or LPS stimulation for 4 h. Figure 4*A* shows a representative experiment in which the induction of the transcriptional rate of IL-12 p40 and TNF-α induced by *S. aureus* in monocytes is significantly inhibited by IL-10. *IL-12 p35* gene transcription appeared to be constitutive with negligible induction, and the effect of IL-10 was also insignificant (Fig. 4*A* and Table II). As shown in Table II, PBMCs and monocytes treated with *S. aureus* showed a 14- $\pm$ 13-fold and an 8- $\pm$ 3-fold increase in the transcription rates of *IL-12 p40* and *TNF-α* genes, respectively, and IL-10 inhibited their transcription by 72 and 59%, respectively. Moreover, LPS-treated PBMCs and monocytes showed a 13- $\pm$ 10-fold and a 3- $\pm$ 0.81-fold increase in the transcription rates of *IL-12 p40* and *TNF-α* genes, respectively, and IL-10 inhibited their transcription by 69 and 49%, respectively (Table II). PBMCs cultured under the same experimental conditions of stimulation as those described above but pretreated with IFN-γ for 16 h showed a significant enhancement of the gene transcriptional level for all three genes, i.e., *IL-12 p40*, *IL-12 p35*, and *TNF-α* (Fig. 4*B*). IL-10 inhibited the gene transcription of IFN-γ-primed *S. aureus*- or LPS-induced genes, including *IL-12 p35*, by 53 to 98% (Table II).

Effect of IL-10 on the stability of S. aureus*-induced IL-12 p40 and TNF-α mRNA*

PBMCs were incubated with or without IL-10 for 1 h, and *S. aureus* or LPS were subsequently added. Act D (5 μg/ml) was added after 4 h to stop further RNA synthesis, and total RNA was extracted at the times indicated after Act D addition and analyzed by Northern blotting (Fig. 5). The calculated $t_{1/2}$ of IL-12 p40 mRNA and TNF-α mRNA (Fig. 5*A*) in PBMCs stimulated with *S. aureus* was 4 h and 1.8 h, respectively; however, the $t_{1/2}$ of p40 was reduced to 2.8 h and that of TNF-α to 0.4 h when LPS was used as an inducer (Fig. 5*B*). The stability of *S. aureus*-induced IL-12 p40 or TNF-α mRNA was not altered by treating PBMCs with IL-10 (Fig. 5*A*). The $t_{1/2}$ of IL-12 p40 mRNA in LPS-stimulated, IL-10-treated cells (Fig. 5*B*) was not measurable because of the low IL-12 p40 mRNA steady-state levels.

Induction of IL-12 p40 mRNA, but not that of IL-12 p35 mRNA, requires de novo protein synthesis

We examined the effect of the protein synthesis inhibitor CHX on the *S. aureus*- or LPS-induced accumulation of cytokine mRNA in PBMCs (Fig. 6*A*), monocytes (Fig. 6*B*), and THP-1 cells (Fig. 6*C*). In these three cell populations, the CHX (10 μg/ml) that was added at 2 h before induction with *S. aureus* or LPS partially blocked the induction of IL-12 p40 mRNA, indicating that IL-12 p40 requires de novo protein synthesis for induction. The observation that IL-12 p35 mRNA accumulation is not inhibited instead is superinduced by CHX (Fig. 6*A*) suggests a different mechanism(s) of regulation for the two components of the heterodimeric IL-12. Consistent with previous reports (14, 15), the accumulation of TNF-α did not require a

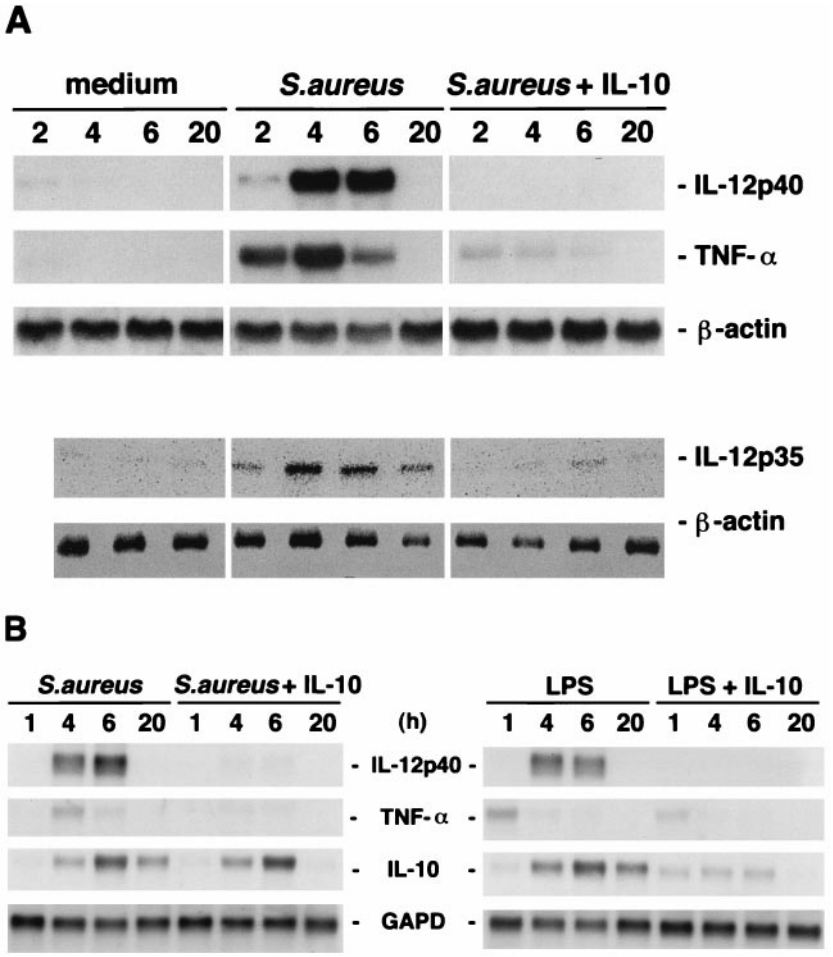

FIGURE 2. Kinetics of cytokine mRNA accumulation in *S. aureus*- or LPS-stimulated cells. PBMCs (*A*) or monocytes (*B*) (5×10^6 cells/ml) were preincubated for 1 h at 37°C in medium supplemented with or lacking rIL-10 (50 U/ml) before the inducers *S. aureus* ($1/10^4$ w/v) or LPS (1 μg/ml) were added. Total RNA was extracted at the time points indicated after the addition of the inducers, and mRNA steady-state levels were determined by Northern blot hybridization with [^{32}P]-labeled IL-12 p40, TNF-α, IL-10, and β-actin cDNA probes. The amount of total RNA loaded/lane was 7 μg (monocytes) or 15 μg (PBMCs). For IL-12 p35, total RNA (20 μg) in solution was hybridized with a [^{32}P]-labeled riboprobe transcribed from IL-12 p35 cDNA, and mRNA accumulation was analyzed by an RNase protection assay. The filters were exposed and quantitated using a PhosphorImager (Molecular Dynamics). Results are from one of four experiments performed with similar results.

newly synthesized protein(s) for induction. Nuclear run-on experiments in PBMCs treated with CHX at 2 h before *S. aureus* stimulation revealed a decreased rate of transcription of *IL-12 p40* but no changes in the transcription rates of the *IL-12 p35* or *TNF-α* genes (Fig. 7). These results show that de novo protein synthesis is required for the optimal induction of *IL-12 p40* gene transcription, but that the superinduced, steady-state mRNA levels of IL-12 p35 and TNF-α, observed in CHX-treated cells, are due to posttranscriptional mechanisms (28–30).

It has been suggested that endogenous IFN-γ plays a positive feedback role in IL-12 p40 mRNA induction (4, 31). Therefore, it was important to determine whether the inhibitory effect of CHX on *IL-12 p40* gene expression was a result of the inhibition of IFN-γ secretion in the cultures. The addition of neutralizing

Table I. *Effect of IL-10 on the steady-state mRNA accumulation of IL-12 p40, IL-12 p35, and TNF-α in* S. aureus- *or LPS-stimulated cells*

	Inducer[a]			
	S. aureus		LPS	
Gene	*n*	% Inhibition by IL-10[b]	*n*	% Inhibition by IL-10[b]
IL-12 p40	17	90 ± 6	7	82 ± 26
IL-12 p35	6	74 ± 20	ND[c]	ND[c]
TNF-α	10	84 ± 6	5	68 ± 11

[a] PBMCs or monocytes (5×10^6 cells/ml) were preincubated for 1 h at 37°C in medium that had been supplemented with rIL-10 (50 U/ml) or left unsupplemented before *S. aureus* or LPS were added. Cells were cultured with *S. aureus* for 4 h to obtain an optimal induction of IL-12 p40, IL-12 p 35, and TNF-α mRNA steady-state levels, and with LPS for 4 h or 1 h to detect IL-12 p40 or TNF-α, respectively. tRNA was extracted, and mRNA steady-state levels were determined by either Northern blotting (IL-12 p40 and TNF-α) or an RNase protection assay (IL-12 p35). The filters were exposed and quantitated using a PhosphorImager. ImageQuant values for IL-12 p40, IL-12 p35, and TNF-α in the indicated number of experiments (*n*) were normalized for RNA loading against the values of accumulated GAPD mRNA.

[b] Percent inhibition by IL-10 was calculated according to the following formula:

$$\frac{\text{Values (}S.\ aureus\text{- or LPS-treated)} - \text{Values (}S.\ aureus\text{- or LPS + IL-10-treated)}}{\text{Values (}S.\ aureus\text{- or LPS-treated)}} \times 100.$$

[c] ND = not done.

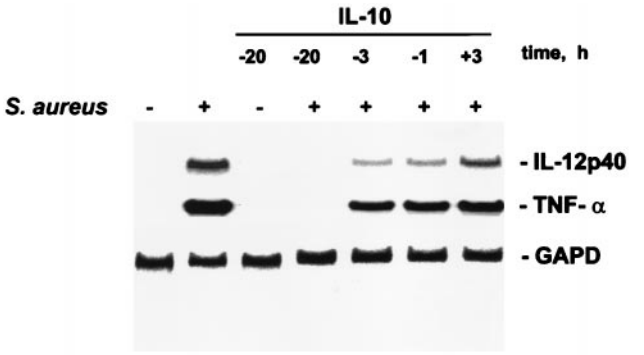

FIGURE 3. Time-dependent suppression of IL-12 p40 and TNF-α mRNA by IL-10. Total RNA was extracted from PBMCs that had been incubated for 4 h in medium alone or in the presence of *S. aureus* ($1/10^4$ w/v) with or without rIL-10 (50 U/ml). IL-10 was added at different times relative to *S. aureus* addition from 20 h before to 3 h afterward. RNA (15 μg/lane) was hybridized with $]^{32}$P[-labeled IL-12 p40, TNF-α, or β-actin cDNA probes. Results are from one of four experiments performed with similar results.

anti-IFN-γ Abs to PBMC cultures at the same time as the CHX addition and 2 h before *S. aureus* stimulation showed that the accumulation of IL-12 p40 mRNA and its inhibition by CHX was similar in the presence or absence of anti-IFN-γ Abs, indicating that the inhibitory effect of CHX on IL-12 p40 induction was not due to the inhibition of endogenous IFN-γ secretion (Fig. 8). Furthermore, the addition of exogenous IFN-γ could not override the inhibitory effect of CHX (data not shown).

Inhibition of IL-12 *and* TNF *gene expression by IL-10 requires de novo protein synthesis*

CHX was added 2 h before *S. aureus* stimulation to determine whether de novo protein synthesis is required for the inhibitory action of IL-10. Figure 6, *A* and *B* shows a representative experiment performed with PBMCs and monocytes, respectively, indicating that CHX completely suppresses the ability of IL-10 to inhibit the *S. aureus*-induced accumulation of IL-12 p40 and p35 and TNF-α mRNA. In nuclear run-on experiments, the ability of IL-10 to inhibit the transcription of the *IL-12 p40* and *TNF-α* genes was similarly suppressed by CHX (Fig. 7). Interestingly, even when CHX was added after IL-10, i.e, at the time of or at 2 h after the addition of *S. aureus*, a marked suppression of the inhibitory effect of IL-10 on IL-12 p40 mRNA accumulation was observed (Fig. 9).

Effect of IL-10 on the hIL-12 p40 promoter in RAW 264.7 cells

In an attempt to search for an in vitro model system whereby the molecular mechanism(s) of the inhibition of *IL-12* gene expression by IL-10 in monocytic cells could be further investigated, we stably transfected an hIL-12 p40 promoter-luciferase construct into the murine monocytic cell line RAW 264.7. This promoter-reporter construct contains ~3.3-kb sequences upstream of the transcription start site that are linked to a luciferase cDNA, and it has been shown previously to recapitulate faithfully the regulation of the endogenous *p40* gene in RAW 264.7 cells in response to IFN-γ and LPS stimulation (26). G418-resistant clones were generated, and one of the clones (no. 3) and a mixed population were tested for their response to IFN-γ/LPS stimulation as well as to IL-10. As shown in Figure 10 (*upper panels*), the endogenous IL-12 p40 production was stimulated significantly by the combination of IFN-γ and LPS treatment and was strongly inhibited by IL-10 added either at 12 h or 2 h before LPS stimulation in both clone no. 3 and the mixed population. The stably integrated hIL-12 p40 promoter was also highly inducible by IFN-γ and LPS treatment, as measured by luciferase activity, but was not affected significantly by IL-10 treatment (Fig. 10, *lower panels*), suggesting that the transcriptional element(s) responsible for the inhibition of the *IL-12 p40* gene by IL-10 is not present in this promoter construct.

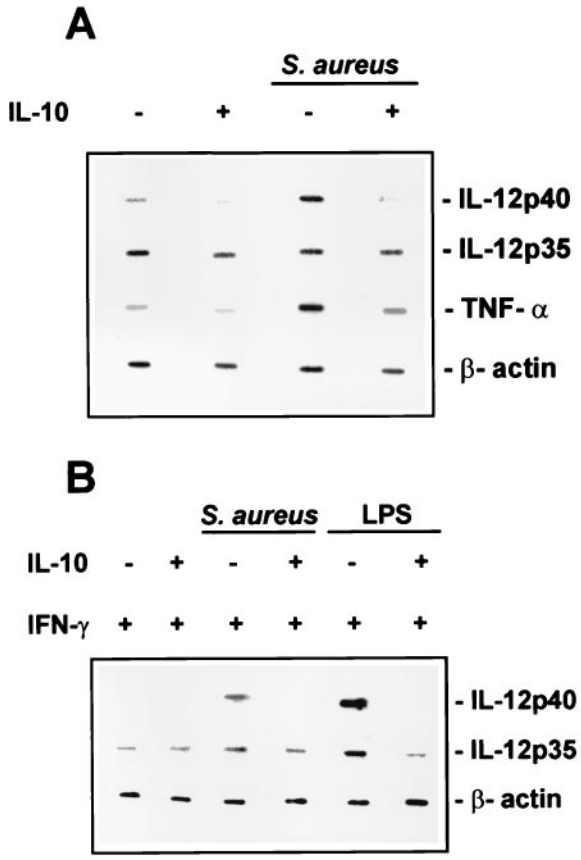

FIGURE 4. Transcription rate of *IL-12 p40*, *IL-12 p35*, and *TNF-α* genes in nuclei from *S. aureus*- or LPS-activated cells. *A*, Monocytes (10×10^6) were cultured for 4 h at 37°C in medium alone or with *S. aureus* ($1/10^4$ w/v) with or without IL-10 (50 U/ml). Treatment with IL-10 was started at 1 h before inducer stimulation. *B*, PBMCs (1×10^8) were pretreated for 16 h with IFN-γ and cultured in medium alone, with *S. aureus*, or with LPS (1 μg/ml) either in the presence or absence of IL-10. The transcription rates of cytokine genes were determined by nuclear run-on and by hybridization of the elongated, labeled RNA transcripts to the indicated slot-blotted cytokine gene fragments amplified by PCR (500 ng/slot). The filters were exposed and quantitated using a PhosphorImager (Molecular Dynamics). Results are from one of four experiments performed with similar results.

Discussion

IL-10 is a potent inhibitor of the expression of several proinflammatory cytokines in monocytes and macrophages. The literature on the mechanisms of action of IL-10 are somewhat contradictory. One study reported that IL-10 exerted its suppressive effect on TNF-α, IL-1β, IL-6, and IL-8 mRNA expression mainly at the level of transcription with a process depending upon de novo protein synthesis (14). By contrast, other data suggested that IL-10 acts mainly at the posttranscriptional level to promote the rapid degradation of TNF-α, IL-1β (15), and IL-8 (32) mRNA. IL-10 inhibition of IL-12 production is accompanied by reduced mRNA steady-state levels of the two IL-12 components, p40 and p35 (4). However, it was not known whether this reduction in IL-12 mRNA accumulation was a consequence of the inhibition of transcription, an enhancement of mRNA

Table II. *Effect of IL-10 on the transcription rate of* IL-12 p40, IL-12 p35, *and* TNF-α *genes in PBMCs and monocytes*

IFN-γ Pretreatment	Gene	Inducer[a]			
		S. aureus		LPS	
		Fold induction[b]	% Inhibition by IL-10[c]	Fold induction	% Inhibition by IL-10
(−)	*IL-12 p40*	14 ± 13 (n = 13)	72 ± 24 (n = 10)	13 ± 10 (n = 3)	69 ± 15 (n = 3)
(−)	*IL-12 p35*	2 ± 0.9 (n = 8)	29 ± 14 (n = 4)	ND[d]	ND[d]
(−)	*TNF-α*	8 ± 3 (n = 13)	59 ± 18 (n = 11)	3 ± 0.8 (n = 4)	49 ± 17 (n = 3)
(+)	*IL-12 p40*	22 ± 12 (n = 3)	89 (n = 2)	51 ± 33 (n = 3)	98 (n = 2)
(+)	*IL-12 p35*	2 ± 0.1 (n = 3)	53 (n = 2)	3 ± 0.6 (n = 3)	65 (n = 2)
(+)	*TNF-ω*	18 ± 17 (n = 3)	57 ± 4 (n = 3)	17 ± 14 (n = 3)	82 ± 17 (n = 3)

[a] PBMCs (2×10^8) or monocytes (10×10^6) were cultured for 4 h at 37°C in medium alone or with *S. aureus* ($1/10^4$ w/v) or LPS (1 μg/ml) with or without IL-10 (50 U/ml). Treatment with IL-10 was started at 1 h before inducer stimulation. Pretreatment for 16 h with IFN-γ was performed only in PBMCs and cultured under the aforementioned conditions. Transcription rates of cytokine genes were determined by nuclear run-on and hybridization of RNA transcripts to the indicated slot-blotted cytokine gene fragments that had been amplified by PCR. The filters were exposed and quantitated using a PhosphorImager. Values for IL-12 p40, IL-12 p35, and TNF-α in the indicated number of experiments were normalized to the levels of β-*actin* gene transcription in the same experimental samples.

[b] Fold induction was calculated according to the following formula: Densitometric values (treatment with *S. aureus* or LPS)/Densitometric values (untreated cells).

[c] Percent inhibition by IL-10 was calculated according to the following formula:

$$\frac{\text{Values (}S.\ aureus\text{- or LPS-treated)} - \text{Values (}S.\ aureus\text{- or LPS + IL-10-treated)}}{\text{Values (}S.\ aureus\text{- or LPS-treated)}} \times 100.$$

[d] ND = not done.

degradation, or both. Our present results show that IL-10 suppressed *S. aureus*- and LPS-induced *IL-12 p40* and *p35* gene expression mainly at the level of transcription in human PBMCs and monocytes, without significant modulation of mRNA stability and regardless of whether they had been primed by IFN-γ.

Wang et al. (33) suggested that the inhibition of NFκB activation is an important mechanism for IL-10 suppression of cytokine gene transcription in human monocytes, while several other transcriptional factors (e.g., NF-IL-6, activator protein-1, activator protein-2, glucocorticoid receptor, cAMP response element-binding protein, Oct-1, and

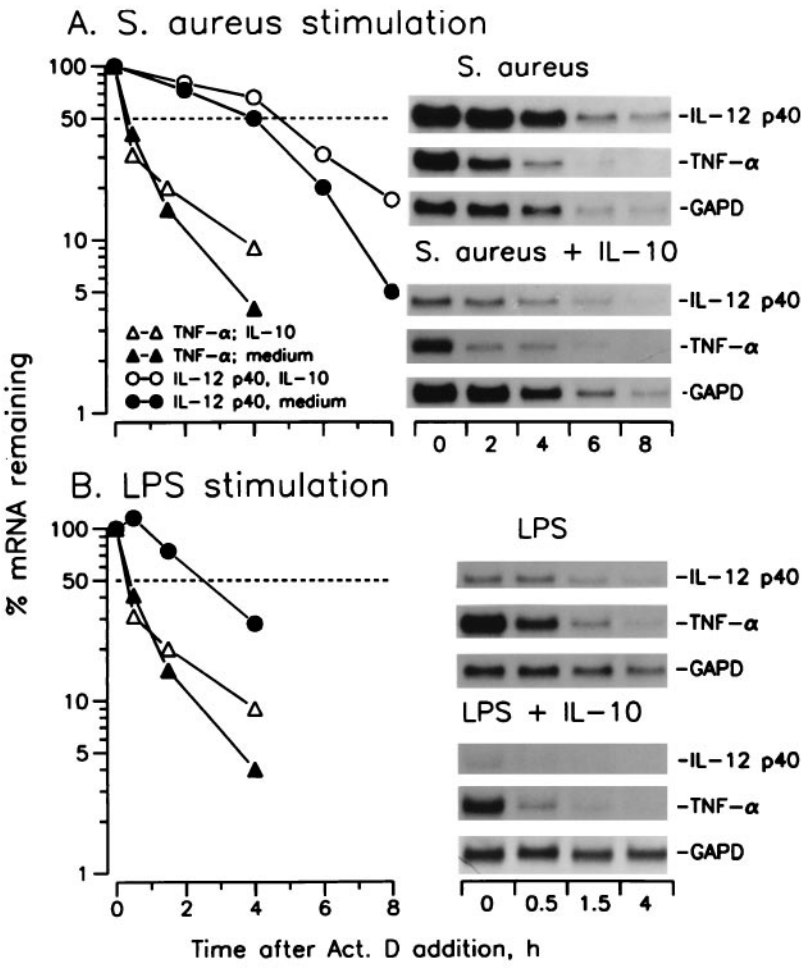

FIGURE 5. Effect of IL-10 on the stability of IL-12 p40 and TNF-α mRNA in *S. aureus*- or LPS-stimulated PBMCs. Cells were incubated for 4 h at 37°C with either *S. aureus* ($1/10^4$ w/v) (*A*) or LPS (1 μg/ml) (*B*), in the presence (open symbols) or absence (closed symbols) of rIL-10. IL-10 was added at 1 h before stimulation. Act D (5 μg/ml) was added at 4 h after stimulus addition. Total RNA was extracted at the time points indicated after Act D addition and analyzed by Northern blot hybridization to IL-12 p40 and TNF-α [^{32}P]-labeled cDNA probes (15 μg total RNA/lane). Hybridization was quantitated by PhosphorImager scanning (Molecular Dynamics). $t_{1/2}$ was calculated from data plotted as the percentage of steady-state mRNA of the initial values at the time addition (Act D) vs decay time. The plotted values in the graphs represent the average of separate experiments, whereas Northern blots show one representative experiment of 3 or 4 when either LPS or *S. aureus*, respectively, were used as inducers. For IL-12 p40 mRNA, the mean and SE of the $t_{1/2}$ for each stimulation were as follows (in h): *S. aureus*, 4.0 ± 0.4 (n = 8); *S. aureus* plus IL-10, 4.8 ± 0.6 (n = 5); LPS, 2.8 (n = 2); and LPS plus IL-10, ND. For TNF-α mRNA, the mean and SE of the $t_{1/2}$ for each stimulation were as follows (in h): *S. aureus*, 1.8 ± 0.6 (n = 7); *S. aureus* plus IL-10, 1.2 ± 0.3 (n = 4); LPS, 0.4 ± 0.03 (n = 3); and LPS plus IL-10, 0.3 ± 0.04 (n = 3).

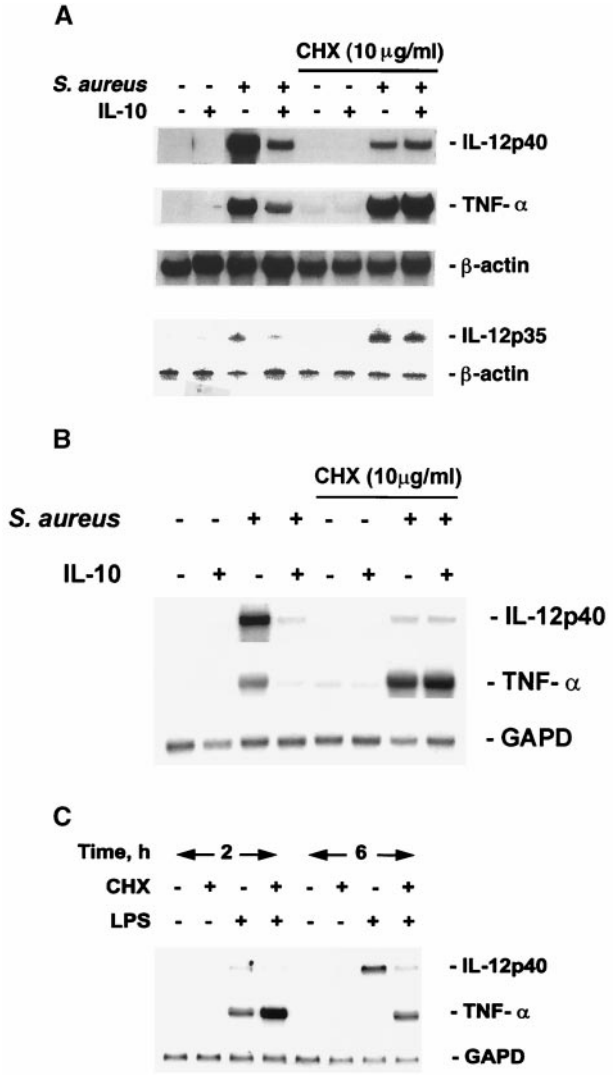

FIGURE 6. Protein synthesis requirement for the induction of IL-12 and its inhibition by IL-10. PBMCs (*A*) or monocytes (*B*) were incubated for 4 h at 37°C in medium alone or in the presence of *S. aureus* ($1/10^4$ w/v) with or without CHX (10 μg/ml) and/or IL-10 (50 U/ml). Cells incubated in the presence of CHX and/or IL-10 were pretreated for 2 h and 1 h, respectively, before stimulation with *S. aureus*. *C*, THP-1 cells pretreated with 1.2% DMSO for 18 h were incubated at 37°C for the indicated times in medium alone or in the presence of LPS (1 μg/ml) with or without CHX (10 μg/ml) and/or IL-10 (50 U/ml), according to procedure described above. Total RNA was extracted, and mRNA levels were determined by Northern blot sequential hybridization with [^{32}P]-labeled IL-12 p40, TNF-α, and GAPD cDNA probes. The amount of RNA loaded was 10 μg/lane. For IL-12 p35, total RNA (20 μg) in solution was hybridized with a [^{32}P]-riboprobe transcribed from IL-12 p35 cDNA, and mRNA accumulation was analyzed by an RNase protection assay. Both Northern blot hybridization and the RNase protection assays were analyzed using a PhosphorImager (Molecular Dynamics) and represent one experiment of four performed with identical results.

Sp-1) are not affected by IL-10 (33). The transcriptional factors that induce IL-12 p40 and p35 expression in monocytes are not defined completely as of yet. The hIL-12 p40 gene promoter contains a consensus sequence binding site for the ets family of transcriptional factors, located at −211 to −207, that is critical to the induction of the promoter by IFN-γ and LPS in the murine macrophage cell line RAW 264.7 (26). The extended region in and around the ets site binds a large nuclear complex, F1, consisting of ets-2, IFN regulatory factor-1, c-Rel, and a novel 109-kDa protein, which is absent in unstimulated cells but is highly inducible by IFN-γ and LPS (34). An "NFκB-half site," originally identified in the mIL-12 p40 promoter (between 121 and −131) (35), is also critical for the response to IFN-γ and LPS. In an attempt to identify the element in the IL-12 p40 promoter that is responsible for IL-10 inhibition, we tested whether IL-10 could act as a suppressor in transient transfection experiments of the promoter activity of several constructs containing up to 3.3 kb of 5′ upstream sequences of the *IL-12 p40* gene and/or the first intron joined to the luciferase reporter gene. No inhibition of the transcriptional activity of the IL-12 p40 promoter was ever observed in these experiments (data not shown), suggesting that the element(s) responsible for IL-10 inhibition may reside outside the DNA regions analyzed. However, because the electroporation procedure used in the transient transfection experiment may have affected the ability of IL-10 to act on the cells, we analyzed the ability of IL-10 to affect the

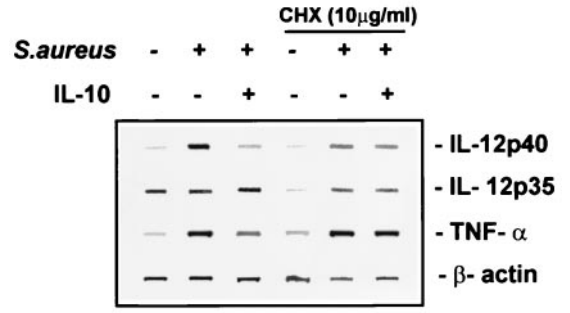

FIGURE 7. Effect of CHX on the transcription rate of cytokine genes in *S. aureus*-activated PBMCs in the presence or absence of IL-10. PBMCs (1×10^8) were incubated for 4 h at 37°C in medium alone or in the presence of *S. aureus* ($1/10^4$ w/v) with or without CHX (10 μg/ml) and/or IL-10 (50 U/ml). Cells stimulated in the presence of CHX and/or IL-10 were pretreated for 2 h and 1 h, respectively, before *S. aureus* addition. The transcription rate for the cytokine genes was determined by nuclear run-on as described in *Materials and Methods*. The filters were exposed and quantitated using a PhosphorImager (Molecular Dynamics). Data are from one experiment of two performed with identical results.

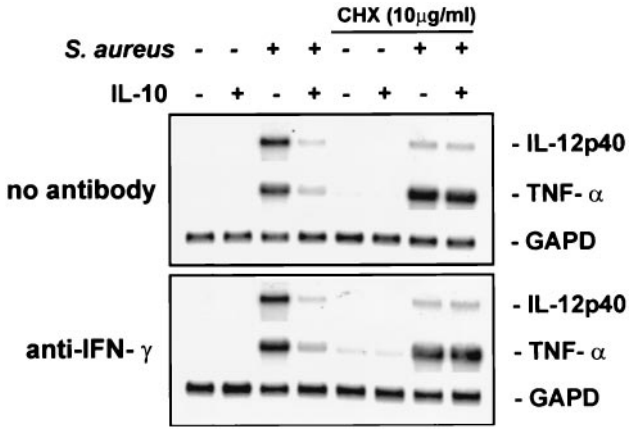

FIGURE 8. Effect of inhibition of endogenous IFN-γ on the *S. aureus* induction of cytokine genes in PBMCs. Cells pretreated for 2 h at 37°C without or with anti-IFN-γ were incubated for 4 h at 37°C in medium alone or in the presence of *S. aureus* ($1/10^4$ w/v) with or without CHX (10 μg/ml) and/or IL-10 (50 U/ml). Cells stimulated in the presence of CHX and/or IL-10 were pretreated for 2 h and 1 h, respectively, before challenge with *S. aureus*. Total RNA was extracted, and mRNA levels were determined by Northern blot hybridization with [^{32}P]-labeled IL-12 p40, TNF-α, and GAPD cDNA probes. The amount of RNA loaded was 10 μg/lane. Data are from one experiment of four performed with identical results.

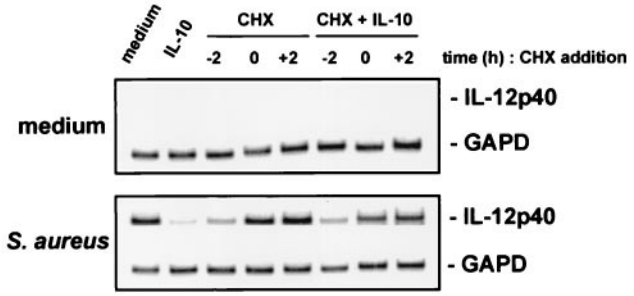

FIGURE 9. Effect of the time of addition of CHX on the up-regulation of IL-12 p40 mRNA induced by *S. aureus*. PBMCs were incubated for 4 h at 37°C in medium alone or in the presence of *S. aureus* ($1/10^4$ w/v) with or without CHX (10 μg/ml) and/or IL-10 (50 U/ml). IL-10 was added 1 h before *S. aureus* addition. Cells were treated with CHX at 2 h before, together with, or 2 h after *S. aureus* challenge. Total RNA was extracted, and mRNA levels were determined by Northern blot hybridization with [^{32}P]-labeled IL-12 p40, and GAPD cDNA probes. The amount of RNA loaded was 10 μg/lane. Date are from one experiment of two performed with identical results.

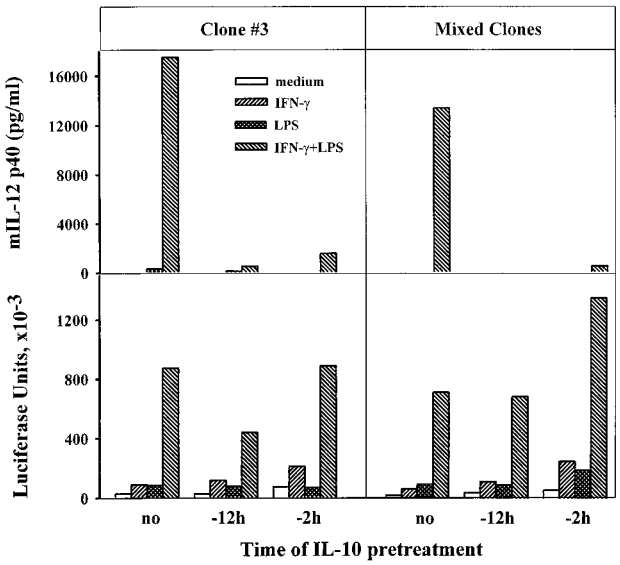

FIGURE 10. IL-10 inhibits the endogenous *IL-12 p40* gene but not the expression of the luciferase gene under the control of a 3.3-kb human p40 promoter in a stably transfected RAW 264.7 cell line. Clone no. 3 and a mixed population of RAW 264.7 cells containing a stably integrated hIL-12 p40 promoter-luciferase construct were pretreated with murine rIFN-γ (1000 U/ml) for 12 h before LPS stimulation (1 fg/ml) for an additional 24 h. rmIL-10 (50 U/ml) was added either at the same time as IFN-γ (-12 h) or 2 h before LPS stimulation (-2 h). The cell-free supernatants were harvested at 24 h after LPS treatment, and the production of endogenous mIL-12 p40 was measured by RIA (*upper panels*). A portion of the cells were harvested at 8 h, and cell lysates (normalized on the basis of total protein content in the lysates) were assayed for luciferase activity, (*lower panels*).

activity of the 3.3-kb IL-12 p40 promoter in stably transfected RAW 264.7 cells. Again, no inhibitory activity of mIL-10 was observed in these experiments on the transfected IL-12 promoter, although IL-10 almost completely suppressed the expression of the endogenous *IL-12 p40* gene in the same cells.

The *S. aureus*- or LPS-induced increase in IL-12 p40 mRNA levels and in *p40* gene transcription were nearly abrogated when cells were pretreated with CHX at 2 h before addition of the inducer, whereas IL-12 p35 mRNA accumulation was superinduced. Because CHX did not block the induction of *IL-12 p35* or *TNF-α* transcription by *S. aureus* or LPS, it is likely that the transcription of those genes depends upon the activation of preexisting transcriptional factors, while the decrease in *IL-12 p40* gene expression may be explained by de novo protein synthesis of a transcriptional factor(s). Although most of the identified transcriptional factors involved in *IL-12 p40* gene transcription are known to be posttranslationally activated, it is possible that other limiting factors may need to be synthesized de novo for the induction of *IL-12 p40* gene transcription; it is also possible that the inhibition of the synthesis of these factors is responsible for the suppressive effect of CHX. Alternatively, CHX may block the secretion of soluble factors in PBMC cultures, which may enhance or be required for the optimal induction of IL-12 p40 in monocytes. In particular, IFN-γ is known to enhance the induction of IL-12 p40 by *S. aureus* or LPS by several-fold (26), and IFN-γ produced in PBMC cultures or by contaminant T and NK cells in enriched monocyte preparations may amplify the expression of the *IL-12 p40* gene. However, it is unlikely that CHX inhibits IL-12 p40 induction by suppressing IFN-γ production, as inhibition was still observed when endogenous IFN-γ was inhibited by anti-IFN-γ Abs and also in the human macrophagic cell line THP-1, which does not produce IFN-γ. The requirement for de novo protein synthesis in the induction of *IL-12 p40* gene expression clearly distinguishes its regulation from that of other proinflammatory cytokines and from IL-12 p35. Interestingly, the induction of another phagocytic cell gene activated by LPS, inducible nitric oxide synthase (iNOS), also requires de novo protein synthesis, and it is blocked by CHX treatment (36). In this case, the inhibition of protein synthesis does not block the NFκB-dependent binding of nuclear protein in the macrophage cell line RAW 264.7 but does block the formation of a second DNA binding complex when a sequence downstream to the iNOS promoter NFκB element is included in the oligonucleotide probe (36). This sequence contains an NF-IL-6 (C/EBPβ) site immediately downstream of the NFκB site (37). Because a C/EBPβ site is also present in the IL-12 p40 promoter downstream of the NFκB site, and CAT enhancer-binding protein β-deficient mice have been shown to have a strong impairment of IL-12 production (38), the possibility that a mechanism similar to that demonstrated for iNOS is involved in the regulation of IL-12 p40 promoter needs to be investigated.

The superinduction of some cytokine genes, such as *TNF-α*, *IL-1β* (28), *IFN-γ*, and *IL-2*, (29, 39), is observed when cells are induced in the absence of CHX for ~2 h, followed by the addition of CHX. This superinduction is attributed to the stabilization of the mRNA, since earlier studies of the c-*fos* gene (40) demonstrated that the mRNA degradation process is dependent upon protein synthesis (28–30). In the present study, we showed that, although the induction of IL-12 p40 transcription, unlike that of IL-12 p35 and TNF-α, requires de novo protein synthesis, its steady-state mRNA level could undergo superinduction if CHX is added 2 h after the inducer, i.e., at a time when transcription is already activated by *S. aureus*. In this respect, the *IL-12 p40* gene is similar to other proinflammatory cytokine genes, including *IL-12 p35* and *TNF-α*.

Our observation, consistent with other reports (14, 15, 32), that the protein synthesis inhibitor CHX abolished the suppressive effect of IL-10 on the induction of IL-12 p40, IL-12 p35, and TNF-α mRNA, suggests that IL-10 may exert its negative effect on cytokine gene transcription through a newly synthesized repressor protein(s). The inhibition of protein synthesis is unlikely to affect the expression of IL-10R on the cells, because the ability of IL-10 to inhibit IL-12 expression is immediately recovered upon washing the cells free of CHX (data not shown). Our data also show that IL-10 acts rapidly to inhibit IL-12 p40 mRNA accumulation even

when added at 1 h postinduction, and its effect was at least partially prevented by blocking protein synthesis even when CHX was added at 2 h after the inducer. Thus, IL-10 is still effective even if added at a time when transcription is already activated; however, its effect needs to be maintained throughout the induction period to achieve efficient inhibition. These findings are not easily reconciled with the demonstration that the inhibitory effect of IL-10 is at the transcriptional level, and this effect is likely to be mediated by a second proteinaceous molecule that is de novo-synthesized upon IL-10 treatment. Furthermore, the nature of this putative second messenger, as well as the promoter element responsible for IL-10 inhibition, remains undetermined. Thus, although this and other studies are starting to shed light on the molecular mechanism of action of IL-10, many aspects are not yet understood. The understanding of the reciprocal regulation of IL-12 and IL-10 may provide some insight into the regulation of cellular and humoral immune responses and may form the basis for therapeutic intervention.

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