

Regulation of Monocyte IL-10 Synthesis by Endogenous IL-1 and TNF- α : Role of the p38 and p42/44 Mitogen-Activated Protein Kinases¹

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IL-10 is an anti-inflammatory cytokine with potent immunomodulatory effects, including inhibition of cytokine production. However, regulation of monocyte IL-10 production is poorly understood. In this report we have investigated the mechanisms of LPS-induced IL-10 production by human peripheral blood monocytes and demonstrate that IL-10 synthesis is uniquely dependent on the endogenous proinflammatory cytokines IL-1 and/or TNF- α . LPS signal transduction in monocytes has been shown to involve activation of the p38 and p42 mitogen-activated protein kinase (MAPK) cascades. The results in this paper indicate that inhibition of p38 MAPK potently inhibited the production of IL-10, IL-1 β , and TNF- α , whereas blockade of the p42/44 MAPK pathway, while partially inhibiting TNF- α and IL-1 β production, had no effect on monocyte secretion of IL-10. Furthermore, neither the inhibition of monocyte TNF- α induced by IL-10 nor the stimulation of soluble TNF receptor production was affected by inhibition of the p42/44 MAPK pathway, suggesting that this signaling event is not involved in either monocyte production of or anti-inflammatory responses to IL-10. These data raise the interesting possibility that proinflammatory TNF- α -mediated effects may be selectively blocked without modulating the induction or the response to IL-10, whereas the signaling events associated with the anti-inflammatory events induced by IL-10 remain to be elucidated. *The Journal of Immunology*, 1998, 160: 920–928.

IL-10 has been shown to be an important regulator of myeloid cells (1, 2). This cytokine is considered to have an anti-inflammatory role, potentially inhibiting the capacity of monocyte/macrophage cells to secrete inflammatory mediators, including TNF- α , IL-1, and IL-6 (3, 4), and down-regulating their capacity to serve as accessory cells for stimulation of T cell function (3–5). In addition, IL-10 has been shown to stimulate monocyte expression of soluble TNF receptors (sTNFR)⁵ (6), the natural inhibitors of TNF- α , and to enhance the production of IL-1R antagonist (IL-1Ra) (7, 8), which competitively inhibits IL-1 binding to the membrane receptor. In vivo studies have shown that mice become more sensitive to endotoxic shock following treatment with anti-IL-10 Abs, while administration of IL-10 suppressed lethal endotoxemia and reduced serum TNF- α (9, 10). Further, IL-10-deficient mice develop chronic enterocolitis, arising from un-

controlled immune responses in the bowel, and marked irritant and contact hypersensitivity responses in the skin (11, 12). As a result of such anti-inflammatory properties, IL-10 has been suggested as a therapeutic agent for inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease, and clinical trials to test this hypothesis are in progress.

IL-10 is produced by a variety of immune cell types, including cells of the monocyte/macrophage lineage (1, 2); nevertheless, little is known concerning the regulation of its synthesis by monocytes/macrophages. A number of studies suggest that both soluble factors and cell-cell contact-mediated signals are involved in the stimulation of monocyte IL-10 production (13–16). Notably, several groups have shown that TNF- α plays a role in the induction of IL-10 by stimulated monocytes (17, 18). To date however, bacterial endotoxin (LPS) has been the most consistent and best characterized signal for IL-10 production by monocytic cells. Activation of human monocytes by LPS in vitro results in the rapid production of proinflammatory cytokines, including TNF- α , IL-1, and IL-6, followed later by the secretion of the anti-inflammatory IL-10, sTNFR, and IL-1Ra, which down-regulate or antagonize the proinflammatory mediators. The kinetics of cytokine production has led to the suggestion that different regulatory mechanisms are involved in the expression of the pro- and anti-inflammatory cytokines.

LPS signal transduction in monocytes involves binding to cell surface CD14 (19) and has been shown to include activation of tyrosine kinases (20, 21), protein kinase C (22–24), and the mitogen-activated protein kinases (MAPK), p38, p42/44(ERK) and p54(stress-activated protein kinase/JNK) (23, 25, 26). While the role of the MAPKs in LPS-induced signaling is probably the best characterized, the relationship between the activation of these signaling molecules and induced cytokine expression is still obscure. The only exception to this is the observation that inhibition of the

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⁵ Abbreviations used in this paper: sTNFR, soluble tumor necrosis factor receptor; IL-1Ra, interleukin-1 receptor antagonist; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein ERK kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; MAPKAP, mitogen-activated protein kinase-activated protein kinase; cA2, chimeric anti-TNF Ab, A2.

p38 MAPK with specific imidazole inhibitors (e.g., SB203580) prevents translation of the TNF- α mRNA (25). Activation of the p42/44 MAPK pathway has also been implicated in TNF- α expression, but these studies have been performed in cell lines transfected with various mutant forms of Raf-1 kinase, a proximal activator of the p42/44 MAPK pathway (27). There is little information on the role of either of these kinases in regulating the expression of other cytokines in monocytes in general, and none regarding IL-10 synthesis in particular. A recent report has suggested that IL-10 production is dependent on protein tyrosine kinase and protein kinase C activation (28), while several studies suggest that factors that elevate cAMP are involved in the regulation of monocytic IL-10 production, primarily at the mRNA level (18, 29, 30). In the present study we have investigated the regulation of monocyte IL-10 production following stimulation by LPS. The results demonstrate the involvement of endogenous monocyte-derived IL-1 in LPS-induced IL-10 production and in addition confirm the importance of endogenous TNF- α in the secretion of IL-10. We further demonstrate that the p38 and p42/44 MAPK pathways differentially regulate monocyte production of the cytokines IL-10, IL-1 β , and TNF- α . Moreover, data suggest that monocyte anti-inflammatory responses to IL-10 and monocyte production of IL-10 do not involve the p42/44 MAPK cascade.

Materials and Methods

Reagents

Human recombinant cytokines were gifts: TNF- α from Prof. W. Stec (Center of Molecular and Macromolecular Studies, Lodz, Poland), IL-10 from Dr. S. Smith (Schering Plough, Kenilworth, NJ), IL-6 from Dr. F. Di Padova (Sandoz, Basel, Switzerland), IL-1 β from Dr. J. Kenny (Syntex, Palo Alto, CA), and human recombinant IL-1Ra from Dr. A. Berger (Upjohn Laboratories, Kalamazoo, MI). The IL-1Ra was tested in a functional assay that measures neutralization of IL-1 activity, as previously described (31). *Salmonella abortus equi* LPS was a gift from Dr. C. Galanos (Max Planck Institute, Freiberg, Germany). PD98059 MEK1 inhibitor was obtained from Calbiochem-Novabiochem Ltd. (Nottingham, U.K.). SB203580 was a gift from Dr. J. Lee (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). Cell culture medium consisted of RPMI 1640 containing 300 μ g/ml L-glutamine (BioWhittaker, Verviers, Belgium) supplemented with 100 U/ml penicillin (BioWhittaker), 100 μ g/ml streptomycin (BioWhittaker), and 10% heat-inactivated FCS (Sigma Chemical Co., Poole, U.K.). All monocyte reagents and culture media were shown to contain <0.1 U/ml endotoxin as measured using the chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker).

Antibodies

Whole Ab and Fab of the chimeric mouse Fv/human IgG1,k anti-TNF- α mAb cA2 were gifts from Dr. J. Ghraieb (Centocor, Malvern, PA) (32). The high affinity cA2 mAb has previously been shown to efficiently neutralize TNF- α in a number of in vitro bioassays at concentrations of 1 to 4 μ g/ml (32, 33) (data not shown). The neutralizing anti-human GM-CSF sheep polyclonal Ab Sh7 was a gift from the Genetics Institute (Cambridge, MA). Anti-human IL-8 mouse mAb A5.12.14 (IgG2a) was a gift from Genentech, Inc. (South San Francisco, CA). Neutralizing anti-human IFN- α mouse mAb MT3/B4 (IgG1), anti-human IFN- α sheep polyclonal Ab H51, and anti-human IFN- β sheep polyclonal Ab H60 were gifts from Dr. A. Meager (National Institute for Biologic Standards and Control, London, U.K.). The neutralizing anti-human IL-6 sheep polyclonal Ab was a gift from Dr. L. Aarden (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service). Isotype control Abs were mouse IgG1 and IgG2a (anti-TNP; PharMingen, San Diego, CA) and sheep IgG (Sigma Chemical Co.).

Monocyte purification and culture

Human PBMC were isolated from single donor plateletpheresis residues purchased from the North London Blood Transfusion Service (Colindale, U.K.). Mononuclear cells were isolated by Ficoll/Hypaque centrifugation (specific density, 1.077 g/ml; Nycomed Pharma A.S., Oslo, Norway) before separation in a Beckman JE6 elutriator (High Wycombe, U.K.). Elutriation was performed in culture medium with 1% heat-inactivated FCS. Monocyte purity was assessed by flow cytometry using fluorochrome-con-

jugated anti-CD45, anti-CD3, anti-CD14, and anti-CD19 mAbs (Becton Dickinson, Oxford, U.K.) and routinely consisted of >80% CD14-expressing cells, <0.5% CD19-expressing cells, and <3% CD3-expressing cells. Monocytes were cultured at 6×10^6 cells/ml in monocyte medium in flat-bottom 96-well culture plates (Nunc Life Technologies Ltd., Paisley, Scotland). At the start of the culture period cells either were left unstimulated or were cultured with the following reagents as indicated in the text: LPS (10 ng/ml), IL-1Ra (20 μ g/ml), IL-10 (10 ng/ml), whole Ab or Fab of anti-TNF- α mAb cA2 (10 μ g/ml), anti-IL-6 polyclonal Ab, anti-GM-CSF polyclonal Ab Sh7, anti-IFN- α polyclonal Ab H51, anti-IFN- β polyclonal Ab H60, and the isotype control sheep polyclonal IgG (all at a 1/100 dilution of stock concentration), anti-IFN- α 2 mAb MT3/B4 (1/100 dilution of ascetic fluid), anti-IL-8 mAb A5.12.14, and the isotype-matched IgG1 and IgG2a control mAb (10 μ g/ml). In some experiments monocytes were treated for 1 h with PD98059 or SB203850 at the indicated concentrations before stimulation with LPS. After 24-h incubation at 37°C with 5% CO₂, culture supernatants (200 μ l/well, three wells per condition) were harvested and stored at -20°C until used. All experiments were performed at least three times, and the figures show representative examples of these experiments.

Measurements of cytokines and sTNFR by sandwich ELISA

Reagents for the IL-10 ELISA were gifts from Dr. K. Moore (DNAX, Palo Alto, CA) and Dr. S. Smith (Schering Plough), and the ELISA was performed as previously described (34). The range of the assay was from 0.04 to 10 ng/ml. Reagents for the TNF- α ELISA were provided by Dr. W. Buurman (Rijks Universiteit Limburg, Maastricht, The Netherlands). The ELISA was performed as described previously (35) using the coat anti-TNF- α mAb 61E71 and was developed using a rabbit polyclonal anti-TNF- α Ab. The rabbit polyclonal Ab was detected using a peroxidase-conjugated goat anti-rabbit IgG(H+L) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) followed by an appropriate substrate. The range of the assay was from 0.004 to 10 ng/ml. Reagents for the IL-1 β ELISA were purchased from BioSource (distributed by Lifescreen Ltd., Watford, U.K.). The limit of detection in this assay is 40 pg/ml. All results are expressed as the mean concentration of cytokines \pm SD obtained per condition. Reagents for the IL-6 ELISA were gifts from Dr. F. Di Padova (Sandoz, Basel, Switzerland), and the ELISA was performed using the paired anti-IL-6 Abs LN314-14 and LN1 14-110 as previously described (36). The range of the assay was from 0.1 to 10 ng/ml. The sTNFR were assayed in the cell supernatants by ELISA as previously described (37) using anti-p75 sTNFR mAb 4C8 as the capture Ab and a rabbit anti-p75 sTNFR polyclonal Ab as the detection Ab (gifts from Dr. W. Buurman, Rijks Universiteit Limburg, Maastricht, The Netherlands). Results are expressed as the mean of triplicate samples, and the detection limit of the ELISA was 20 pg/ml.

p42 MAPK and MAPKAP-2 kinase assays

Monocytes (3×10^6) were stimulated with LPS (100 ng/ml) for 10 min. Following stimulation, cells were lysed in 400 μ l of lysis buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β -glycerol phosphate, 200 mM NaCl, 1% Triton-X-100, 1 mM DTT, 10 mM sodium fluoride, 1 mM sodium ortho-vanadate, 1 mM DTT, 1 mM PMSF, 3 μ g/ml aprotinin, 2.5 μ g/ml pepstatin, and 1 μ g/ml leupeptin). Postnuclear lysates were incubated with 2 μ l of rabbit anti-p42 MAPK (Dr. P. Cohen, Dundee, U.K.) or 3 μ l of anti-MAPKAP-2 (Upstate Biotechnology, Inc., Lake Placid, NY), and 20 μ l (50 μ l for the MAPKAP-2 kinase assay) of a 50% suspension of protein G in lysis buffer and rotated for 2 h at 4°C. The beads were washed twice in RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 25 mM β -glycerol phosphate, 10 mM Na tetra-pyrophosphate, 1 mM sodium ortho-vanadate, 1 mM DTT, 1 mM PMSF, 3 μ g/ml aprotinin, 2.5 μ g/ml pepstatin, and 1 μ g/ml leupeptin) and then twice in assay buffer (20 mM HEPES (pH 7.2), 2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, and 0.1 mM sodium ortho-vanadate).

For the p42/44 MAP kinase assay, after the last wash the beads were left as a 1/1 suspension in assay buffer. Kinase reactions were conducted at room temperature (under constant agitation) for 20 min after the addition of 20 μ l of 350 μ g/ml myelin basic protein (Calbiochem), 10 μ l of 180 μ M ATP, and 5 μ Ci of [γ -³²P]ATP. Reactions were stopped by the addition of 25 μ l of 4 \times gel sample buffer and boiled for 3 to 5 min. Samples were fractionated on a 12.5% SDS-polyacrylamide gel. After the gel had been fixed in a mixture of water/methanol/acetic acid (5/4/1) and then dried, the phosphorylated myelin basic protein was detected by autoradiography using Hyperfilm MP (Amersham, Aylesbury, U.K.).

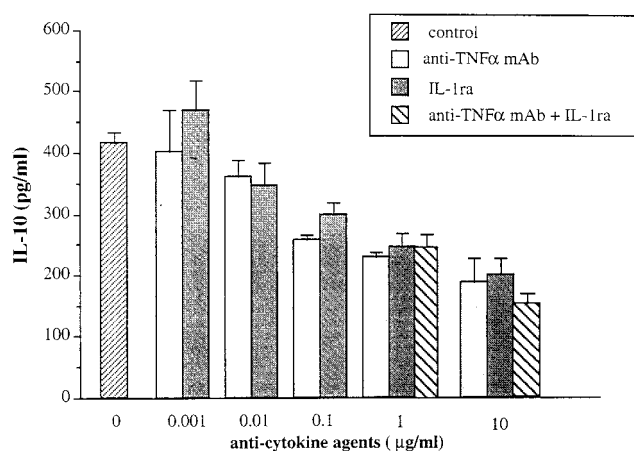


FIGURE 1. Neutralization of endogenous IL-1 and/or TNF- α partially inhibits LPS-stimulated monocyte IL-10 production. PBMC were cultured with LPS for 24 h in the presence or the absence of neutralizing anti-TNF- α mAb cA2 or IL-1Ra, either alone or together at the indicated concentrations. Culture supernatants were assayed for IL-10. Data are means of triplicate culture supernatants \pm SD; a representative example of 10 replicate experiments is shown.

For the MAPKAP-2 kinase assay, after the last wash the beads were resuspended in 50 μ l of kinase assay buffer containing 30 μ M (final concentration) heat shock protein-27 peptide sequence KKLNRTSVA. (38). Reactions were initiated by the addition of 10 μ l of 180 μ M ATP containing [γ - 32 P]ATP (0.5 μ Ci; Amersham). The reaction was allowed to proceed for 20 min before termination. This was achieved by spotting the assay mixture onto squares of p81 paper and then placing them in 0.75% ortho-phosphoric acid. The squares were washed three times in the acid and once in acetone before scintillation counting.

Results

Endogenous IL-1 is required for LPS-stimulated monocyte IL-10 production: confirmation of the role of endogenous TNF- α

LPS is a commonly used stimulus for monocyte cytokine production in vitro, inducing the production of cytokines, including IL-10, IL-1, TNF- α , and IL-6. Monocyte IL-10 is detectable 8 h following LPS stimulation, with peak production detected at 24 h and maintained to at least 96 h (4). In contrast, monocyte production of proinflammatory cytokines such as TNF- α , IL-1, and IL-6 is detected within 2 h of LPS stimulation. We wished to investigate whether the delayed stimulation of monocyte IL-10 production by LPS was mediated by such endogenous inflammatory cytokines. To this end, monocytes were cultured with LPS in the presence or the absence of neutralizing concentrations of Abs or antagonists to monocyte-derived cytokines. A representative experiment is shown in Figure 1, where both anti-TNF- α mAb (cA2) and IL-1Ra potently inhibited LPS-induced monocyte IL-10 production in a dose-dependent manner. In a series of 10 experiments illustrated in Figure 1, a maximally neutralizing concentration of cA2 (Centocor, Malvern, PA) (10 μ g/ml) resulted in $55 \pm 9.4\%$ inhibition of LPS-induced IL-10 production. A maximally neutralizing concentration of IL-1Ra (10 μ g/ml) resulted in $52 \pm 6\%$ inhibition of IL-10 production. Addition of both cA2 and IL-1Ra to monocyte cultures enhanced ($62 \pm 3.8\%$) the inhibition observed with either inhibitor alone, but was routinely less than additive. It was noted, however, that neutralizing concentrations of cA2 and IL-1Ra added together did not completely abrogate LPS-stimulated IL-10 production.

Neutralizing Abs to other monocyte-derived cytokines, including Abs to IL-6, GM-CSF, IL-8, IFN- β , or IFN- α 2, or isotype-

matched control Abs had no effect on monocyte IL-10 production (data not shown), indicating that the endogenous cytokines IL-1 and TNF- α play a unique role in LPS-induced IL-10 production. A Fab preparation of the anti-TNF- α mAb also markedly inhibited monocyte IL-10 production, demonstrating that the effect of anti-TNF- α Ab is specific and not mediated by ligation of monocyte Fc receptors (data not shown).

Addition of exogenous rTNF- α or IL-1 α and IL-1 β , either alone or in combination, to monocyte cultures did not induce IL-10 production unless the monocytes also were primed with LPS.

p42 MAPK and MAPKAP-2 kinase assays: demonstration of inhibitor specificity

There is strong evidence that LPS, IL-1, and TNF- α all activate the p42/44 MAP kinase pathway in a number of cell types (39), and it was therefore of interest to investigate whether p42/44 MAP kinase activation is required for the LPS-mediated induction of monocyte IL-10 production. PD98059 is a specific inhibitor of MEK1 (MAPKK), the dual specificity kinase that activates p42/44 MAPK via phosphorylation on both threonine and tyrosine residues (40). Inhibition of MEK1 by PD98059 prevents activation of p42/44 MAPK and the subsequent phosphorylation of MAPK substrates (41). In Figure 2A, we show that PD98059 inhibits LPS-induced p42 MAPK activation in human monocytes in a dose-dependent manner, with an IC_{50} between 1 and 10 μ M. This is in agreement with the reported in vitro MEK inhibitory activity of PD98059 (41). In contrast, PD98059 at 50 μ M had no effect on p38 MAPK activity as demonstrated in Figure 2B, whereas LPS-induced p38 MAPK activity, as measured by activation of its substrate kinase (MAPKAP-2), was inhibited to background levels by the bicyclic imidazole SB203580 (10 μ M).

Differential role for p42/44 MAP kinase in monocyte production of proinflammatory and anti-inflammatory cytokines

Monocytes were treated for 1 h with PD98059 before stimulation with LPS, and the culture supernatants were harvested at both 4 and 24 h and then assayed for the presence of TNF, IL-1, and IL-10. Significant levels of all three cytokines were only detected after 24 h in culture; therefore, only the results from the 24 h points are illustrated. Furthermore, monocytes that were left unstimulated (no LPS) did not produce detectable amounts of any cytokine (TNF- α , IL-1, or IL-10) at any time point examined, indicating that adherence to plastic is not sufficient to activate the relevant pathways to initiate cytokine production.

PD98059 (0.1 to 50 μ M) inhibited LPS-induced TNF- α and IL-1 β in a dose-dependent manner, although complete inhibition of either cytokine was not seen in any experiment. In contrast, PD98059 did not inhibit LPS-induced IL-10 production at any dose from 0.1 to 50 μ M. A representative experiment is shown in Figure 3, A, B, and C. In additional experiments, it was noted that PD98059 partially inhibited LPS-induced production of IL-6 in a dose-dependent manner, with an IC_{50} of 5 μ M (data not shown). Concentrations of PD98059 >50 μ M were not used, as such concentrations reduced monocyte viability (as determined by exclusion of 1% nigrosin). Addition of DMSO vehicle alone did not inhibit the production of any cytokine (data not shown). While the addition of PD 98059 has no effect on IL-10 synthesis (Fig. 3C), IL-10 levels were reduced when TNF or IL-1 activity was blocked by the further addition of neutralizing concentrations of cA2 (10 μ g/ml) or IL-1Ra (10 μ g/ml; Fig. 3D). Thus, LPS-stimulated monocytes produced 475 ± 49 pg/ml of IL-10 and were unaffected by the addition of PD98059 (455 ± 47 pg/ml; not significant), but IL-10 production was reduced in the presence of cA2 (156 ± 18

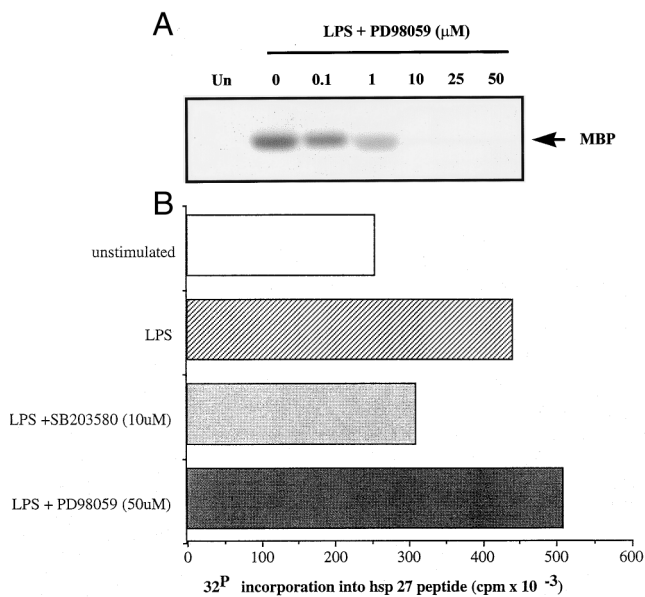


FIGURE 2. p42 MAPK and MAPKAP-2 kinase assays. Monocytes were stimulated with LPS for 10 min with or without PD98059 (0–50 μ M) or SB203580 (10 μ M). Postnuclear lysates were then incubated with a suspension of protein G with rabbit anti-p42 MAPK or anti-MAPKAP-2 as described in *Materials and Methods*. For the p42/44 kinase assay (A), the immunoprecipitated activity was assessed via [γ -³²P]ATP incorporation into myelin basic protein. Phosphorylated products were visualized by autoradiography using Hyperfilm. For the MAPKAP-2 kinase assay (B), immunoprecipitated activity was assessed via [γ -³²P]ATP incorporation into the heat shock protein-27 peptide sequence. Phosphorylated products were assessed by scintillation counting. The figures are representative samples from experiments performed at least three times.

pg/ml; $p < 0.01$) or IL-1Ra (255 ± 28 pg/ml; $p < 0.01$) alone and was reduced to almost background levels when cA2 and IL-1Ra were included together in the presence of PD98059 (54 ± 4 pg/ml; $p < 0.001$).

In light of the observations that PD98059 inhibition of the p42/44 MAPK cascade does not inhibit monocyte production of the anti-inflammatory IL-10, it was of interest to determine whether the monocyte response to IL-10 was also independent of this signaling pathway. One potent anti-inflammatory effect mediated by IL-10 is inhibition of monocyte production of proinflammatory cytokines, including TNF- α (3, 4). Addition of 10 ng/ml IL-10 to LPS-stimulated monocyte cultures resulted in >90% inhibition of TNF- α production as illustrated in Figure 3A; however, treatment of monocytes with PD98059 (1–50 μ M) before addition of IL-10 did not reverse this effect, suggesting that the p42/44 MAPK pathway is not involved in this IL-10-mediated anti-inflammatory effect. Furthermore, IL-10-induced release of the native inhibitor of TNF, sTNFR (6), was not blocked by the inclusion of PD98059 at concentrations ranging from 1 to 50 μ M in a total of three experiments (Fig. 4A), whereas LPS-induced p75 sTNFR release (Fig. 4B) was inhibited in a dose-dependent manner by the p42 MAPK inhibitor.

LPS-induced IL-10 production is dependent on the p38 MAPK pathway

It has previously been demonstrated (42) that the pyridinyl imidazole compounds that inhibit p38 MAPK inhibit LPS-induced TNF and IL-1 production. We sought to determine whether LPS-induced IL-10 production was likewise inhibited. Treatment of monocytes for 1 h with SB203580 resulted in a marked dose-

dependent inhibition of LPS-induced IL-10 production, with concentrations >5 μ M reducing IL-10 levels to below the minimum sensitivity of the ELISA. A representative experiment is shown in Figure 5, where SB203580 induced dose-dependent down-regulation of IL-10 production, with an IC₅₀ of 44 nM. We also confirmed the potent dose-dependent inhibition of both IL-1 β and TNF- α production, with IC₅₀ values of 35 and 70 nM, respectively, in agreement with published results (42). We noted, however, that there was some variation in IC₅₀ values for SB203580 inhibition between experiments. This variation may be related to the use of primary monocytes rather than cell lines, with different donors having different sensitivities to the drug or different responses to the LPS activation signal.

We also tested whether IL-10 synthesis, which was blocked following the addition of the p38 MAPK inhibitor to LPS-stimulated monocytes, could be rescued by the addition of exogenous IL-1 (10 ng/ml) or TNF- α (10 ng/ml), either alone or together, after pretreatment of monocytes with SB 203580. Figure 6 illustrates that LPS-induced IL-10 production in this experiment was enhanced by the further addition of rTNF- α /IL-1 β . However, IL-10 synthesis was once again inhibited in a dose-dependent manner by the addition of the p38 inhibitor, SB 203580, and this blockade was not rescued by the addition of TNF- α or IL-1 alone or together. Taken together, this suggests that the p38 MAPK pathway is involved in IL-10 synthesis, which itself is both dependent on and independent of IL-1 and TNF- α synthesis.

In other experiments, monocytes were treated with a combination of PD98059 and SB203580 (Fig. 7). As shown above, 10 μ M PD98059 did not inhibit LPS-induced IL-10, and in addition, PD98059 did not enhance SB203580-mediated inhibition of IL-10 production at any concentration of SB203580 tested (0.016 to 50 μ M). In contrast, PD98059 induced marked inhibition of LPS-stimulated TNF- α production and had an additive effect with SB203580 to inhibit TNF- α production.

Discussion

In the present study we show that endogenously produced IL-1, a potent primary inflammatory cytokine, plays an important role in LPS-stimulated IL-10 production by human monocytes. Furthermore, in agreement with previously published studies (17, 18), we demonstrate the involvement of endogenous TNF- α in LPS-stimulated monocyte IL-10 production. As IL-10 inhibits monocyte production of IL-1 and TNF- α , it is apparent that these three cytokines form an autoregulatory feedback loop.

Addition of a combination of anti-TNF- α mAb cA2 and IL-1Ra to LPS-stimulated monocyte cultures did not markedly enhance the inhibition caused by either cA2 or IL-1Ra alone, suggesting that IL-1 and TNF- α regulate IL-10 production by a common mechanism. Previous studies have suggested that the TNF- α primarily regulates IL-10 transcription, and it is of interest that the human IL-10 promoter contains an activating protein-1 recognition site (18), as both IL-1 and TNF- α can induce activating protein-1 (43, 44). However, further investigation of IL-10 production at the levels of transcription, translation, and secretion is necessary to determine the precise roles of IL-1 and TNF- α in these processes.

The involvement of both endogenous IL-1 and TNF- α in monocyte IL-10 production was not unexpected, as these two cytokines have a similar broad range of physiologic effects. A previous report has suggested that IL-1 did not regulate IL-10 production (17); however, in those studies either an anti-IL-1 α Ab or an anti-IL-1 β Ab, but not a combination, was used, and it may not have sufficiently neutralized the high levels of endogenous IL-1 that monocytes are capable of secreting. In the present study IL-1Ra

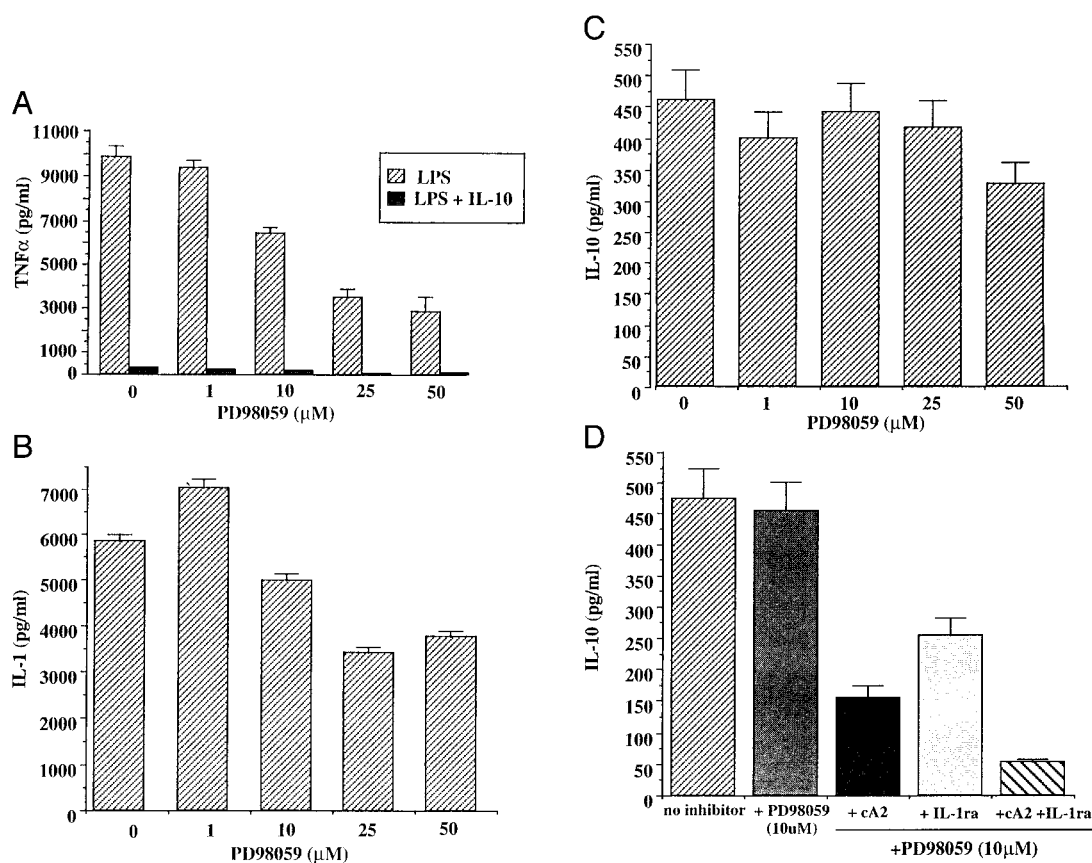


FIGURE 3. PD98059 reduces LPS-induced TNF- α and IL-1 β release but does not block IL-10 synthesis. Monocyte cultures were treated with PD98059 (0–50 μ M) for 1 h before stimulation with LPS in the absence or the presence of IL-10 (10 ng/ml), cA2 (10 μ g/ml), and IL-1Ra (10 μ g/ml). Culture supernatants were harvested 24 h later and assayed for TNF- α (A), IL-1 β (B), and IL-10 (C and D) by ELISA. Data are means of triplicate culture supernatants \pm SD; a representative example of three replicate experiments is shown.

was used to prevent the effects of both IL-1 α and IL-1 β . We also noted that addition of LPS-free, exogenous recombinant IL-1 α , IL-1 β , or TNF- α (in contrast to previous studies (17)) did not directly induce LPS-stimulated monocyte IL-10 production (data not shown), although on some occasions these cytokines did augment LPS-induced IL-10 synthesis (Fig. 6). It is possible that the high levels of endogenous cytokines present in the cultures maximally stimulate monocytes, and addition of exogenous cytokines has no further effect. Several studies, including the present work, suggest that monocyte cytokine production is regulated in an autocrine/paracrine network of stimulatory and inhibitory cytokines such as IL-1, TNF- α , and IL-10 (17, 18, 28). This suggests the existence of both early common and late specific signal transduction events. The latter may be mediated at least partly by LPS-induced mediators such as IL-1 and TNF- α . The data shown here also indicate that IL-1/TNF- α -independent mechanisms of LPS-mediated IL-10 production exist, as IL-10 was routinely detected in cultures treated with maximally neutralizing concentrations of IL-1Ra in combination with anti-TNF- α mAb. Further, addition of IL-1 α , IL-1 β , and/or TNF- α to monocytes in the absence of LPS did not induce IL-10 production (data not shown). This LPS-induced signal may involve additional endogenous cytokines, inducible contact-mediated signals between monocytes, or a distinct biochemical pathway. Indeed, we have recently demonstrated that TCR-activated T lymphocytes can induce monocyte IL-10 production in a contact-dependent, LPS-independent manner (16).

The signaling pathways involved in LPS-stimulated monocyte production of proinflammatory or anti-inflammatory cytokines are

not yet fully understood. LPS has previously been shown to activate all three (known) mammalian MAPK pathways, p42/44 (ERK1/2), p38, and p54/JNK (stress-activated protein kinases), in monocytes or macrophages (23, 25, 26). Until recently, p42/44 was the only cloned and well-characterized mammalian MAPK; however, the discovery of two other MAP kinases, p38 and p54, revealed the existence of parallel MAPK cascades that can be activated individually and simultaneously (39, 42, 45), suggesting independent signaling roles for these MAPK cascades.

The data presented here demonstrate that the p42/44 MAPK pathway is not involved in LPS-stimulated monocyte IL-10 production. LPS-stimulated TNF- α and IL-1 β production was partially inhibited by PD98059, an inhibitor of MEK1, indicating that these proinflammatory cytokines are to some extent dependent on the p42/44 MAPK pathway. The PD98059 inhibitor abrogated p42/p44 activity, as illustrated in Figure 2A, with an IC₅₀ between 1 and 10 μ M, whereas the IC₅₀ of this drug for inhibition of TNF- α and IL-1 β was slightly higher (\sim 10 μ M; Fig. 3, A and B). This most likely reflects the difference between the kinetics of drug activity in the *in vitro* kinase assay (cells harvested after 10 min) compared with those in the biologic assay (cells harvested after 18 h). However, it cannot be excluded that the PD98059 inhibitor has a target other than MEK, the upstream kinase in the p42/44 pathway, although to date there is no evidence for nonspecificity of this drug (55). Furthermore, a number of recent reports have shown that IL-1 and TNF- α activate p42/44 MAPK in a variety of cell types, including fibroblasts, vascular endothelial cells, and chondrocytes (39), and in a recent study it was demonstrated that

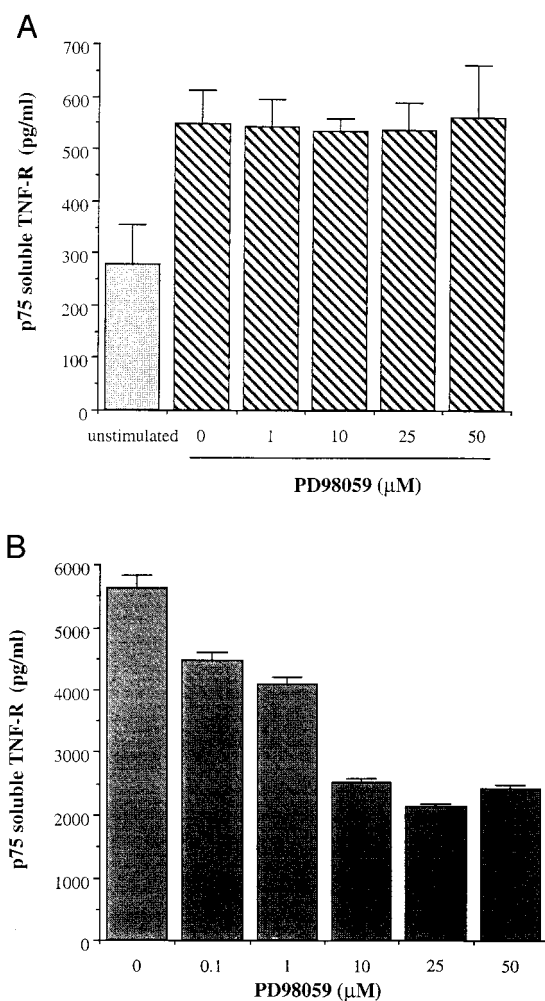


FIGURE 4. PD98059 does not inhibit IL-10-induced p75 sTNFR shedding. Monocyte cultures were pretreated with PD98059 (0–50 μM) for 1 h before stimulation with 10 ng/ml IL-10 (A) or LPS (B). Supernatants were harvested after 18 h in culture, and sTNFR levels were measured by ELISA. Data are means of triplicate culture supernatants \pm SD; a typical example from three experiments is shown.

cross-linking Fc γ receptors on mouse macrophages results in p42/44 MAPK activation and TNF- α production, which was inhibited by the PD98059 drug (46). However, the lack of effect of PD98059 on monocyte IL-10 production suggests that LPS-induced IL-10 synthesis is not mediated by a p42/44-mediated pathway, although IL-10 synthesis was reduced in those LPS-stimulated monocyte cultures pretreated with PD98059, if additionally all TNF- α and IL-1 activities were neutralized using cA2 and IL-1Ra, similar to that seen with cA2 and IL-1Ra alone (data not shown). This indicates that IL-10 production is independent of the p42/44 MAPK pathway, but that endogenous TNF and IL-1 clearly play a role in the IL-10 synthetic pathway.

Despite the wealth of papers demonstrating the importance of IL-10 as an immunoregulator, the mechanisms by which it exerts these effects remain poorly understood. To date, IL-10 has been shown to activate two distinct pathways, the JAK/STAT pathway (47, 48) and the phosphatidylinositol 3-kinase/S6 kinase pathway (49). However, neither of these pathways has been shown to mediate the anti-inflammatory actions of IL-10. We have shown that the p42/44 MAPK pathway is not involved in IL-10 synthesis in monocytes, and it was of interest to determine whether IL-10-mediated signaling events in monocytes were likewise indepen-

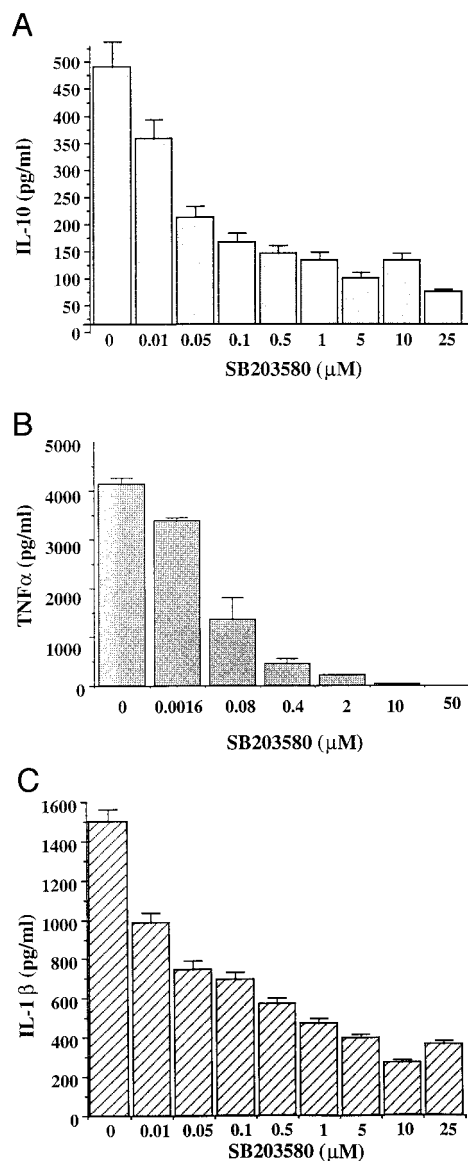


FIGURE 5. SB203580 inhibitor of p38 MAPK reduces LPS-stimulated monocyte production of IL-10, IL-1 β , and TNF- α . Monocytes were treated with SB203580 at the indicated concentration for 1 h before stimulation with LPS for 24 h. Culture supernatants were assayed for IL-10 (A), TNF- α (B), and IL-1 β (C) by ELISA. Data are means of triplicate culture supernatants \pm SD; a representative example from six replicate experiments is shown.

dent of this kinase. Results show that neither IL-10-induced inhibition of TNF- α production nor stimulation of p75 sTNFR was affected by PD98059 inhibitor, thus indicating that the p42/44 MAPK pathway is not involved in these IL-10-mediated anti-inflammatory effects. In contrast to the IL-10 effects, the induction of p75 sTNFR by LPS was sensitive to the p42/44 inhibitor. In keeping with the cellular data, which indicate that IL-10-induced anti-inflammatory effects are not mediated by the p42/44 MAPK pathway, we have shown that IL-10 does not induce MAPK phosphorylation (as assessed by gel retardation) and hence did not activate p42 MAPK (data not shown). Furthermore, LPS-induced p42 MAPK activation, as measured by *in vitro* kinase assay and by gel retardation, was not inhibited by IL-10 (data not shown). This is in contrast to an earlier report (50) in which IL-10 was reported

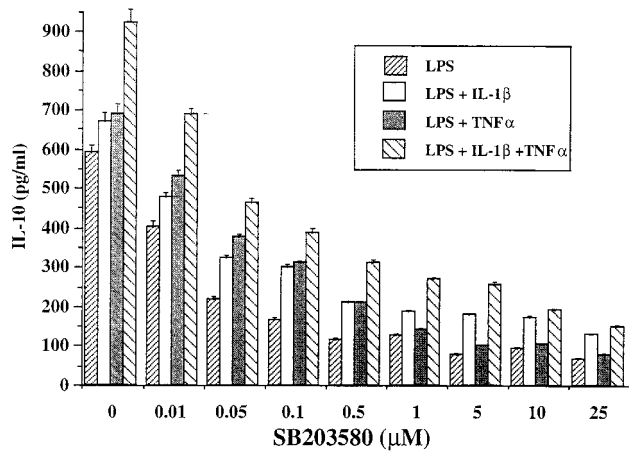


FIGURE 6. SB203580 inhibition of LPS-induced IL-10 synthesis is not reversed by the addition of exogenous TNF- α or IL-1 β . Monocytes were treated with SB203580 at the indicated concentration for 1 h before stimulation with LPS \pm TNF- α (10 ng/ml) and IL-1 β (10 ng/ml), alone or together, for 24 h, and culture supernatants were assayed for IL-10 by ELISA. Data are means of triplicate culture supernatants \pm SD.

to inhibit LPS-induced tyrosine phosphorylation of p56^{l^{ym}} and p56^{vav}, with a subsequent decrease in Ras and p42 MAPK activation. In addition, IL-10 had no effect on LPS-induced activation of either p38 or p54 MAPK (data not shown), as assessed using an in vitro kinase assay as measured by [γ -³²P]ATP incorporation into the relevant substrate (activating transcription factor-2 or myelin basic protein). The inability of IL-10 to modify p38 activity was surprising, as this enzyme is clearly a strong candidate for IL-10 deactivation because it has been shown to be critically involved in TNF production. IL-10 also did not inhibit LPS-induced tyrosine phosphorylation of p38 MAPK, nor was the activity of MAPKAP-2 (a downstream substrate for activated p38 MAPK) inhibited by IL-10 (data not shown). Thus, despite the importance of IL-10 as a potent immunoregulatory cytokine, the signaling events associated with the anti-inflammatory effects remain to be elucidated.

p38 MAPK has previously been shown to be activated by LPS, IL-1, and TNF- α (42). In this report we show for the first time that inhibition of p38 MAPK with SB203580 results in complete abrogation of LPS-induced IL-10 production. In addition, we confirm that SB203580 inhibits LPS-induced TNF- α , IL-1 β , and IL-6, in agreement with earlier studies using related bicyclic imidazoles (25, 51, 52). These results suggest that activation of the p38 MAPK cascade is an early common signal necessary for LPS-stimulated monocyte cytokine production. While the signaling events leading to IL-10 synthesis are complex, we hypothesize that IL-10 production requires at least two signals; the first is provided by LPS (or its physiologic equivalent), and the second by endogenous TNF- α and/or IL-1. The fact that the addition of exogenous rTNF (10 ng/ml) or IL-1 β (10 ng/ml), either alone or together, failed to rescue IL-10 synthesis in those LPS-stimulated cultures that had been pretreated with SB203580 indicates that IL-10 production could be dependent upon p38 MAPK at different levels in terms of endogenous TNF- α /IL-1 synthesis as well as at the LPS-induced and/or TNF- α /IL-1 signaling level.

Inhibition of the p42/44 MAPK cascade did not augment the SB203580 inhibition of IL-10 production, providing further evidence that p42/44 MAPK is not involved in LPS-induced IL-10. In contrast, both PD98059 and SB203580 inhibited LPS-induced TNF- α singly and acted additively to reduce monocyte TNF- α .

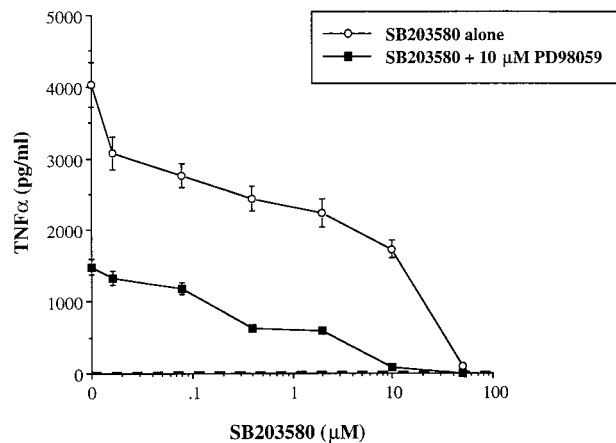
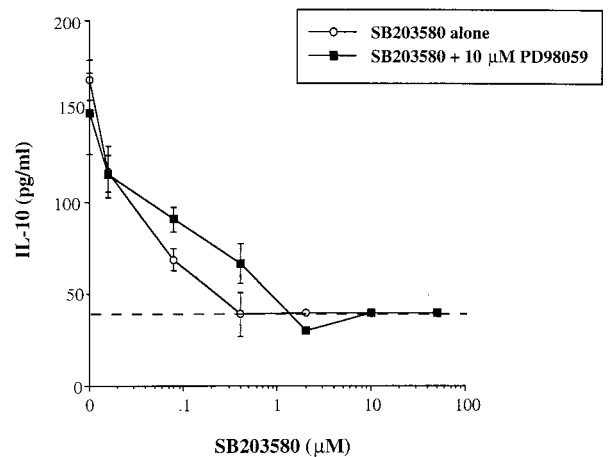


FIGURE 7. PD98059 acts with SB203580 to further inhibit LPS-induced TNF- α release, but has no effect on SB203580 inhibition of IL-10. Monocytes were treated with PD98059 (10 μ M) and SB203580 (0–50 μ M) for 1 h before stimulation with LPS. Supernatants were assayed for IL-10 and TNF- α by ELISA. Data are means of triplicate culture supernatants \pm SD; an example from three replicate experiments is shown. Dashed lines indicate the minimum sensitivity of the ELISA for each cytokine.

The Ras/Raf/ERK kinase pathway has been suggested to be primarily responsible for LPS-induced transduction of the TNF- α gene (53). The partial inhibition of TNF- α production induced by PD98059 observed in the present study might suggest that several parallel pathways regulate TNF- α gene transcription, and that these alternative pathways may be sufficient to stimulate the TNF- α promoter despite effective blockade of the p42 MAPK cascade. The LPS-induced signal leading to TNF- α production is believed to branch, ultimately yielding both transcriptional and translational activation of TNF- α biosynthesis (54). Our data would support this view, as a combination of PD98059, leading to transcriptional block of TNF- α synthesis, and SB203580, leading to translational block of TNF- α synthesis, resulted in complete abrogation of TNF- α production, suggesting that the p38 and p42/44 MAPK pathways are both necessary for LPS-induced monocyte TNF- α production.

In conclusion, the results demonstrate the different effects of the MAPK inhibitors on monocyte production of IL-1, TNF- α , and IL-10, indicating that at least two MAPK cascades are involved in LPS-stimulated production of these cytokines. Further, the differential susceptibility of pro- vs anti-inflammatory production to PD98059 or SB203580 indicate the differing importance of p42/44

and p38 MAPK in the signaling events leading to the production of a given cytokine. TNF- α and IL-1 are considered to be key mediators in many inflammatory conditions, including arthritis and inflammatory bowel disease, whereas IL-10 is believed to play an important anti-inflammatory role. Clearly, there is great therapeutic potential for any compound that interferes with the production or action of TNF- α or IL-1 but does not modulate either the production of IL-10 or the anti-inflammatory effects mediated by IL-10.

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