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Mesangial Cell-Derived Interleukin-10 Modulates Mesangial Cell Response to Lipopolysaccharide

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Interleukin (IL)-10 is a novel cytokine produced by a variety of cells, including monocytes/macrophages, upon exposure to lipopolysaccharide (LPS). Recent observations indicate that, in turn, IL-10 exerts suppressive effects on macrophage response to LPS. Because mesangial cells are also a target for LPS, we have examined the potential role of IL-10 in the regulation of mesangial cell response to LPS. To this aim, we have studied the synthesis and the autocrine/paracrine function of IL-10 in cultured mouse mesangial cells. IL-10 mRNA expression and IL-10 protein secretion were determined by a reverse transcription polymerase chain reaction technique and a specific enzyme-linked immunosorbent assay, respectively. No IL-10 mRNA expression was detectable in unactivated cells. LPS induced IL-10 mRNA expression in a dose-dependent fashion (1 to 100 μg/ml). In addition, LPS induced IL-10 protein release that was both dose dependent (1 to 100 μg/ml) and time dependent (24 to 72 hours). We have also studied the effect of IL-10 on the production of inflammatory mediators by LPS-activated mouse mesangial cells. Whereas recombinant IL-10 inhibited the generation of tumor necrosis factor-α (TNF-α) and IL-1β by 90 and 60%, respectively, it did not affect the formation of nitric oxide-derived nitrite (NO_2^-) and nitrate (NO_3^-). As shown by the use of anti-IL-10 monoclonal antibody, endogenously produced IL-10 affected the generation of TNF-α but neither that of IL-1β nor that of NO_2^- and NO_3^-. Finally, we have examined whether conditions known to also reduce the generation of TNF-α modified the expression of IL-10. Of all the conditions tested, only the addition of desferrioxamine and transforming growth factor-β were found to increase IL-10 release. Together, these data demonstrate that mesangial cell-derived IL-10 has important regulatory effects on the inflammatory response of these cells to LPS because of its capacity to blunt TNF-α generation. (Am J Pathol 1995, 147:176–182)

In inflammatory diseases of the glomerulus, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1 are produced by both resident cells and infiltrating monocytes/macrophages[1,2] and participate in destructive processes. TNF-α reduces glomerular blood flow and glomerular filtration rate by altering the balance between vasoconstrictive and vasodilatory mediators, increases the formation of thrombi, and promotes the recruitment and activation of inflammatory cells by enhancing the expression of adhesion molecules and of chemotactic cytokines.[3,4] However, in addition to these proinflammatory cytokines, an anti-inflammatory response can contain the inflammation and limit glomerular destruction. For instance, the results we obtained in *in vitro* studies suggest an important role for prostaglandin (PG)E_2 and IL-6 in limiting TNF-α release in lipopolysaccharide (LPS)-induced acute renal failure.[5,6] Other potential anti-inflammatory cytokines include IL-4 and IL-10. In support of this concept, preliminary studies demonstrated that IL-4 and IL-10 mRNA were increased in glomeruli after the induction of immune injury and that IL-4 mRNA levels were inversely related to those of IL-1.[7]

IL-10 was first described as a cytokine produced by the T helper type 2 subset of $CD4^+$ T lymphocytes that inhibited the synthesis of interferon-γ by T helper type 1 cells.[8] Recent studies have shown that IL-10 was produced by a variety of cells, including thymocytes, cytotoxic T cells, mast cells, B cells, and LPS-

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activated macrophages. In addition, IL-10 has been found to limit the cytotoxic function of activated macrophages, to inhibit *in vitro* macrophage production of cytokines, including TNF-α and IL-1[8] and, hence, to prevent lethality in experimental endotoxemia.[9] Because mouse mesangial cells possess macrophage-like characteristics,[10] we hypothesized that LPS promoted IL-10 production by these cells as well and that, in turn, IL-10 was responsible for a regulatory feedback loop. Accordingly, in this study, we measured mRNA expression and IL-10 protein secretion in mouse mesangial cells using a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) technique and IL-10 specific enzyme-linked immunosorbent assay (ELISA), respectively. We found that (1) LPS induced IL-10 mRNA expression and IL-10 secretion in these cells, (2) IL-10 generation provided negative feedback to TNF-α, and (3) two conditions responsible for TNF-α suppression, namely the addition of transforming growth factor-β (TGF-β) and the addition of desferrioxamine (DFX) determined amplification of LPS-induced IL-10 secretion.

Materials and Methods

Materials

Mouse recombinant IL-10 was obtained as culture supernatants from CHO-K1 cells stably transfected with the corresponding cDNA, as previously described.[9] Supernatant collected from mock-transfected cells was used as control. The JES5–2A5 mAb, a rat immunoglobulin (Ig)G1 neutralizing mouse IL-10, was kindly given by T. Mosmann (Department of Immunology, University of Alberta, Edmonton, Canada). Monoclonal antibody (MAb) was purified on thiophyllic agarose column (AFFI-T, Kem En Tec, Copenhagen, Denmark) according to recommendations of the manufacturer. The LO-DNP MAb, a rat IgG1 used as control, was kindly given by H. Bazin (Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium). The endotoxin levels of mIL-10 and MAb preparations, as determined by the limulus assay, were less than 1 ng/ml and 10 pg/mg, respectively. LPS from *Escherichia coli* 026B6, PGE_2, and dexamethasone were from Sigma Chemical Co., St. Louis, MO. DFX was from Ciba, Rueil-Malmaison, France, and TGF-β from Genzyme, Cambridge, MA. IL-1β and IL-10 levels were measured with commercially available sandwich ELISA, with a sensitivity of 10 pg/ml (Intertest-1β, Genzyme) and 7 pg/ml (Titerzyme, PerSeptive Diagnostics, Cambridge, MA), respectively.

Mesangial Cell Culture

Mouse mesangial cells were grown from glomerular explants with minor modifications of the previously published procedure.[11] Kidneys from 8-week-old male BALB/C mice were removed and glomeruli were isolated from cortices with a series of sieves of decreasing pore size (180, 106, and 50 μm). The final preparation, which was resuspended in Hank's balanced salt solution was checked for purity under light microscopy. Glomeruli were free of Bowman's capsule, and virtually no afferent or efferent arterioles could be detected. After treatment by 300 U/ml collagenase (grade I collagenase from *Clostridium histolyticum*, Sigma Chemical Co.) for 30 minutes at 37°C, isolated glomeruli were rinsed twice, resuspended in culture medium (RPMI 1640 buffered with 10 mmol/L HEPES to pH 7.4, and supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L glutamine), and then plated onto plastic Petri dishes (Nunc, Roskilde, Denmark) and cultured at 37°C in 5% CO_2 in air. Under these conditions, mesangial cells appeared after 7 days in culture and reached confluency by day 21. They were used for experimentation after passages 2 to 4. The preparations were free of detectable macrophage contamination, as previously reported.[11]

Incubation of Glomeruli

Both 8-week-old male BALB/C mice and C3H/HeJ mice (Institut Pasteur, Paris, France), were injected intraperitoneally with a 100-μl volume containing 250 μg of LPS. After 1 hour, renal glomeruli were isolated as indicated above, resuspended in culture medium (15,000 glomeruli/ml) and incubated for 4 hours in a 37°C humid atmosphere with 5% CO_2. After the incubation, the media were harvested and centrifuged. The supernatants were then frozen at −70°C for IL-10 analysis.

mRNA Isolation and PCR Analysis

After incubation with LPS (0 to 100 μg/ml), cells were exposed to a lysing buffer (10 mmol/L Tris/HCl, pH 7.4, containing 100 mmol/L NaCl, 1 mmol/L EDTA, 2% sodium dodecyl sulfate, and 250 μg/ml proteinase K) for 45 min at 37°C. Total RNA was then extracted by the phenol-chloroform method, precipitated with isopropanol and, after dissolution in 10 mmol/L Tris/HCl buffer, pH 7.4, containing 1 mmol/L EDTA, precipitated again with 3 mol/L LiCl. After two supplementary precipitations with ethanol, RNA concentration and purity were determined by obtaining the A260 and

A280 readings.[5] Preparation of cDNA and PCR for IL-10 and hypoxanthine phosphoribosyl transferase (HPRT) were performed by standard procedures.[12] Briefly, 1 μg of total RNA was incubated for 10 minutes at 65°C with 1 μg of oligo (dT) and was further incubated for 60 minutes at 37°C with 120 U of RNAsin (Promega Corp., Madison, WI), 1 mmol/L dNTPs, 200 U of Moloney murine leukemia virus reverse transcriptase, 0.01 mg/ml acetylated bovine serum albumin, and RT buffer (7.5 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 50 mmol/L Tris/HCl, pH 8.3) in a final volume of 20 μl.

PCR was performed with aliquots of the resulting cDNA (equivalent to 50 and 250 ng of total RNA for HPRT and IL-10 assays, respectively). To this was added 0.1 mmol/L dNTP, 2.5 U of Taq DNA polymerase, 1 μg of each sense/antisense primer, and PCR buffer (1 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.001% gelatin, 10 mmol/L Tris/HCl, pH 8.3) in a total volume of 25 μl. Primers used for mouse HPRT mRNA were as follows: sense primer 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and antisense primer 5′-GATTCAACTTGCGCTCATCTTAGGC-3′ and for mouse IL-10 mRNA were as follows: sense primer 5′-TCAAACAAAGGACCAGCTGGACAACATACTGC-3′ and antisense primer 5′-CTGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3′. Reactions were incubated in a Perkin-Elmer (Norwalk, CT) Gene Amp PCR system 9600 for 28 cycles (denaturation, 10 seconds at 91°C; annealing, 25 seconds at 55°C; extension, 25 seconds at 72°C). PCR products were run on a 2% agarose gel and stained with ethidium bromide.

Determination of Cytokine Production by Mouse Mesangial Cells

Cells that were cultured in 24-well plates were exposed to 500 μl of culture medium containing bacterial LPS together with the agent to be tested. After 24 to 72 hours of incubation, cell-free supernatants were removed. Adherent cells were placed on ice, washed with cold phosphate-buffered saline, freeze-thawed three times and scraped into 200 μl of culture medium. After sonication, the homogenate was centrifuged for 3 minutes at 15,000 × *g* and 4°C, and the pellet was discarded. Cytokine concentration was determined in both the cell-free supernatants and/or the cell extracts.

The concentration of IL-10 was measured by Titer Zyme mouse IL-10 enzyme immunoassay. The lower detection limit of the EIA was 7 pg/ml. The concentration of IL-1–1β was measured by Intertest mouse IL-1β ELISA. The lower detection limit of the ELISA was 10 pg/ml. The activity of TNF-α was measured by an L-929 fibroblast lytic assay as previously described.[13] Specificity for TNF-α was demonstrated by neutralization of TNF-α-induced cytotoxicity with anti-mouse TNF-α. Results were expressed as percent specific cytotoxicity.

Determination of NO Production by Mouse Mesangial Cells

Inducible NO synthase activity of mouse mesangial cells was assayed indirectly by measuring NO_2^-/NO_3^- production. NO_2^- was measured by a colorimetric assay based on the Griess reaction, and NO_3^- was measured after reduction of NO_3^- to NO_2^- with nitrate reductase, as previously described.[14] Results were expressed as nanomoles of NO_2^- and NO_3^- per milliliter cell-free supernatant.

Statistics

Results are presented as mean ± SEM. Statistical significance ($P < 0.05$) was determined by Student's *t*-test.

Results

IL-10 is Produced by Cultured Mouse Mesangial Cells

Mouse mesangial cells were grown to subconfluence in the presence of 10% FCS. Under these conditions, there was no IL-10 mRNA levels detectable by PCR analysis (Figure 1). Stimulation of cells with LPS for 72 hours led to a strong induction of IL-10 mRNA. The PCR primers we used generated a single band of PCR-amplified product with the expected 420-bp size.

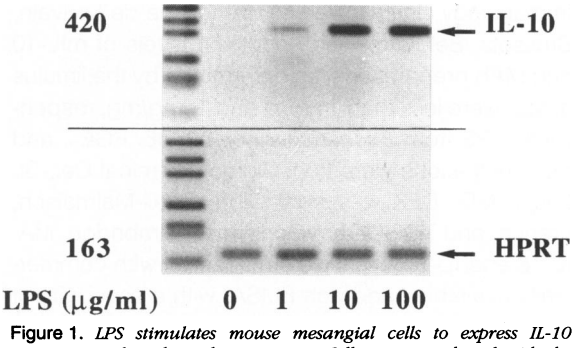

Figure 1. *LPS stimulates mouse mesangial cells to express IL-10 mRNA in a dose-dependent manner. Cells were incubated with the indicated concentration of LPS for 72 hours before total RNA extraction and RT-PCR analysis for HPRT and IL-10 mRNA expression.*

To demonstrate the effects of LPS on IL-10 production at the protein level, cultured mouse mesangial cells were incubated for 24, 48, or 72 hours with 10 μg/ml LPS, and the culture supernatant fluid was assayed for IL-10 protein with an IL-10-specific ELISA. As shown in Figure 2A, the release of IL-10 within 24 hours was below the detection limit of the ELISA. Upon stimulation with LPS for 48 and 72 hours, high amounts of IL-10 protein were released.

Dose-dependent induction of IL-10 release by LPS was also analyzed (Figure 2B). IL-10 was barely detectable in the culture medium of untreated cells, and LPS increased IL-10 release in a dose-dependent fashion. At all of the LPS doses tested, the cell-associated fraction of IL-10 represented less than 15% of the released fraction of IL-10.

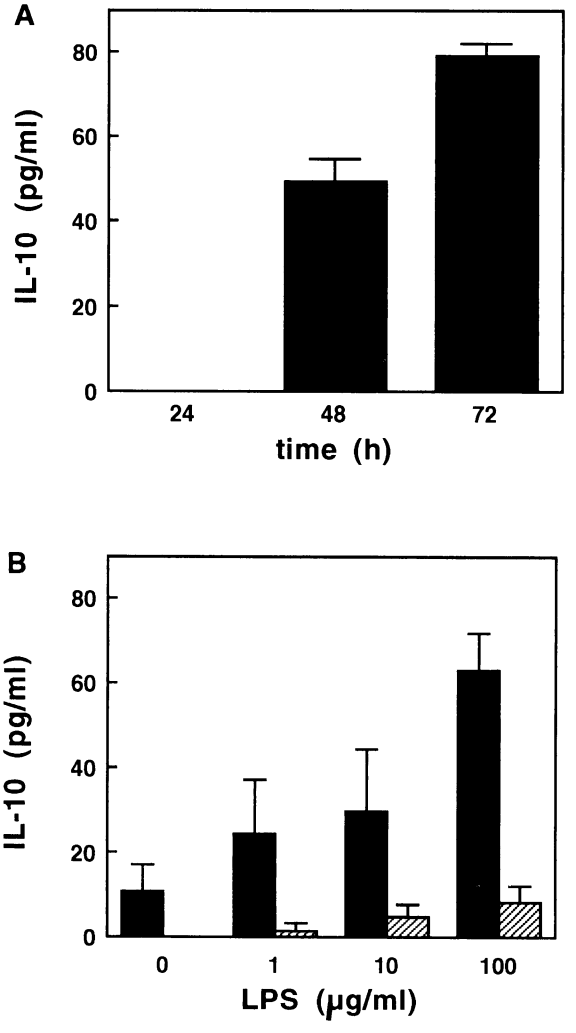

Figure 2. *LPS stimulates mouse mesangial cells to produce IL-10 in a time-dependent and dose-dependent manner. IL-10 levels were quantified by ELISA in both culture supernatant fluid (solid bars) and cell extract (hatched bars).* A: *Time course. Cells were incubated with LPS (10 μg/ml) for the indicated periods of time. The data presented in the figure are the means ± SEM of three experiments.* B: *Dose response. Cells were incubated with the indicated concentration of LPS for 48 hours. The data are the means ± SEM of four experiments.*

Comparable amounts of IL-10 were observed in the incubation medium of glomeruli isolated from the kidney cortex of BALB/C mice 1 hour after *in vivo* LPS challenge (78.8 ± 9.4 pg/ml, n = 5). Glomeruli from C3H/HeJ mice, a LPS-nonresponsive genetic mutant strain of C3H/HeN, released barely detectable amounts of IL-10 under these conditions (11.5 ± 5.3 pg/ml, n = 4, $P < 0.001$).

IL-10 Inhibits Cytokine Production by Cultured Mouse Mesangial Cells

IL-10 has been shown to inhibit the monocyte/macrophage production of NO and cytokines, including IL-1α and -β, IL-6, IL-8, TNF-α and colony-stimulating factors.[8] To analyze IL-10 effects on the production of such inflammatory mediators by mesangial cells, mouse mesangial cells were exposed to 10 μg/ml LPS for 24 hours in the presence or absence of IL-10 (25 U/ml; Table 1). Activation of cells by LPS resulted in production of high levels of IL-1β, TNF-α, and NO-derived NO_2^- and NO_3^-. Interestingly, IL-10 modulated these productions to various extents. The strongest inhibitory effect of IL-10 was observed on the production of TNF-α that was inhibited by 90%. The inhibition of IL-1β production was less pronounced (60%) whereas that of NO_2^- and NO_3^- was not affected by IL-10. The inhibitory effects of endogenously produced IL-10 on inflammatory mediator production by LPS-activated mouse mesangial cells was further analyzed by measuring the levels of IL-1β, TNF-α, and NO_2^- + NO_3^- in the presence of neutralizing anti-IL-10 MAb (Table 1). Exposure of cells to 20 μg/ml anti-IL-10 MAb resulted in increased production of TNF-α, whereas the production of IL-1β and NO_2^- + NO_3^- was not affected. No significant alteration of TNF-α production was detected after incubation with irrelevant control IgGI (data not shown). In addition, the regulatory effects of both recombinant and endogenously produced IL-10 on TNF-α production by mesangial cells was observed at all of the doses of LPS tested (Figure 3).

Conditions Responsible for TNF-α Suppression Determine Modulation of IL-10 Production by Cultured Mouse Mesangial Cells

Having found that IL-10 affected the production of TNF-α by mouse mesangial cells, we examined whether conditions known to also reduce the produc-

Table 1. *Effects of Exogenous IL-10 and Endogenously Produced IL-10 on the Production of IL-1β, TNF-α, and NO_2^- plus NO_3^- by Mouse Mesangial Cells*

Addition	IL-1β (pg/well; n = 3)	TNF-α (% cytotoxicity; n = 8)	NO_2^- + NO_3^- (nmol/ml; n = 4)
None	26.5 ± 15.3	3.2 ± 2.0	ND
LPS	64.7 ± 8.0	32.4 ± 4.2	117.4 ± 5.6
LPS + IL-10	23.9 ± 8.0*	3.2 ± 1.6*	103.9 ± 13.2
LPS + anti-IL-10 MAb	55.5 ± 10.0	49.1 ± 5.5*	112.3 ± 7.1

Mouse mesangial cells were activated by LPS (*10 μg/ml*) *in the absence and presence of IL-10 (25 U/ml) or anti-mouse IL-10 MAb (20 μg/ml IgG_1) for 24 hours, and production of cytokines and NO metabolites was determined by cytokine-specific ELISA and colorimetric assay, respectively. Data are the means ± SEM. *P < 0.05 versus* LPS alone. ND, not determined.

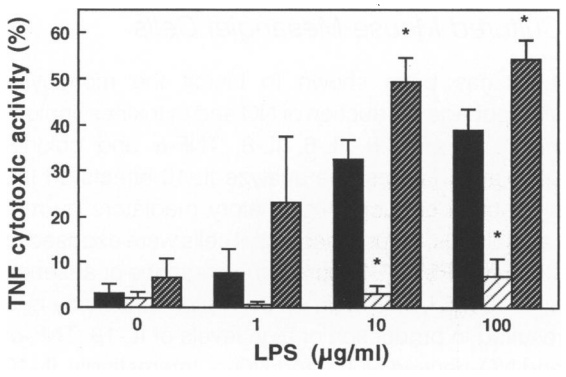

Figure 3. *Effects of exogenous IL-10 and endogenously produced IL-10 on TNF-α production by mouse mesangial cells. Cells were incubated for 24 hours with the indicated concentration of LPS alone (solid bars) and in the presence of recombinant mouse IL-10 (25 U/ml; white hatched bars) or neutralizing anti-mouse IL-10 MAb (20 μg/ml IgGl; black hatched bars). TNF-α cytotoxic activity was measured in culture supernatant fluid by the L-929 fibroblast lytic assay. The data are the means ± SEM of eight determinations.* *P < 0.05 versus *LPS alone.*

tion of TNF-α modified the expression of IL-10. These include the exposure of mesangial cells to PGE_2,[5] dexamethasone, TGF-β (Baud and Fouqueray, unpublished observations), DFX,[15] or heat shock.[16] Of all these conditions we tested, DFX and TGF-β appeared to be unique in their ability to increase IL-10 release (Table 2). In addition, as shown in Figure 4, TGF-β increased IL-10 in a dose-dependent fashion.

Discussion

Previous *in vitro* studies demonstrated that, upon stimulation with LPS or immune complexes, mesangial cells express genes and secrete newly synthesized IL-1, IL-6, IL-8, TNF-α, and colony-stimulating factors.[17] These cytokines have a critical role in amplifying the glomerular inflammatory reaction.

IL-10 is a recently characterized cytokine involved in the control of the inflammatory response, mainly because of its ability to suppress the synthesis of proinflammatory mediators by monocytes/macrophages.[8] In this study, we have demonstrated that cultured mouse mesangial cells synthesized and released IL-10, which in turn modulated their response to LPS. We specifically identified IL-10 mRNA transcripts by RT-PCR analysis in LPS-stimulated mesangial cells but not in unstimulated cells. IL-10 mRNA expression was associated with IL-10 protein production. Similarly to monocytes, LPS stimulated mesangial cell IL-10 secretion with a peak occurring at 48 hours.[18] However, the stimulatory effect of LPS on IL-10 production was less pronounced in mesangial cells than in monocytes as this led to much lower levels of IL-10 in the cell culture medium (~50 pg/ml *versus* ~50 ng/ml).[18]

Interestingly, even when the production of IL-10 by LPS-activated mouse mesangial cells was weak, this was sufficient to exert autoregulatory effects on TNF-α production by these cells. Indeed, the addition of neutralizing anti-IL-10 MAb to the culture medium of LPS-activated mouse mesangial cells resulted in higher levels of bioactive TNF-α release as compared with activation by LPS alone. Our findings are in accordance with the observation that IL-10 has strong down-regulatory effects on the secretion of TNF-α by monocytes.[18] It remains to be determined whether this inhibition occurred at the transcriptional level. In

Table 2. *Conditions Responsible for TNF-α Suppression Determine Amplification of IL-10 Production by Mouse Mesangial Cells*

Stimulus	n	IL-10 (pg/ml)
LPS	6	39.5 ± 8.3
LPS + PGE_2 (0.1 μmol/L)	3	56.4 ± 9.3
LPS + dexamethasone (1 μmol/L)	3	39.5 ± 22.9
LPS + DFX (5 mmol/L)	4	182.4 ± 46.0*
LPS + TGFβ (10 ng/ml)	6	100.7 ± 21.8*
LPS + heat shock (41°C, 30 minutes)	3	0

Mouse mesangial cells were activated by LPS (10 μg/ml) under the indicated conditions. After 48 hours of incubation, IL-10 concentration was determined by specific ELISA. Data are the means ± SEM of n experiments. *P < 0.05 *versus* LPS alone.

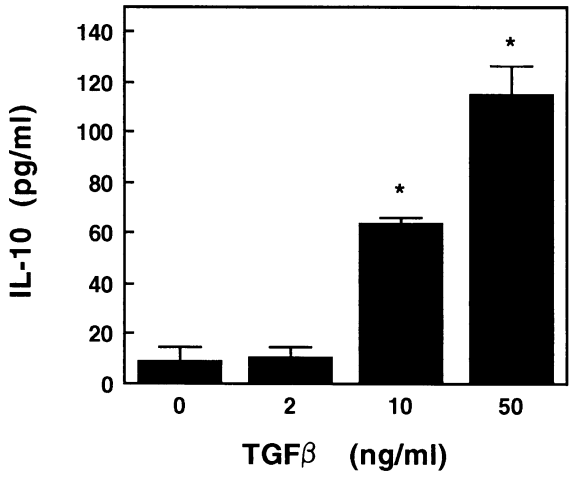

Figure 4. *Effects of TGF-β on IL-10 production by mouse mesangial cells. Cells were incubated for 48 hours with 10 μg/ml LPS together with the indicated concentration of TGF-β. IL-10 levels were quantified by ELISA in culture supernatant fluid. The data are the means ± SEM of three experiments. *P < 0.05 versus LPS alone.*

contrast, no autoregulatory role of endogenously produced IL-10 was observed on IL-1β production. This could be related to the fact that, as already shown,[18] the inhibitory effects of IL-10 were less pronounced on IL-1β than on TNF-α production. Therefore, IL-10 levels in the culture medium of mouse mesangial cells might be insufficient to have a significant feedback activity on IL-1β response to LPS. Nevertheless, endogenously produced IL-10 could locally affect IL-1β biological activity by up-regulating the production of IL-1 receptor antagonist, a protein that competes with IL-1β for binding to the IL-1 receptor.[19]

Autoregulatory effects of IL-10 on TNF-α production is of potential importance because TNF-α contributes to glomerular injury in numerous experimental models of glomerulonephritis.[20] This has been suggested by the observation that administration of anti-TNF-α antiserum or soluble TNF-α receptors provides protection.[21] On the basis of the work reported herein, the possibility should be considered that IL-10 is expressed in glomeruli after the induction of immune or nonimmune injury and that its levels are inversely related to those of TNF-α.

By extension, our results suggest protective effects of IL-10 in experimental models of glomerulonephritis. Therefore, it would be important to identify all of the processes resulting in up-regulated expression of IL-10 in glomerular cells. In this context, a new aspect of the IL-10 regulatory pathway is revealed by the finding that both TGF-β and DFX amplified LPS-induced IL-10 generation. Whereas the IL-10 effect on TGF-β synthesis was previously analyzed,[18,22] TGF-β effect on IL-10 synthesis was not. Such a loop of amplification is of theoretical importance, because TGF-β and IL-10 are both considered as anti-inflammatory cytokines exhibiting identical effects on target cells.[23] Thus, the possibility exists that TGF-β modulates cell functions indirectly via the generation of IL-10. However, such a mechanism would be restricted to some TGF-β effects as TGF-β and IL-10 usually deactivate LPS target cells by different mechanisms.[24] A second IL-10 amplification pathway relates to the effect of DFX. The precise molecular mechanisms that account for DFX-induced IL-10 production are unknown. Because this drug is known to suppress hydroxyl radical toxicity by interfering with its generation,[25] one likely possibility is that DFX-induced reduction of hydroxyl radical toxicity is involved. These and other possible mechanisms require additional examination and will be the subject of additional reports.

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