The Journal of Immunology

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J Immunol 2001; 166:6358-6366; ;
doi: 10.4049/jimmunol.166.10.6358
http://www.jimmunol.org/content/166/10/6358

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IL-10 Up-Regulates Macrophage Expression of the S100 Protein S100A8[1]

Ken Xu, Tina Yen, and Carolyn L. Geczy[2]

The murine calcium binding protein S100A8 (A8) is a leukocyte chemoattractant, but high levels may be protective and scavenge hypochlorite. A8 is induced by LPS, IFN-γ, and TNF in elicited macrophages. Th2 cytokines generally suppress proinflammatory gene expression, and IL-4 and IL-13 partially decreased A8 induction in macrophages and endothelial cells stimulated by LPS or IFN. In contrast, IL-10 synergized with LPS and IFN to increase mRNA levels ≥9-fold and secreted A8 levels ~4-fold. IL-10 decreased the optimal time of mRNA expression induced by LPS from 24 to 8 h. Blocking experiments indicated that endogenous IL-10 contributes to gene induction by LPS. Cooperation between IL-10 and LPS was not due to altered mRNA stability but was dependent on de novo protein synthesis. Transfection analysis with A8 luciferase constructs confirmed that synergy was due to increased transcription. The region of the promoter involved was localized to a 178-bp fragment flanking the transcription start site of the gene. This region was also responsible for the suppressive effects of IL-4 and IL-13. Forskolin, CTP-cAMP, and PGE_2 also enhanced LPS- and IFN-induced A8 mRNA, whereas indomethacin significantly reduced synergy between IL-10 and LPS. Mitogen-activated protein kinase/cyclooxygenase 2/cAMP pathways involving CCAAT-enhancing binding protein, located within the active promoter, may mediate A8 gene up-regulation in a manner mechanistically distinct to genes regulated by IL-10 via the STAT pathway. A8 exhibits pleiotropic effects, and the high levels secreted as a result of IL-10 synergy may regulate untoward inflammatory damage by virtue of its an antioxidant capacity. *The Journal of Immunology,* **2001, 166: 6358–6366.**

Recent studies implicate S100 proteins in processes involved in inflammation. Human S100A8 (A8)[3] and S100A9 (A9) are associated with neutrophil and monocyte activation processes (1, 2) and adhesion (3) and have antimicrobial properties (4). The proteins are elevated in plasma from patients with chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, in lungs of patients with cystic fibrosis, and in psoriatic epidermis (reviewed in Refs. 5, 6). Murine A8 is expressed at high levels by neutrophils and macrophages (Mac) in bacterial abscesses (7), and lavage fluid from mice with a bleomycin lung contains high levels of the protein (8). Several other S100 proteins are chemotactic (9–11) and have been located at inflammatory sites (11, 12). In the mouse, A8 (formerly known as CP-10) is a potent chemoattractant for neutrophils and monocytes in vitro and in vivo (13–15) and may influence leukocyte margination and transmigration into tissues by increasing leukocyte deformability (16). Up-regulation of the A8 gene by LPS (17), IFN-γ, IL-1, and TNF in elicited Mac (18) and by LPS and IL-1 in microvascular endothelial cells (MEC; 19) suggested a proinflammatory role.

Pleiotropic effects of some cytokines are not uncommon, and TGF-β is a particular example. Like A8, TGF-β is chemotactic at picomolar levels (16), but higher amounts are anti-inflammatory, and it is a key regulator of wound healing and repair (20). Both mediators play a role in development (20, 21). Our recent experiments suggest that A8 may be protective when released at high concentrations, and, in acute inflammatory responses, it may efficiently scavenge hypochlorite anions produced by activated neutrophils (22). Neutrophils contain enormous amounts of A8 (~20% of total cytoplasmic protein), which is released following activation (6). A8 is only expressed in elicited Mac following activation. LPS- (17), IFN-, and TNF-induced gene expression is modulated by Ca^{2+} and by pathways involving protein kinase (PK)C, leading to activation of mitogen-activated (MA)PK (18).

In keeping with the negative regulatory role of Th2 cytokines on proinflammatory properties of activated Mac, we investigated the regulation of the A8 gene by IL-4, IL-10, and IL-13. Although Th2 cytokines have some overlapping effects, they also exhibit distinct actions on Mac. For example, IL-10, but not IL-4 or IL-13, down-regulates MHC class II expression and Ag presentation by monocytes (23) and up-regulates monocyte chemoattractant protein (MCP)-1 production by blood monocytes and alveolar Mac (24, 25), although other chemokines are negatively affected (26, 27). Moreover, IL-10 positively regulates several chemoattractant receptors (28–30). The immunoregulatory mechanisms involving IL-10 appear complex and involve proinflammatory and suppressive properties, although, in general, studies using IL-10-deficient mice indicate that its prime function is to protect the host from overzealous immune/inflammatory responses (31).

Here we present evidence that IL-4 and IL-13 partially suppressed A8 induction in elicited Mac and a microvascular endothelial cell line (MEC). In stark contrast, IL-10 strongly synergized with LPS or IFN to markedly enhance A8 secretion. Synergy may involve a cAMP-dependent pathway, acting principally at the level of transcription, and was confined to a small region of the proximal

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Received for publication December 20, 2000. Accepted for publication March 15, 2001.

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[1] This work was supported by a grant from the National Health and Medical Research Council of Australia. K.X. holds an Australian postgraduate award.

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[3] Abbreviations used in this paper: A8, S100A8; A9, S100A9; Mac, macrophages; MEC, microvascular endothelial cells; PK, protein kinase; MA, mitogen-activated; MCP, monocyte chemotactic protein; C/EBP, CCAAT/enhancer binding protein; TG, thioglycollate; ActD, actinomycin D; CHX, cycloheximide; COX-2, cyclooxygenase 2.

0022-1767/01/$02.00

promoter containing binding sites for Ets and CCAAT/enhancer binding protein (C/EBP) transcription factors.

Materials and Methods

Reagents

RPMI 1640 and DMEM were obtained from Life Technologies (Grand Island, NY), and antibiotics and HBSS were obtained from Sigma (St. Lois, MO). Bovine calf serum, obtained from HyClone Laboratories (Logan, UT), was heated at 56°C before use. Plastic flasks and plates were obtained from Falcon (Lincoln Park, NJ). Thioglycollate (TG) broth and LPS (*Escherichia coli*, 055:B5) were obtained from Difco (Detroit, MI). IFN was obtained from Genentech (San Francisco, CA; 0.032 endotoxin units/mg; specific activity 0.5×10^7 U/mg). Forskolin was obtained from Sigma, IL-4, IL-10, IL-13, TGF-β, and monoclonal anti-mouse IL-10 Ab from R&D Systems (Minneapolis, MN), and PGE_2, CTP-cAMP, Br-cAMP, Br-cGMP, SB202190, and PD98059 from Calbiochem (Croydon, Victoria, Australia). For inhibition of mRNA-synthesis, 5 mg/ml actinomycin D (ActD; Calbiochem) in ethanol was diluted into medium. Cycloheximide (CHX; Sigma) was used as protein synthesis inhibitor.

Cell culture

TG-elicited Mac were obtained as described (18), and washed cells (5×10^6) in 60-mm tissue culture plates were incubated for 1 h at 37°C in 5% CO_2 in air, washed three times with warm (37°C) HBSS to remove nonadherent cells, and equilibrated in culture medium for 18 h. Culture medium (3 ml) was replenished before activation, and populations contained >98% Mac (~98% viable by trypan blue exclusion) and <0.3% neutrophils. Mac were stimulated for up to 96 h with the agents indicated.

The murine monocyte-Mac cell line RAW 264.7 (TIB 71; American Type Culture Collection, Manassas, VA) and the murine edothelioma cell line (MEC) derived from brain (bEND-3) were cultured as described (17, 19). MEC were stimulated once they had reached postconfluence (6–7 days).

Northern analysis

Total cellular RNA (from ~5×10^6 Mac) was size fractionated and transferred onto membranes as described (17). Hybridizations with A8 and A9 riboprobes were for 16 h at 58°C and at 36°C for the 18S rRNA oligoprobe in formamide-containing buffer as described (19). Membranes were washed twice at 48°C for 10 min in 2× standard saline citrate phosphate/EDTA with 0.1% SDS, then twice with 0.1× standard saline citrate phosphate/EDTA with 0.1% SDS at 65°C for 30 min. Phosphor imager analyses were performed with a Bio-Rad Molecular Imager GS-525 system (Bio-Rad, Hercules, CA). The relative magnitude of expression for each gene was determined using software packages and normalized to the level of 18S RNA on the same blot. Blots were stripped according to the manufacturer's instructions.

Quantitation of A8 protein

A8 in supernatants or cell lysates was quantitated using a double-sandwich ELISA and rabbit polyclonal anti-A8 IgG as described (7, 17) using recombinant A8 (0.1–50 ng/ml) as standard. The lower limit of detection was ~30 pg/ml.

Reporter plasmids

Truncated promoter fragments of the A8 gene were produced either by using conveniently located restriction endonuclease sites or by nested deletion of a PCR-amplified product. In brief, two fragments extending from *Eco*RI and *Xbo*I sights (−917 and −665, respectively) to an *ASP*700I site at +465 (8 bp before the start codon in exon 2) were excised from pCP110, end-filled, and placed upstream of the luciferase reporter gene in pGL2-basic vector (Promega, Madison, WI) at an end-filled *Hin*dIII site. The constructs were designated pCP-917/+465 and pCP-665/+465, respectively. A 783-bp fragment spanning −316 to +465 was amplified by PCR using pUC/M13 forward sequencing primer (GTTTTCCCAGTCACGAC) and exon primer II (TGTCagatctGATTTCCTTTCAACTGA). PCR was performed as described (32) but using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Following *Bam*HI/*Bgl*II digestion, the fragment was subcloned into *Bgl*II sites of pGL2-basic producing pCP-316/+465. The 5′ deletion mutants (with a common 3′-end at +465) generated using the double-stranded Nested Deletion kit (Amersham Pharmacia Biotech, Uppsala, Sweden) yielded the pCP-*x*/+465 construct series (*x* represents different 5′ ends). The extent of deletion was verified by sequencing.

Transient transfections

RAW 264.7 cells were transiently transfected as described (18) using 2×10^5 cells/well seeded into 12-well plates 24 h before transfection, and then 0.5 μg luciferase reporter plasmid or 0.1 μg reference plasmid (pRL-TK; Promega) was transfected in the presence of DEAE-dextran (Sigma; 300 μg/ml). After 24 h, cells were stimulated for 20 h with the agents indicated, and firefly and Renilla luciferase activities assayed with 20 μl extract using Promega reagents according to the manufacturer's instructions. Results are expressed as mean ± SD of luciferase activity from three separate experiments. Data was analyzed using the Student *t* test, and differences were considered statistically significant when $p < 0.05$.

Results

IL-4 and IL-13 suppress A8 mRNA induction

IFN rapidly and maximally induces A8 mRNA 12 h after stimulation of elicited Mac, whereas LPS responses are optimal after 24 h (18). To compare the effects of Th2 cytokines on A8 mRNA expression, TG-elicited Mac were treated with IL-4 and IL-13 in the presence or absence of IFN or LPS using these time points. IL-4 and IL-13 did not alter basal A8 mRNA levels (Fig. 1*A*) but suppressed positive responses by ~50% (Fig. 1*B*). Higher doses (30 ng/ml IL-4 or 30 ng/ml IL-13) did not potentiate inhibition (data not shown). Suppression at the mRNA and protein levels was observed with LPS-activated RAW 264.7 cells. Secreted A8 produced in response to LPS was reduced by ~90% by IL-4; IL-13 caused ~66% suppression (Table I).

Murine A8 is also induced in MEC by LPS and IL-1β (19). IL-4, -10, and -13 did not alter basal A8 mRNA levels (Fig. 2*A*), and IL-4 suppressed the LPS-induced response by 50%. Although not as potent as IL-4, when IL-13 was incubated with LPS, gene induction decreased by ~20% (Fig. 2*B*). IL-10 weakly reduced mRNA levels in LPS-activated MEC, and suppression was additive when IL-10 was mixed with IL-4 or IL-13.

IL-10 and TGF-β amplify inducible A8 in Mac

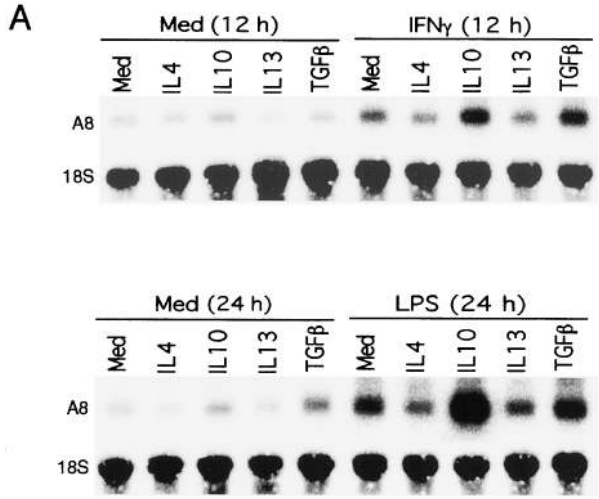
IL-10 did not induce A8 mRNA directly (Fig. 1*A*) but increased the LPS-activated response >5-fold after 24 h stimulation, and mRNA levels of IFN plus IL-10-stimulated cells harvested at 12 h were 2-fold higher (Fig. 1*B*). Similar results were observed with RAW 264.7 cells (data not shown) and were reflected by ~3-fold higher A8 in supernatants following LPS plus IL-10 stimulation (1.78 ± 0.14 ng A8/ml) compared with levels induced with LPS alone (0.59 ± 0.06 ng/ml; Table I).

TGF-β is also involved in resolution of inflammation, and responses to TGF-β were compared with those provoked by the Th2 cytokines. Fig. 1*B* shows moderate A8 mRNA induction at 24 h, but not at 12 h (Fig. 1*A*), after addition of TGF-β, and responses to IFN at 12 h and LPS at 24 h increased ~1.5-fold when TGF-β was included (Fig. 1*B*). A8 in supernatants of LPS-activated RAW 264.7 cells increased from 0.59 ± 0.06 ng/ml to 0.75 ± 0.26 ng/ml when LPS and TGF-β were cocultured.

Because IL-10 generally down-regulates proinflammatory responses in Mac, the marked up-regulation of the A8 gene is unusual, and mechanisms involved in potentiation of LPS responses were investigated more fully.

Characterization of IL-10-mediated synergy of A8 expression

To confirm synergy between IL-10 and LPS, increasing amounts of LPS were cultured with a constant amount of IL-10 (10 ng/ml). In agreement with our previous data (18), elicited Mac responded to 0.5–50 ng/ml LPS with little potentiation with higher amounts (500 ng/ml; Fig. 3*A*). When IL-10 was included, a linear increase in mRNA was evident with doses of LPS between 0.5 and 50



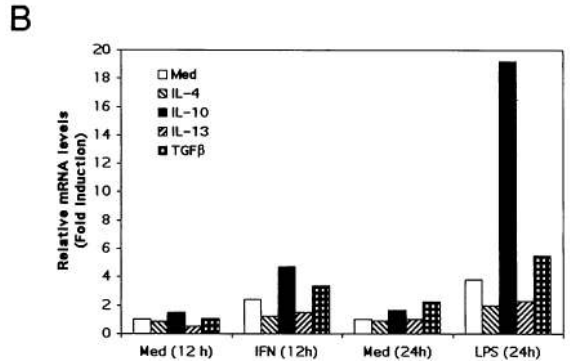


FIGURE 1. Th2 cytokines and TGF-β regulate A8 gene expression in elicited Mac. *A*, Mac were untreated or treated with IL-4 (10 ng/ml), IL-10 (10 ng/ml), IL-13 (10 ng/ml), or TGF-β (5 ng/ml) in the presence/absence of IFN (300 U/ml) for 12 h (*upper panel*) or in the presence/absence LPS (100 ng/ml) for 24 h (*lower panel*). A8 and 18s RNA was analyzed by Northern blotting. *B*, Northern blots were quantitated by phosphor image analysis. Relative mRNA levels are presented as fold increases above controls at 12 h (for IFN) or 24 h (for LPS). Results are representative of three experiments.

ng/ml, with maximal synergy at 50 ng or greater. An LPS dose of 100 ng/ml was used in all subsequent experiments.

IL-10 showed a dose-dependent induction of A8 mRNA in the presence of LPS, with marked amplification at 10 ng/ml (Fig. 3*B*). IL-4 suppressed the synergy, reducing A8 mRNA levels almost to those of LPS alone (Fig. 3*B*). A8 in supernatants of RAW 264.7 cells stimulated with LPS and IL-10 was 1.78 ng/ml, and IL-4 or IL-13 reduced this to 0.01 $\pm$ 0.01 or 0.07 $\pm$ 0.02 ng/ml, respectively (Table I).

IL-10 markedly decreased the optimal time of A8 mRNA induction by LPS. Low mRNA levels were evident 6 h after addition of LPS; levels peaked at 24–48 h and declined slowly thereafter (Fig. 4*A*). The dramatic increases in mRNA (~9-fold) in the presence of IL-10 were maximal by 8–12 h and declined rapidly to levels below those induced by LPS alone after 48 h. A8 in supernatants reflected this rapid increase and remained 3- to 4-fold higher than levels in supernatants from Mac stimulated by LPS alone until 72 h after stimulation (Fig. 4*B*).

Because IL-10 is secreted by LPS-activated Mac, induction of the LPS-induced A8 gene in the presence of neutralizing anti-

Table I. *IL-4, IL-10, IL-13, and TGF-β regulate A8 production*[a]

Treatment	A8 (ng/10^6 cells)
LPS	0.59 $\pm$ 0.06
LPS + IL-4	0.07 $\pm$ 0.02
IL-10	1.78 $\pm$ 0.14
IL-13	0.20 $\pm$ 0.03
IL-4 + IL-10	0.01 $\pm$ 0.01
IL-10 + IL-13	0.07 $\pm$ 0.02
IL-4 + IL-10 + IL-13	[Not detected]
TGF-β	0.75 $\pm$ 0.25

[a] RAW 264.7 cells were stimulated with LPS (100 ng/ml) for 24 h together with IL-4 (10 ng/ml), IL-10 (10 ng/ml), IL-13 (10 ng/ml), or their combinations, or with TGF-β (5 ng/ml). A8 levels in supernatants were quantitated by ELISA. Results are expressed as nanograms A8 generated by 10^6 cells and represent the means + SD of duplicate values from three experiments. A8 was not detected in supernatants from cells cultured either without LPS or with the cytokines or their combinations without LPS.

IL-10 mAb was examined. Relative mRNA levels were reduced by ~50% 24 and 48 h after stimulation with LPS (Fig. 4*C*).

Mechanisms of IL-10-mediated A8 gene expression

Many IL-10-mediated effects are via induction of new genes. If IL-10-increased LPS-induced A8 was due to new protein synthesis, synergy may be enhanced in Mac pretreated with IL-10 and/or blocked in Mac costimulated with a protein synthesis inhibitor. A8 mRNA in Mac preincubated with IL-10 for 1–3 h before addition of LPS were greater (12-fold increased mRNA levels compared with LPS alone) than preincubation for 24 h (3-fold increase) (Fig. 5*A*). When LPS and IL-10 were added together at the start of the culture, mRNA levels were ~10-fold more than those produced by LPS alone. Synergy was reduced by ~60% when IL-10 was added 3 h after LPS and became less obvious after later addition. Mac treated with LPS $\pm$ IL-10 and a concentration of CHX which inhibits protein synthesis by >95% (33) did not express A8 mRNA levels significantly above controls (Fig. 5*B*), confirming a requirement for new protein synthesis.

The IL-10-mediated activation of genes may result from alterations in transcriptional activity (28) or in the stability of primary transcripts and/or of mature cytoplasmic RNA or from both (34, 35). To elucidate mechanism(s), Mac incubated with LPS for 24 h or with LPS plus IL-10 for 12 h were treated with ActD for various times to block further transcription. As reported earlier, ActD transiently increased LPS-induced A8 mRNA within the first 4 h and then declined gradually, with a half-life of 16 h (18). IL-10 diminished the early increase in mRNA levels, and half-life was reduced to 10 h (Fig. 6). Although steady-state levels of A8 mRNA were different in LPS plus IL-10- and LPS-treated Mac, degradation rates were comparable, indicating little effect of IL-10 on the stability of LPS-induced A8 mRNA.

To determine whether IL-10 synergy involved A8 gene transcription and to locate LPS and IL-10 response elements in the region 5′ to the transcription start site, a series of 5′ deletion fragments of this sequence linked to a luciferase reporter gene were transiently transfected into RAW 264.7 cells. LPS treatment increased luciferase activity 7- to 10-fold in cells transfected with the pCP-178/+465 A8 promoter construct (Fig. 7*A*). IL-10 alone also caused a small but reproducible increase (~3-fold), and, when used with LPS, luciferase activity was potentiated to 14- to 20-fold (Fig. 7*A*), confirming enhanced transcription.

Analysis of unidirectional deletion mutants of the A8 promoter fragment indicated that responses to LPS or LPS plus IL-10 were

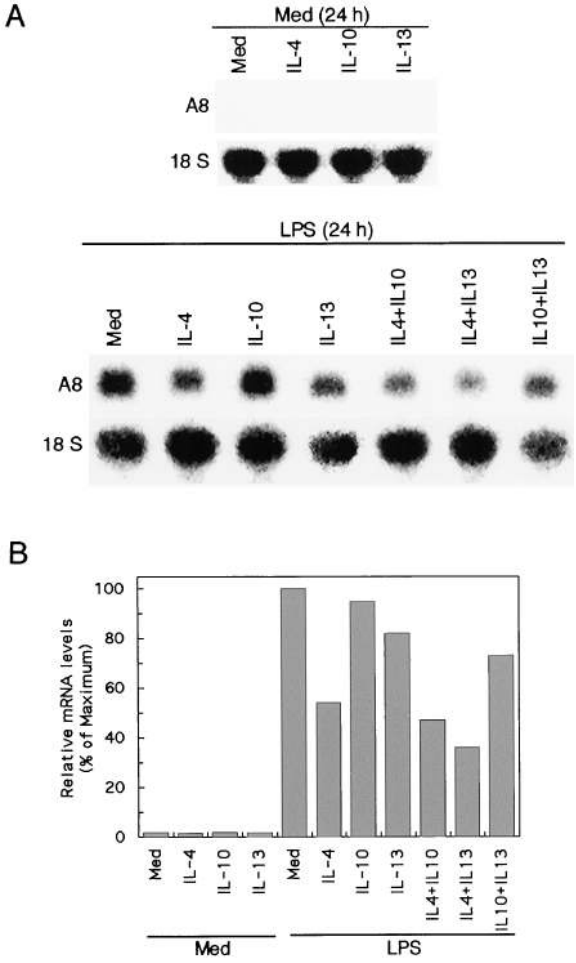


FIGURE 2. Th2 cytokines regulate A8 gene expression in murine microvascular endothelial cells. *A*, The bEND-3 cells were untreated or treated with IL-4 (10 ng/ml), IL-10 (10 ng/ml), or IL-13 (10 ng/ml) or their combinations in the presence or absence of LPS (100 ng/ml) for 24 h before extraction of RNA for Northern blot analysis. *B*, Relative mRNA levels of A8 are presented as the percentage of maximal expression induced by LPS normalized to 18S rRNA. Results are representative of three experiments.

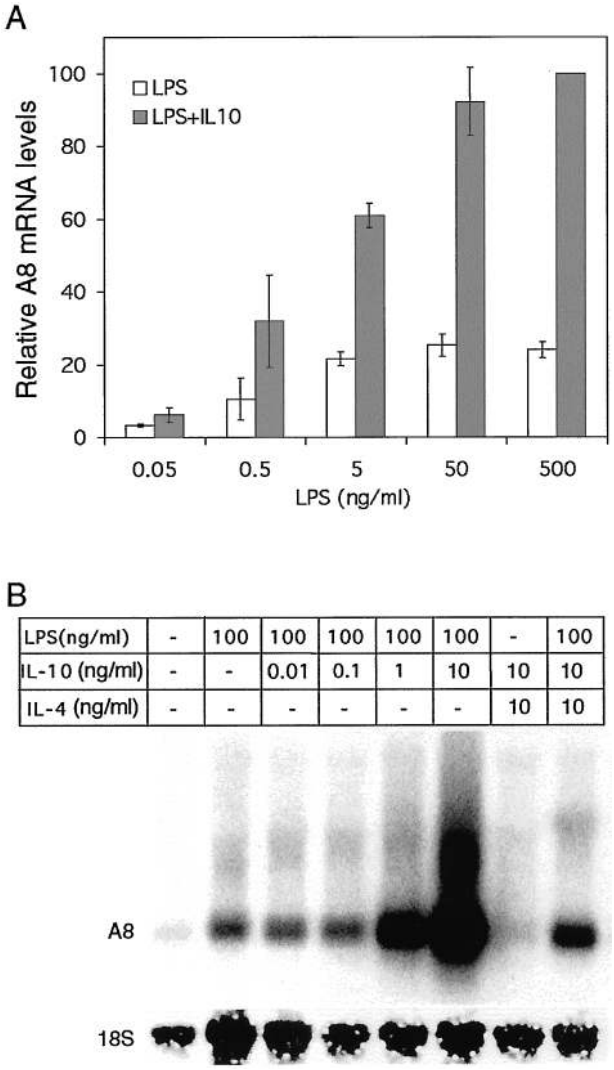


FIGURE 3. LPS and IL-10 synergize to induce high levels of A8. *A*, Induction of A8 mRNA by varying doses of LPS. TG-elicited Mac were cultured with LPS in the presence or absence of IL-10 (10 ng/ml) for 24 h, and A8 mRNA was analyzed by Northern blotting. Relative mRNA levels represent percentage of maximal expression. Data represent means ± SD of three experiments. *B*, Amplification of LPS-induced A8 mRNA by IL-10. Northern blotting of RNA from Mac treated for 24 h with LPS (100 ng/ml) in the presence or absence of increasing concentrations of IL-10. IL-4 (10 ng/ml) was also added with IL-10 (10 ng/ml) in the presence or absence of LPS. Data are representative of two experiments.

located between positions −178 and −94; the 84-bp fragment remained sensitive to LPS and IL-10 (Fig. 7*A*). The minimal construct pCP-178/0 was used to test whether IL-4 and IL-13 suppression of the LPS-inducible A8 gene was also confined to this region. Fig. 7*B* shows that IL-4 and IL-13 reduced LPS-induced responses by 50%. Although IL-4 and IL-13 did not reduce IL-10-induced luciferase activity, they strongly suppressed IL-10-LPS synergy, and results correlated closely with effects on mRNA and protein levels described above. Taken together, IL-4, IL-13, and IL-10 appear to regulate A8 gene expression of LPS-stimulated Mac via transcriptionally mediated processes through DNA elements present in the defined promoter of this gene.

Is synergy with IL-10 via PGE_2 and cAMP?

Some S100 genes are activated via PKC,- PKA-, and/or Ca^{2+}-dependent pathways (18, 36, 37). Preliminary experiments to determine intracellular mechanisms involved in A8 gene regulation by IL-10 were performed. Although not activating alone, PKA activators PMA, (18), Br-cAMP, and CTP-cAMP markedly increased IFN- and LPS-induced A8 mRNA, and the cAMP-elevating agent forskolin potentiated the LPS response, albeit to lower levels than the other agents (Fig. 8*A*). CTP-cAMP did not potentiate A8 mRNA levels induced by LPS plus IL-10. PGE_2 tested at two doses did not alter basal A8 mRNA levels but substantially increased the LPS-induced response (Fig. 8*B*), whereas indomethacin markedly reduced synergy between LPS and IL-10 (Fig. 8*C*).

Weak potentiation of the LPS response by PGE_2 was confirmed using the pCP-178/0 reporter construct (Fig. 8*C*). In three separate experiments, luciferase activity in transfected Mac stimulated with LPS alone increased 1.28 ± 0.06-fold with PGE_2 plus LPS ($p < 0.05$), suggesting a requirement for other elements outside the region of the essential promoter for full enhancement by PGE_2. The synergy evident with LPS plus IL-10 increased from 1.83 ± 0.08-fold (relative to LPS alone) to 2.34 ± 0.17-fold when PGE_2 was included ($p < 0.05$). Conversely, indomethacin suppressed LPS plus IL-10 synergy ($p < 0.01$, compared with LPS plus IL-10) to

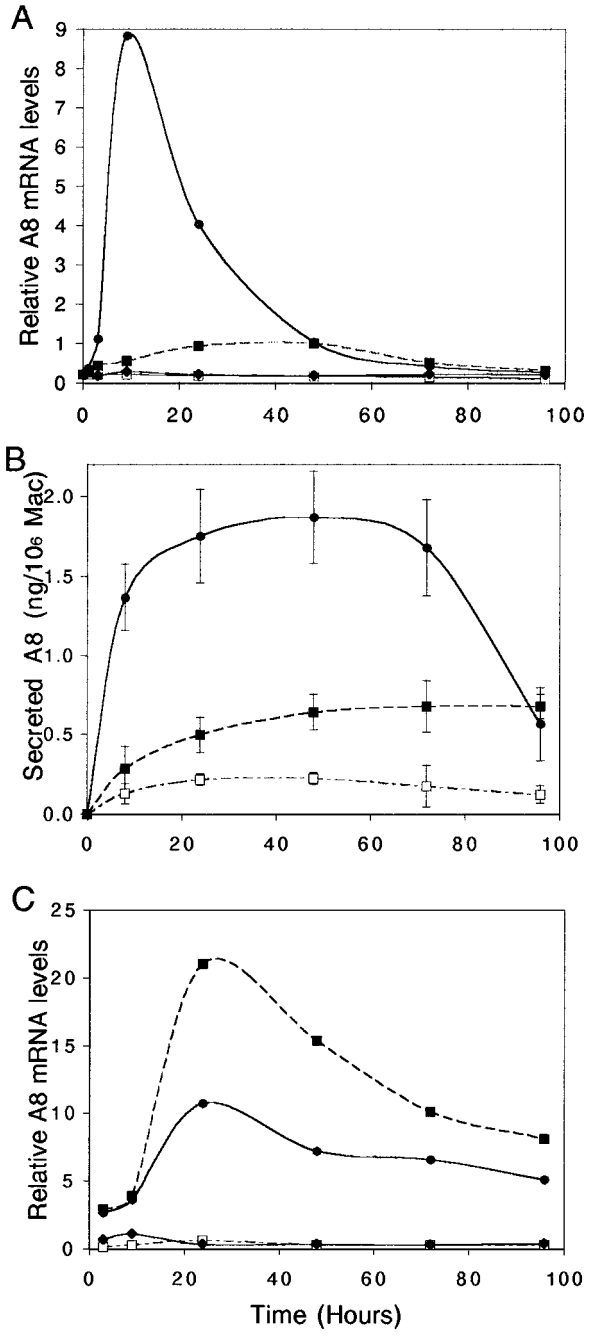


FIGURE 4. IL-10 reduces the optimal time for induction of A8 by LPS and mediates the LPS-induced response. *A*, Mac were untreated (control, □) or stimulated with 10 ng/ml IL-10 (♦), 100 ng/ml LPS (■), or IL-10 and LPS combined (●) for the times indicated before Northern blot analysis. Relative A8 mRNA levels are presented as fold increases of maximal mRNA levels induced by LPS. Results are representative of two experiments. *B*, A8 in supernatants of elicited Mac, untreated (□), treated with LPS (■), or with LPS plus IL-10 (●), was quantitated by ELISA. Protein (nanograms A8 generated by 10^6 Mac) is expressed as the mean ± SD of duplicates from three experiments. *C*, Cells were untreated (□), treated with LPS as in *A* with (●) or without (■) IL-10 mAb (2 μg/ml) and anti-IL10 mAb alone (♦). Relative mRNA levels are plotted against duration of stimulation. Data are representative of two experiments.

levels provoked by LPS alone, although LPS-induced luciferase activity was unaffected. Taken together, IL-10 may amplify LPS-induced A8 via PGE_2 and cAMP produced via the cyclooxygenase (COX)-2 pathway.

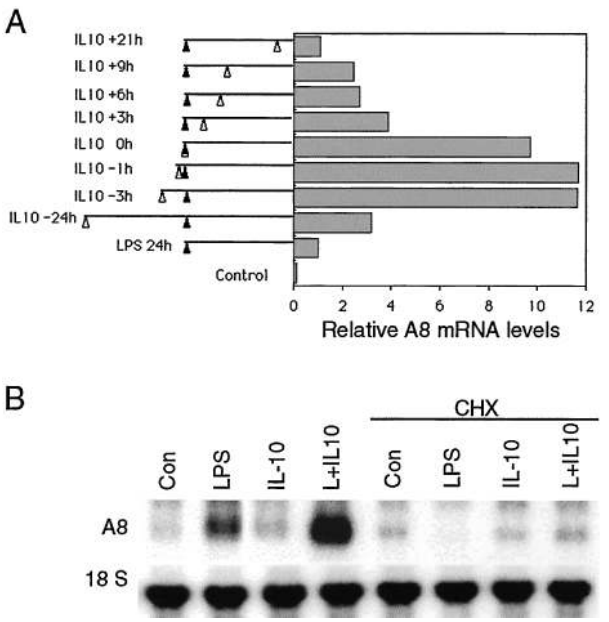


FIGURE 5. New protein synthesis is required for the synergy of A8 mRNA induction. *A*, Effect of time of addition of IL-10 on LPS-induced A8 mRNA expression. Mac were treated with LPS (100 ng/ml) for 24 h (▲). IL-10 (△; 10 ng/ml) was added at the indicated times relative to the addition of LPS (▲). The numbers represent 24, 3, or 1 h before, with, or 3, 6, 9, or 21 h after addition of LPS. Relative mRNA levels are presented as fold increases in A8 mRNA above that induced by LPS alone. Data are representative of two experiments. *B*, Effects of CHX on IL-10-mediated synergy of LPS-induced A8 mRNA expression. Northern blot analysis of A8 mRNA levels in Mac treated with LPS (100 ng/ml) and/or IL-10 (10 ng/ml) in the presence or absence of CHX (2 μg/ml); total RNA extracted after 12 h. Results are representative of three experiments.

Discussion

The roles of A8 in inflammation are still unclear. In the mouse, A8 is induced in Mac by LPS, IFN, and TNF in the absence of A9 (17, 18), whereas the human counterparts are generally coexpressed and the A8/A9 heterodimer is implicated as the functional form (5, 6). Murine A8 is chemotactic for monocytes and neutrophils at picomolar levels in vitro, but the human homologue does not share this function (13). In contrast, A8 from both species is highly susceptible to oxidation by hypochlorite, a property not shared with A9 (22). We suggested that, in acute inflammation, A8 expressed in enormous amounts by neutrophils and released at inflammatory sites (7, 8) may protect the host against excessive oxidative damage. Here we show that induction of murine A8 is differentially regulated by agents that generally down-regulate proinflammatory Mac functions.

IL-4 and IL-13 inhibited mRNA induced by LPS in Mac (Fig. 1) and MEC (Fig. 2) by ~50%. Transient transfection analysis of an A8 reporter construct into RAW 264.7 cells confirmed similar levels of suppression of gene transcription by both cytokines (Fig. 7*B*). Although IL-4 and IL-13 at high levels never reduced mRNA more than 60%, inhibition was additive when the mediators were used together (Figs. 2 and 7*B*), yielding secreted protein levels barely above baseline (Table I). Suppression by IL-4 and IL-13 is not unexpected because they share the IL-4α receptor component, thereby activating common signaling pathways (38). In contrast, IL-10 markedly enhanced A8 gene transcription and A8 protein secreted by Mac cocultured with LPS or IFN (Fig. 1 and Table I). The mRNA levels in MEC were relatively unaffected (Fig. 2), suggesting cell-specific regulation of the A8 gene by IL-4, IL-13,

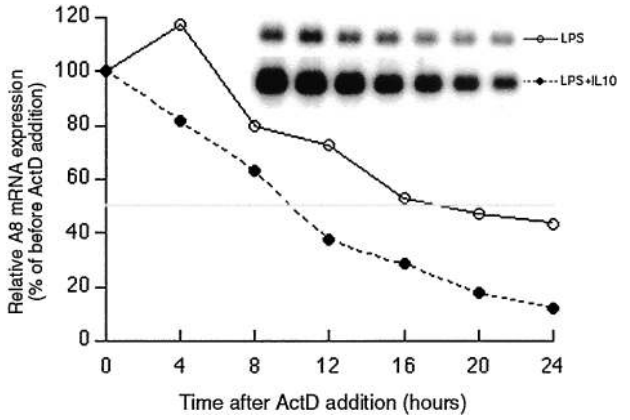


FIGURE 6. Effect of IL-10 on stability of LPS-induced A8 mRNA. Mac were stimulated with LPS (100 ng/ml) in the presence or absence of IL-10 (10 ng/ml) for 12 h. RNA was extracted immediately or after incubation of cells with ActD (1 μg/ml) for the times indicated. Densitometric analysis of signal intensities relative to 18S rRNA from LPS (●)- or LPS plus IL-10 (○)-treated Mac are plotted against time after ActD addition. Insert shows results of Northern blot analysis of A8 mRNA. Results are representative of three experiments.

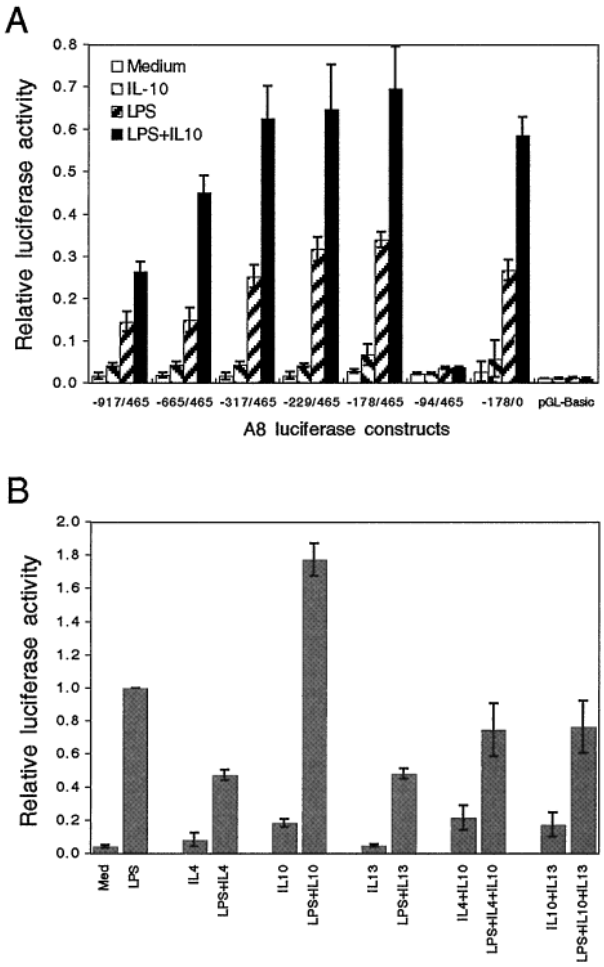


FIGURE 7. Effect of IL-10 on LPS-induced A8 gene transcription and identification of an IL-4-, IL-13-, and IL-10-responsive region in the 5′ flanking sequence. *A*, Serial 5′ deletion fragments of the A8 promoter region linked to a luciferase reporter gene were transiently transfected into RAW 264.7 cells and stimulated 24 h later with IL-10 (10 ng/ml), LPS (100 ng/ml), or LPS plus IL-10 for 16 h. The relative luciferase activity for each transfection was normalized using Renilla luciferase as described in *Materials and Methods*. *B*, The pCP-178/0 A8 promoter luciferase construct was transiently transfected into RAW 264.7 cells that were untreated or stimulated with IL-4 (10 ng/ml) or IL-13 (10 ng/ml) in the presence or absence of LPS and/or IL-10 for 16 h and relative luciferase activity measured. Data are representative of the mean ± SD of three experiments.

and IL-10. In contrast, IL-4 and IL-10 both amplify MCP-1 production by murine MEC (24, 39).

The immunoregulatory roles of IL-10 appear complex and even paradoxical. In addition to positive effects on Ab production, CTL development, and growth-costimulatory activity for thymocytes, mast cells, and B cells (40–42), IL-10, like IL-4 and IL-13, inhibits expression of many proinflammatory Mac-derived genes and some types of inflammation in vivo (31, 43, 44). IL-10 plays a pivotal role in establishing and maintaining T cell anergy, whereas IL-4 and IL-13 direct a Th2 cytokine profile typical of an allergic response or a protective response to parasite infection. Thus, up-regulation of A8 by IL-10 and suppression by IL-4 and IL-13 suggest a role for this mediator in resolution of Th1 T cell-mediated immune responses, particularly because A8 expression by Mac was prolonged and cAMP mediated (Fig. 8). In contrast, early in an inflammatory response, IL-10 may regulate leukocyte recruitment by amplifying the A8 gene. IL-10 affects leukocyte migration in vivo (45, 46), and it augments plalelet-activating factor receptor, FMLP-R (28) and CCR-5 (29, 30) expression on monocytes, and their production of human CC chemokine-4 (47) and MCP-1 chemokines (25) in vitro, strongly supporting a role in leukocyte migration. Other chemokine genes are either down-regulated or not affected by IL-10. Unlike the direct positive effect of IL-10 on chemotactic receptors or human CC chemokine-4 and MCP-1 expression, A8 gene amplification required a positive costimulant, LPS or IFN. TGF-β also has pleiotropic effects and generally suppresses tissue/inflammatory Mac function but activates monocytes (48). TGF-β directly induced A8 mRNA (Fig. 1*A*) in elicited Mac and, although not to the same extent as IL-10, also increased responses provoked by IFN and LPS (Fig. 1*B*).

Because of the unusual nature and potency of the LPS response to IL-10, it was examined in more detail. Dose-response experiments (Fig. 3) confirmed synergy, and the time for optimal A8 mRNA induction by LPS was reduced from 24–48 h to 8–12 h (Fig. 4*A*), and secreted A8 increased ~5-fold within 8 h (Fig. 4*B*). Moreover, endogenous IL-10, which is up-regulated by LPS in monocytes (49), contributed significantly to A8 mRNA levels induced by LPS 24–48 h after stimulation (Fig. 4*C*). In contrast, MCP-1 levels are elevated in human monocytes stimulated with LPS in the presence of anti-IL-10 Ab (25).

Genes induced/enhanced by IL-10 can be regulated by transcriptional (28) and/or post-transcriptional mechanisms (34, 35). IL-10 abolished the early increase in A8 mRNA levels in LPS-stimulated Mac occurring soon after addition of ActD to prevent further transcription (18), suggesting involvement of a suppressor. After 4 h, the degradation rate of LPS/IL-10 mRNA was similar to that of Mac stimulated with LPS alone (Fig. 6), indicating little alteration in mRNA stability. IL-10-mediated mRNA destabilization in murine Mac occurs through AU-rich elements (50), and because the 3′-untranslated region of the A8 gene lacks these (18), destabilization by IL-10 is unlikely. Like A8 mRNA in elicited Mac induced by LPS (18), the IL-10-mediated increase was dependent on protein synthesis (Fig. 5), and synergy was greatest in Mac pretreated with IL-10 for 1–3 h (Fig. 5*A*), suggesting that IL-10-inducible factor(s) mediate enhancement.

PGE_2 produced by LPS-stimulated Mac (51) regulates many functions of these cells, including cAMP and IL-10 generation (52–54). Suppression of COX-2, the enzyme at the rate-limiting

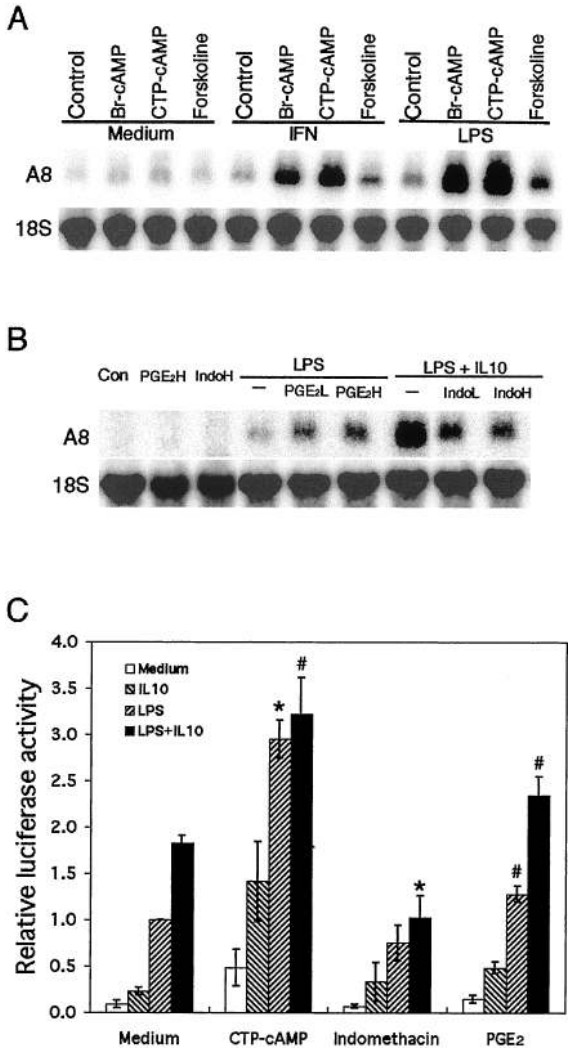


FIGURE 8. PGE_2 and cAMP analogs synergize with LPS and IFN for A8 mRNA induction. *A*, Northern analysis of A8 mRNA induction in Mac stimulated for 12 h with LPS (100 ng/ml) or IFN (500 U) with or without 50 μM Br-cAMP, 50 μM CTP-cAMP, and 50 μM forskolin. *B*, Mac were unstimulated or stimulated with PGE_2 (10 μM), indomethacin (100 μM), or LPS (100 ng/ml) in the presence or absence of PGE_2 (1 μM for PGE_2L, 10 μM for PGE_2H) or costimulated with IL-10 plus LPS with or without indomethacin (10 μM for IndoL, 100 μM for IndoH) for 12 h and RNA analyzed. Results are representative of two experiments. *C*, RAW 264.7 cells were transiently transfected with the pCP-178/0 A8 luciferase promoter construct and stimulated 24 h later with IL-10 (10 ng/ml), LPS (100 ng/ml), or LPS plus IL-10 in the presence or absence of cAMP pathway modulators (50 μM CTP-cAMP, 100 μM indomethacin, 10 μM PGE_2) for 16 h. Relative luciferase activity for each transfection is presented as the mean $\pm$ SD of three experiments. Significant differences in responses of activated cells. $*$, $p < 0.01$; #, $p < 0.05$, compared with activity of control cells.

step of prostanoid production, by indomethacin indicates that, like endogenous IL-10, endogenous PGE_2 contributed to A8 mRNA levels 24 h after stimulation of Mac with LPS or LPS/IL-10 (Fig. 8*B*). PGE_2 and cAMP analogs alone did not initiate or weakly initiated A8 gene expression (Fig. 8*A*). Reporter assays confirmed low transcriptional activity provoked directly by CTP-cAMP in RAW 264.7 cells (Fig. 8*C*). PGE_2 and CTP-cAMP, but not cGMP, analogs (data not shown) strongly amplified LPS-and IFN-stimulated responses (Fig. 8, *A* and *B*) and increased transcription of the luciferase reporter in the presence of LPS or LPS/IL-10 (Fig. 8*C*). These studies suggest indirect regulation of the A8 gene in macrophages by LPS, involving LPS-mediated production of IL-10 and PGE_2, via a cAMP-dependent pathway.

The high A8 mRNA and protein levels induced by LPS plus IL-10 were markedly reduced by IL-4 and IL-13 (Fig. 3*B* and Table I), confirming their important negative regulatory role. Although mechansims are unclear, IL-4 and IL-13 decreased transcription of the minimal A8 promoter in RAW 264.7 cells stimulated with LPS/IL-10 by ~60% (Fig. 7*B*). Constitutive expression of A8 protein in human monocytes is suppressed by IL-4 and IL-10, and decreases are also greatest with the mediators combined (55). Reduced production of IL-10 and PGE_2 by IL-4 and IL-13, as occurs with some monocyte/Mac populations (56–58), may contribute to suppression.

The 178-bp region of the A8 promoter is necessary and sufficient for gene induction by LPS, and transient transfection experiments confirmed the involvement of this region in LPS synergy with IL-10 (Fig. 7*A*). IL-10R signaling involves activation of Jak kinases and phosphorylation of receptor docking sites for members of the STAT family of transcription factors (59, 60). IL-10-mediated potentiation can occur via binding of phosphorylated STAT1 and STAT3 multimeric complexes to the IFN-γ response region, such as in the FcγRI gene promoter (61). However, database searches indicate no STAT binding elements or IFN-γ response region in the essential A8 promoter, although several copies of Ets, E-Box and C/EBPβ consensus sequences were located. C/EBP is strongly associated with cAMP signaling pathways, and its expression is intensified by cAMP (62–64). C/EBP is conserved in the promoter regions of the murine and human A8 genes and is located within an enhancer element in the human A9 gene (65, 66). The human A8 promoter is activated by C/EBP (67). C/EBPβ can be up-regulated following monocyte activation (68) and is involved in induction of the COX-2 gene in LPS-activated RAW 264.7 cells (69), suggesting a mechanism for a potentiating feedback loop in A8 gene regulation in Mac by PGE_2. IL-4 and IL-13 decrease TNF-α-induced C/EBP in synovial fibroblasts, whereas IL-10 up-regulates basal levels (70), suggesting a common mechanism. Examination of the role of this transcription factor in A8 gene regulation by pro- and anti-inflammatory cytokines is underway in our laboratory.

Induction of A8 by LPS is dependent on multiple signals, including changes in intracellular calcium, PKC, and activation of MAPK (18), and early induction of A8 mRNA by LPS/IL-10 (4–8 h) was abolished by the MAPK inhibitors SB202190 and PD98059 (data not shown). Here we show that IL-10, COX-2, and cAMP contribute to the LPS response, and we propose that the MAPK/COX-2/cAMP pathways involving C/EBP may regulate transcriptional synergy between LPS and IL-10 in a manner similar to up-regulation of the arginase gene by LPS and IL-10 (71–74). This differs mechanistically to genes regulated by IL-10 via the STAT pathway. Multiple factors may be involved and may include a newly synthesized protein that regulates release of a suppressor to promote rapid transcription.

A8 may exhibit pleiotropic effects, and the high levels secreted as a result of IL-10 synergy may regulate untoward inflammatory damage by virtue of A8's capacity to act as an antioxidant (22), thereby protecting against acute cytokine-mediated pathology.

Acknowledgments

We are grateful to Drs. Sheng Ping Hu and Robert Passey for constructing the A8 luciferase reporter plasmids.

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