Brief Definitive Report

Evidence for the Involvement of Interleukin 10 in the Differential Deactivation of Murine Peritoneal Macrophages by Prostaglandin E_2

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Summary

Among other effects, prostaglandins (PG) of the E series are known to inhibit several acute and chronic inflammatory conditions in vivo and proinflammatory cytokine production by activated macrophages in culture. The research presented here demonstrates that the inhibitory effect of PGE_2 on tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) production by lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages involves IL-10. In a dose-dependent manner, PGE_2 inhibits LPS-induced release of TNF-α and IL-6, but not of lactate or nitric oxide. The decrease in the level of these cytokines is inversely proportional to the increase in immunoreactive IL-10. This differential inhibitory effect of PGE_2 is mimicked by agents that elevate intracellular levels of cAMP, but not cGMP. Neutralizing anti IL-10 antibody but not neutralizing antibodies against other macrophage secretory products (IL-6, leukemia inhibitory factor, and transforming growth factor β [TGF-β]), significantly reverse the potent inhibitory effect of PGE_2. In vivo, the administration of PGE_2 before LPS challenge significantly reduces circulating TNF-α and IL-6 levels. Anti–IL-10 antibody substantially enhanced the LPS-induced TNF-α and IL-6 levels in mice that received either LPS alone or LPS plus PGE_2. These results suggest that the antiinflammatory effect of PGE_2 on mononuclear phagocytes is mediated in part by an autocrine feedback mechanism involving IL-10.

Mononuclear phagocytes (macrophages) play a central role in the regulation of immune responses as well as in both acute and chronic inflammation. The mechanisms that participate in these activities are believed to be multifactorial and involve macrophage secretory products (for a review see reference 1). TNF-α is an important macrophage inflammatory cytokine that mediates a wide range of biologic functions. TNF-α is believed to play a major role in septic shock, and to contribute to the pathogenesis of AIDS and several inflammatory and autoimmune diseases (for reviews see references 2, 3). Recently, a number of regulatory factors were described as having the capacity to block macrophage functions, including TNF-α release. These molecules, also termed "macrophage-deactivating factors," include PG of the E series (4–8), TGF-β (9), IL-4 (10), and IL-10 (11, 12). In addition to their capacity to induce pain, vascular changes and downregulation of T cell functions, PG have been shown to exhibit antiinflammatory properties on macrophages. For example, stimulation of macrophages by LPS or by TNF-α induces PGE_2 production (13, 14), and the addition of PGE_2 to LPS-stimulated cells inhibits TNF-α mRNA expression

and protein secretion (6–8). This information leads to the hypothesis that the release of PG during LPS-induced inflammation constitutes a negative-feedback mechanism that limits the magnitude of inflammatory cytokine production. In vivo, the exogenous administration of PGE suppresses adjuvant arthritis in rats (15), inhibits the manifestation of interstitial nephritis (16), and prolongs the survival of NZB/W F_1 female autoimmune mice (17).

IL-10 is a 35-kD protein that can be produced by subpopulations of T helper cells, B cells, and macrophages. Among other properties, IL-10 is a potent inhibitor of monocyte and macrophage-derived cytokine synthesis in vitro (for a review see reference 18). In vivo, the neutralization of IL-10 by specific mAb results in increased levels of TNF-α and IL-6 (19), and the administration of IL-10 effectively protects mice from lethal endotoxemia (20).

In the current experiments, we establish a connection between the macrophage inhibitory effect of PGE_2 and IL-10. Evidence is presented to support an important role for IL-10 in the deactivation of murine peritoneal macrophages by PGE_2.

Materials and Methods

Mice. Specific pathogen free, male C57Bl/6 and CB6 (BALB/c × C57Bl/6)F_1 mice 9–12 wk old were purchased from Charles River Breeding Laboratories (Wilmington, MA), and they were housed under conventional conditions.

Reagents and Antibodies. LPS from *Salmonella enteriditis*, PGE_2, 3-isobutyl-1-methylxanthine (IBMX), dibutyryl (db) cAMP, and dbcGMP were purchased from Sigma Chemical Co. (St. Louis, MO). The polyclonal rabbit IgG anti–murine leukemia inhibitory factor (LIF) and anti–murine TNFα, the rat IgG_1 mAb anti–murine IL-6 (designated 20F3), the mAb anti–murine IL-10 (rat IgG1, designated 2A5), and isotype control antibody (GL113) were generated and purified as described before (20–22). Neutralizing rabbit pan-specific TGF-β IgG was purchased from R&D Systems (Minneapolis, MN). Anti-LIF and anti–TNF-α were the kind gifts of Dr. H. R. Alexander (National Cancer Institute, National Institutes of Health, Bethesda, MD).

Macrophage Cultures. Thioglycollate broth–elicited macrophages were prepared as reported previously (23). Peritoneal lavage was performed using 10 ml of cold HBSS containing 10 U/ml heparin. Peritoneal exudate cells were washed and resuspended in culture medium (RPMI 1640 containing 10% heat-inactivated FCS, penicillin, and streptomycin), and were then plated in 12-well tissue culture plates (Corning, Corning, NY) at a cell density of 1.2 × 10^6 per well. After incubation for 90 min, the cell monolayers

were washed three times with complete medium. The cell monolayers were routinely found to contain >95% mononuclear phagocytes. Conditioned medium generated after LPS stimulation for 24 h was removed, centrifuged, and cell-free supernatants were stored at −35°C until further analysis. The macrophage monolayers were washed three times with PBS, solubilized, and cellular protein was quantified by the bicichoninic acid (Pierce Chemical Co., Rockford, IL) method. The cells were treated as described in the text or figure legends.

Bioassays and ELISA. TNF-α and IL-6 bioassays were performed as previously described (23). The addition of anti–TNF-α antibody or anti IL-6 (10 μg/ml) resulted in the neutralization of ∼95% of the bioactivity of the corresponding cytokines in the respective assays. Murine TNF-α and IL-10 ELISA kits were obtained from Endogen (Boston, MA) and Biosource International (Cammarillo, CA), respectively.

Nitrite concentration as a reflection of nitric oxide release was measured using the Griess reagent (24). Lactate concentration was measured using reagents from Sigma Chemical Co.

Endotoxin-induced Shock. Mice were injected with PGE_2 dissolved in ethanol (200 μg/mouse in 0.2 ml) or with the same amount of ethanol diluted in PBS (final ethanol concentration was 9%) 3 h before the administration of endotoxin (from *S. enteriditis*) diluted in sterile PBS (0.1 ml/mouse containing 30 μg LPS). 90 min be-
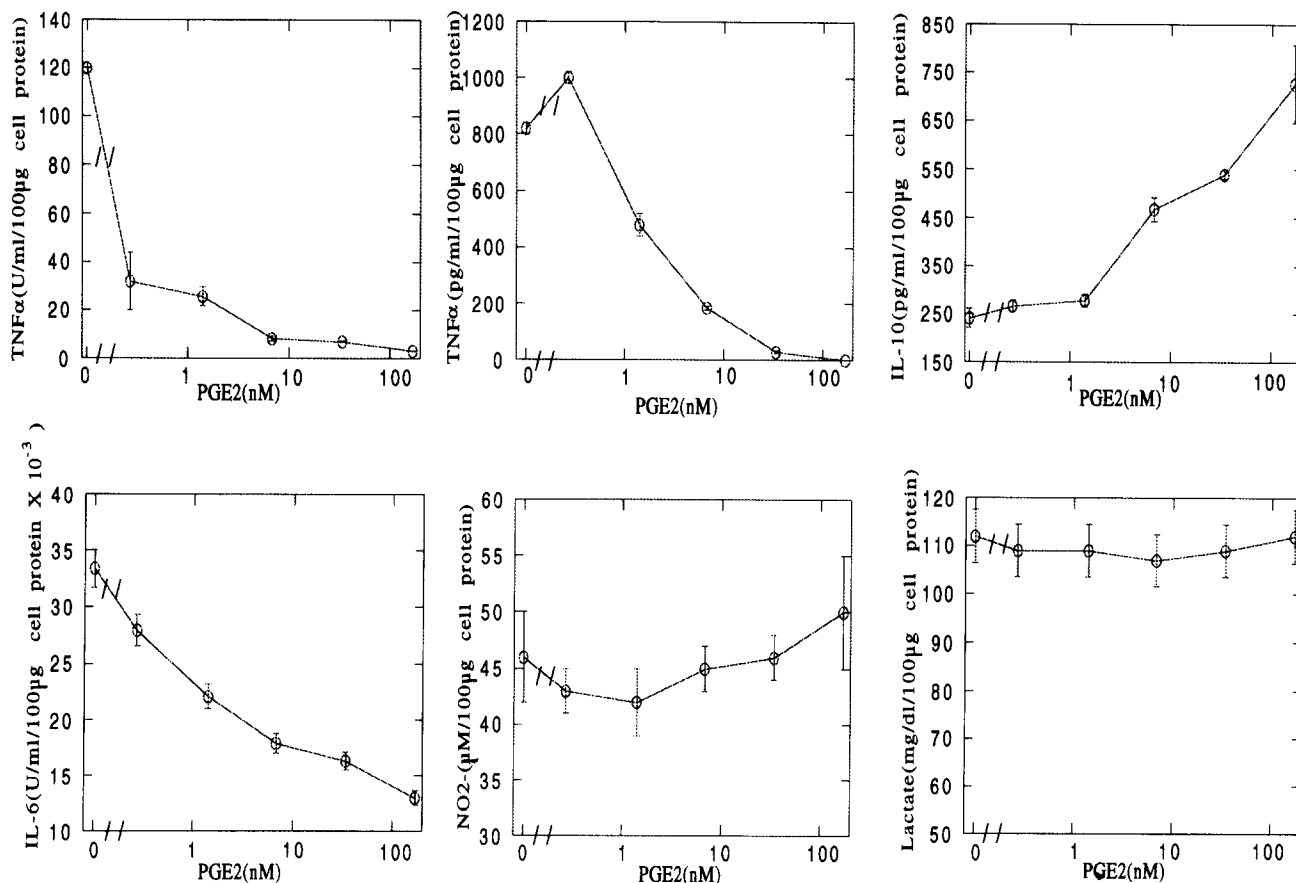

Figure 1. PGE_2 suppresses TNF-α and IL-6, but increases IL-10 release by LPS-stimulated macrophages. Macrophages were pretreated with the indicated concentration of PGE_2, stimulated with LPS (0.5 μg/ml) for 24 h, and cell-free supernatants were subjected to the corresponding assay, as described in Materials and Methods.

fore endotoxin challenge, the mice were injected with anti–murine IL-10 (2A5, 0.9 mg/mouse in 0.1 ml PBS) or isotype control antibody (GL113). All injections were performed via intraperitoneal administration. Blood was obtained by a retro-orbital plexus puncture at 1 and 3 h after LPS challenge, and serum levels of TNF and IL-6 were determined at these time points, respectively.

Data Presentation and Statistical Analysis. Results are expressed as mean ±SD of duplicate determinations of each duplicate culture for every treatment. Results obtained by ELISA and bioassays are shown in picograms per milliliter per 100 μg cell protein, and in units per milliliter per 100 μg cell protein, respectively.

Serum TNF-α and IL-6 expressed as units per milliliter were determined from five mice in each group. Statistical differences were calculated by analysis of variance (ANOVA).

Results and Discussion

Previous reports demonstrated that the addition of PGE₂ to macrophage cultures decreases LPS-induced stimulation of TNF-α mRNA and protein levels (6–8). To determine whether this antiinflammatory effect of PGE₂ involves IL-10, macrophage monolayers were pretreated with increasing concentrations of PGE₂ for 16 h, followed by LPS stimulation (500 ng/ml) for an additional 24 h. In a dose-dependent manner, PGE₂ potently inhibits LPS-induced TNF-α and IL-6 release, with half-maximal inhibition of $\sim 10^{-9}$ M (Fig.

1). In contrast to the PGE₂-induced inhibition of TNF-α and IL-6 release, the quantification of immunoreactive IL-10 in the same cell-free supernatants reveals a PGE₂-dependent augmentation of LPS-induced IL-10 levels. The selective inhibitory action of PGE₂ is further reflected by the inability of this arachidonic acid metabolite to alter LPS-induced release of nitric oxide and of lactate (Fig. 1). These results demonstrate that the addition of PGE₂ to our LPS-stimulated cultures is not toxic to the cells. Nearly identical results were obtained in three separate experiments, irrespective of whether the cells were pretreated with PGE₂ or cotreated with PGE₂ plus LPS. In the absence of LPS macrophages do not produce detectable TNF-α or nitric oxide, but release 10 U/ml per 100 μg cell protein of IL-6 and 45 mg/dl per 100 μg cell protein of lactate.

PGE₂ is known to elevate intracellular cAMP levels via stimulation of adenylate cyclase (25). To determine whether the effect of PGE₂ on IL-10 production might relate to changes in intracellular cAMP, macrophages were treated with LPS in the absence or presence of dbcAMP. Fig. 2 demonstrates that dbcAMP but not dbcGMP can mimic the differential action of PGE₂ in reducing TNF-α and augmenting IL-10 production. Also in the figure, the involvement of intracellular cAMP is further confirmed by using IBMX, which elevates cAMP levels by inhibiting phosphodiesterase and, therefore, indirectly blocks the degradation of cAMP (26).
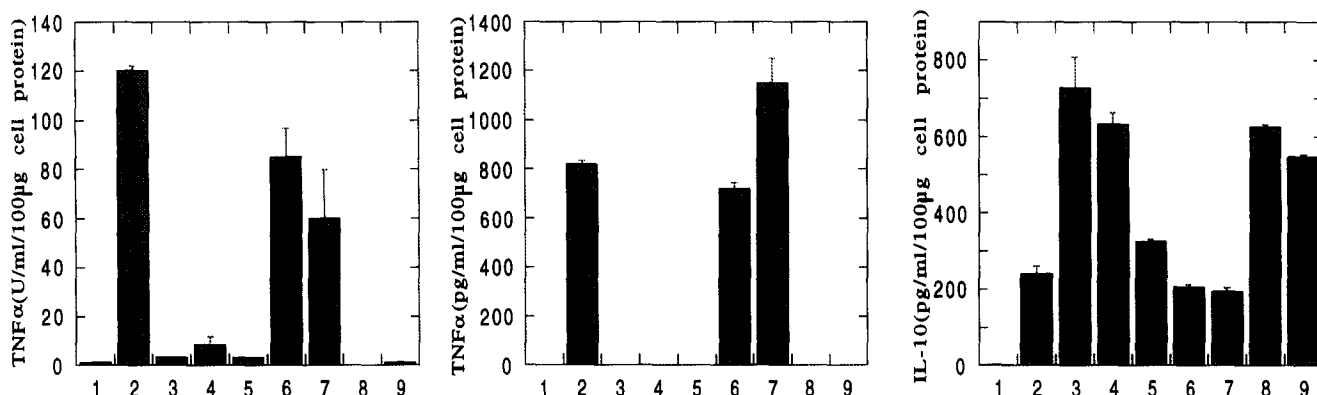
Pretreatment			Treatment
1. Medium			Medium
2. Medium			LPS
3. PGE2	0.17	μM	LPS
4. dbcAMP	100	μM	LPS
5. dbcAMP	30	μM	LPS
6. dbcGMP	100	μM	LPS
7. dbCGMP	30	μM	LPS
8. IBMX	200	μM	LPS
9. IBMX	70	μM	LPS

Figure 2. Agents that increase intracellular cAMP inhibit TNF-α and IL-6, but increase IL-10 release. Macrophages were pretreated with the indicated concentration of dbcAMP, dbcGMP, and IBMX, and were then stimulated with LPS (0.5 μg/ml) for 24 h.

We next evaluated whether the rise in IL-10 level in culture supernatants of PGE₂-treated and LPS-stimulated cells is involved in the subsequent reduction in TNF-α and IL-6 levels. To this end, neutralizing anti–IL-10, as well as antibodies against other macrophage secretory products, including IL-6, LIF, and TGF-β, were added in the presence or absence of LPS after pretreatment of macrophages with PGE₂. None of the above mentioned antibodies stimulates TNF production alone (data not shown). The potent inhibitory effect of PGE₂ on TNF-α and IL-6 production is significantly reversed by anti–IL-10, but not by anti–IL-6, anti–TGF-β, anti-LIF, or isotype-control antibody (Fig. 3).

IL-10 is known to suppress LPS-induced TNF production (18), and anti–IL-10 (2A5) elevates the levels of TNF-α and IL-6 in mice (19). The degree to which anti–IL-10 reverses TNF-α and IL-6 production was, therefore, determined in LPS-stimulated cells in the absence and presence of PGE₂. Fig. 4 shows that whereas TNF-α is significantly elevated in anti–IL-10 and PGE₂-treated cells, the level of the cytokine is considerably less than that observed in cells treated with LPS and anti–IL-10. Determination of IL-10 by ELISA, however, shows that the amount of anti–IL-10 used in these experiments is sufficient to totally inactivate the IL-10 released by macrophages treated with PGE₂ and LPS (Fig. 4). In the same experiments, anti–IL-10 almost completely reversed

PGE₂ inhibition of IL-6 levels (no significant difference between groups 3 and 5).

Finally, we attempted to reproduce our in vitro observations in endotoxemic mice. A preliminary experiment showed that the pretreatment of CB6F1 mice with PGE₂ (200 μg/mouse intraperitoneally) results in a significant decrease (65% reduction, $p < 0.006$) in circulating TNF-α levels 1 h after endotoxin challenge (data not shown). We and others (27) have previously shown that the peak of circulating TNF-α and IL-6 levels can be seen at 1 and 3 h after LPS administration, respectively. The experiment shown in Fig. 5 demonstrates that PGE₂ suppresses TNF-α and IL-6 levels during endotoxin shock by ~70 and 50%, respectively ($p < 0.002$). Anti–IL-10 antibody significantly reversed the inhibitory effect of PGE₂ on TNF-α. However, when compared with the augmented serum TNF-α levels seen in anti–IL-10–treated mice, the reversal of TNF-α levels in PGE₂ plus anti–IL-10–treated animals was incomplete. In contrast, serum IL-6 is almost completely reversed by the anti–IL-10 treatment. These results are, therefore, in agreement with the data obtained in culture. Namely, anti–IL-10 affords a significant but incomplete reversal of the inhibitory effect of PGE₂ on TNF-α release, but it appears to completely reverse the PGE₂ effect on IL-6 production both in vitro and in vivo. Thus, IL-10 is partially responsible for TNF-α inhibition but appears to
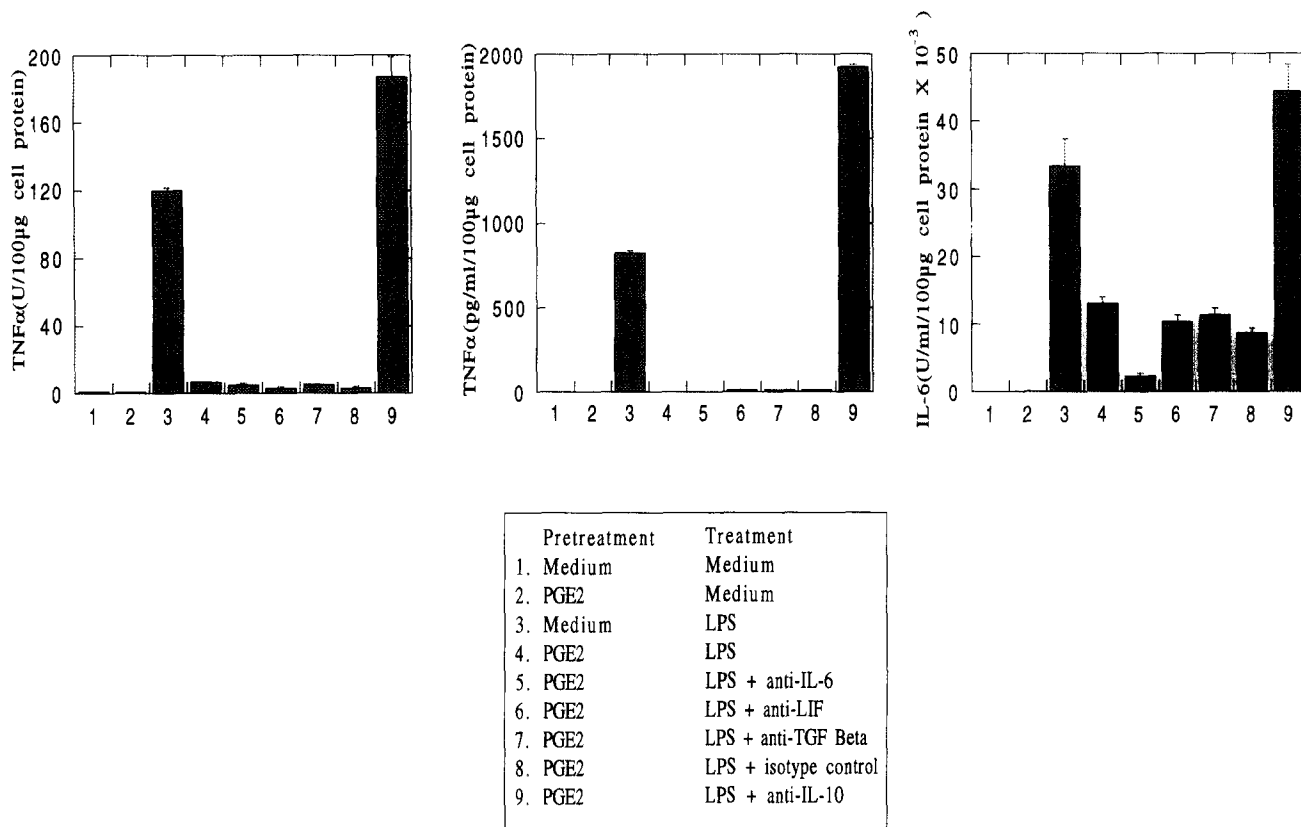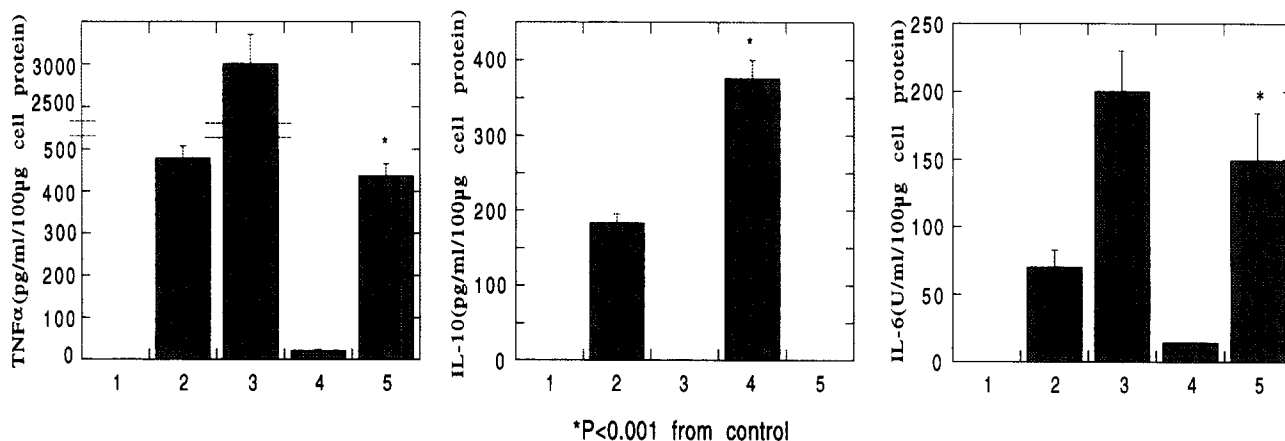

	Pretreatment	Treatment
1.	Medium	Medium
2.	PGE2	Medium
3.	Medium	LPS
4.	PGE2	LPS
5.	PGE2	LPS + anti-IL-6
6.	PGE2	LPS + anti-LIF
7.	PGE2	LPS + anti-TGF Beta
8.	PGE2	LPS + isotype control
9.	PGE2	LPS + anti-IL-10

Figure 3. The inhibitory effect of PGE₂ on TNF-α and IL-6 release can be specifically reversed by anti–IL-10 mAb. Cells were pretreated with medium or PGE₂ (34 nM) and treated with LPS (0.5 μg/ml) plus 20 μg/ml of the indicated antibody. TNF-α and IL-6 were determined by ELISA and bioassay, respectively. In the absence of LPS, this concentration of the antibodies did not induce TNF-α and IL-6 (not shown).

Pretreatment	Treatment
1. Medium	Medium
2. Medium	LPS + GL113
3. Medium	LPS + 2A5
4. PGE2	LPS + GL113
5. PGE2	LPS + 2A5

Figure 4. The reversal of the PGE_2 effect by anti-IL-10 is significant but not complete. Cells were pretreated with PGE_2 (3.4 nM) or medium, and were then treated with LPS plus 2A5 or GL113 (20 $\mu g/ml$), as indicated. TNF-α and IL-10 were determined by ELISA. IL-6 was determined by the B-9 bioassay. Similar results were obtained in three separate experiments.

be the major element responsible for IL-6 inhibition by PGE_2. It is possible that the mechanism of action of PGE_2 involves an additional inhibitory factor(s) that regulates TNF-α release or that PGE_2 directly regulates the synthesis of this cytokine. It remains to be established whether induction of PG synthesis and the subsequent rise in cAMP that regulates IL-10 release is also involved in the negative effect of other "macrophage-deactivating factors," such as IL-4 (10) or IL-13 (28). PG are known to inhibit lymphocyte and macrophage functions. For example, PGE_2 inhibits chemotaxis and chemokinesis of lymphocytes (29). Because IL-10 is produced by subpopulations of T helper cells and B cells (18), it is possible that the inhibitory effect of PGE_2 on lymphocytes also involves IL-10, as is shown here for murine macrophages.

*P<0.001 from control
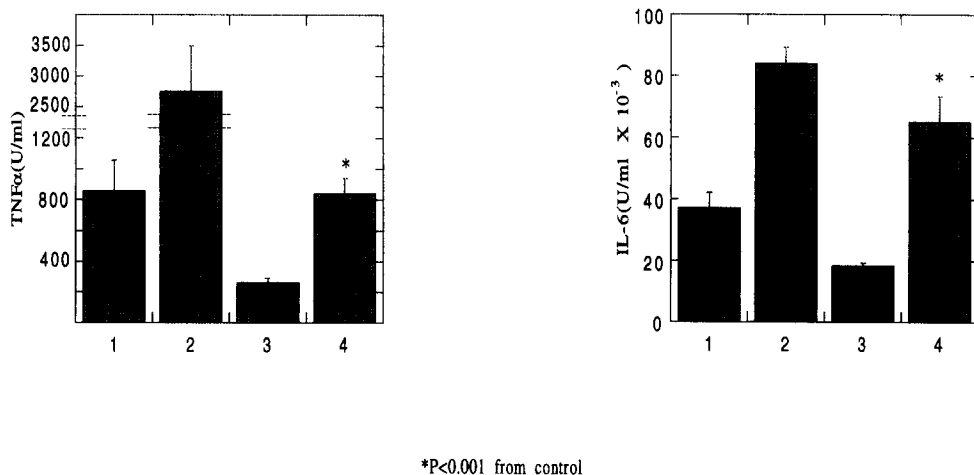
1.	PBS	GL113	LPS
2.	PBS	2A5	LPS
3.	PGE2	GL113	LPS
4.	PGE2	2A5	LPS

Figure 5. Anti-IL-10 partially reverses the inhibitory effect of PGE_2 on TNF-α release in endotoxemic mice. CB6F1 mice were injected with PGE_2 or PBS, followed by the administration of anti-IL-10 or isotype control antibody and LPS, as described in Materials and Methods. There were five mice per group. Serum TNF-α and IL-6 were quantified in L929 and B-9 bioassays, in the absence and presence of the corresponding neutralizing mAb.

In summary, our results provide an explanation to the known inhibitory effect of PG on cytokine synthesis by activated murine peritoneal macrophages in culture (6–8), and they may also explain part of the antiinflammatory effect of PGE in vivo (15–17). Collectively, the data strongly suggest that bioactive IL-10 is produced by a mechanism involving adenylate cyclase during LPS stimulation. In turn, IL-10 contributes to the control of the magnitude of macrophage inflammatory profile by a mechanism involving an autocrine pathway.

References

1. Nathan, C.F., and Z.A. Cohn. 1985. Cellular components of inflammation: monocytes and macrophages. In Textbook of Rheumatology. W. Kelly, E. Harris, S. Ruddy, and C. Sledge, editors. W.B. Saunders, Philadelphia. 144.

2. Beutler, B., and A. Cerami. 1989. The biology of cachectin/TNF: a primary mediator of host response. Annu. Rev. Immunol. 7:625.

3. Vassali, P. 1992. The pathophysiology of tumor necrosis factor. Annu. Rev. Immunol. 10:411.

4. Snyder, D.S., D.I. Beller, and E. Unanue. 1982. Prostaglandins modulate macrophage Ia expression. Nature (Lond.). 299:163.

5. Taffet, S.M., and S.W. Russell. 1981. Macrophage mediated tumor cell killing: regulation of expression of cytolytic activity by prostaglandin E. J. Immunol. 126:424.

6. Kunkel, S.L., M. Spengler, M.A. May, R. Spengler, J. Larrick, and D. Remick. 1988. Prostaglandin E2 regulates macrophage derived tumor necrosis factor gene expression. J. Biol. Chem. 263:5380.

7. Tannenbaum, C.S., and T.A. Hamilton. 1989. Lipopolysaccharide induced gene expression in murine peritoneal macrophages is selectively suppressed by agents that elevate intracellular cAMP. J. Immunol. 142:1274.

8. Kunkel, S.L., R.C. Wiggins, S.W. Chensue, and J. Larrick. 1986. Regulation of macrophage tumor necrosis factor production by prostaglandin E2. Biochem. Biophys. Res. Commun. 137:404.

9. Tsunawaki, S., M. Sporn, A. Ding, and C.F. Nathan. 1988. Deactivation of macrophages by transforming growth factor β. Nature (Lond.). 334:260.

10. Hart, P.H., G.F. Vitti, D.R. Burgess, G.A. Whitty, D.S. Picolli, and J.A. Hamilton. 1989. Potential anti-inflammatory effect of interleukin 4: suppression of human monocyte tumor necrosis factor α, interleukin 1 and prostaglandin E2. Proc. Natl. Acad. Sci. USA. 86:3803.

11. Bogdan, C., Y. Vodovotz, and C.F. Nathan. 1991. Macrophage deactivation by interleukin 10. J. Exp. Med. 174:1549.

12. Fiorentino, D.F., A. Zlotnik, T.R. Mossman, M. Howard, and A. O'Garra. 1991. Interleukin-10 inhibits cytokine production by activated macrophages. J. Immunol. 147:3815.

13. Bachwich, P.R., S.W. Chensue, J.W. Larrick, and S.L. Kunkel. 1986. Tumor necrosis factor stimulates interleukin 1 and prostaglandin E2 production in resting macrophages. Biochem. Biophys. Res. Commun. 136:94.

14. Lehmmann, V., B. Benninghoff, and W. Droge. 1988. Tumor necrosis factor induced activation of peritoneal macrophages is regulated by prostaglandin E2 and cAMP. J. Immunol. 141:587.

15. Zurier, R.B., and F. Quagliata. 1971. Effect of prostaglandin E1 on adjuvant arthritis. Nature (Lond.). 234:304.

16. Kelly, C.J., R.B. Zurier, K.A. Krakauer, N. Blanchard, and E.C. Nielson. 1987. Prostaglandin E1 inhibits effector cell induction and tissue damage in experimental murine interstitial nephritis. J. Clin. Invest. 79:782.

17. Zurier, R.B., D.M. Sayadoff, S.B. Torrey, and N.F. Rothfield. 1977. Prostaglandin E1 treatment of NZB/W mice. Prolonged survival of female mice. Arthritis Rheum. 20:723.

18. Howard, M., and A. O'Garra. 1992. Biological properties of interleukin 10. Immunol. Today. 13:198.

19. Howard, M.A., A. O'Garra, H. Ishida, R. de Waal Malefyt, and J. de Vries. 1992. Biological properties of interleukin 10. J. Clin. Immunol. 12:239.

20. Howard, M., T. Muchamel, S. Andrade, and S. Menon. 1993. Interleukin 10 protects mice from lethal endotoxemia. J. Exp. Med. 177:1205.

21. Block, M.I., M. Berg, M.J. McNamara, J.A. Norton, D.L. Fraker, and H.R. Alexander. 1993. Passive immunization against D factor blocks lethality and cytokine release during endotoxemia. J. Exp. Med. 178:1085.

22. Strassmann, G., M. Fong, J.S. Kenney, and C.O. Jacob. 1992. Evidence for the involvement of interleukin 6 in experimental cancer cachexia. J. Clin. Invest. 89:1681.

23. Strassmann, G., D.R. Bertolini, S.B. Kerby, and M. Fong. 1991. Regulation of murine mononuclear phagocyte inflammatory products by macrophage colony stimulating factor. Lack of IL-1 and prostaglandin E2 production and generation of a specific IL-1 inhibitor. J. Immunol. 147:1279.

24. Ding, A.H., C.F. Nathan, and D. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J. Immunol. 141:2407.

25. Bonney, R.J., S. Burger, P. Davies, F.A. Kuehl, and J.L. Humes. 1980. Prostaglandin E2 and prostacyclin elevate cyclic AMP levels in elicited populations of mouse peritoneal macrophages. Adv. Prostaglandin Thromboxane Res. 8:1681.

26. Wells, J.N., and G.L. Kramer. 1981. Phosphodiesterase inhibitors as tools in cyclic nucleotide research: a precautionary comment. Mol. Cell. Endocrinol. 23:1.

27. Strassmann, G., M. Fong, S. Windsor, and R. Neta. 1993. The role of interleukin 6 in lipopolysaccharide induced weight loss, hypoglycemia and fibrinogen production in vivo. Cytokine. 5:285.

28. Zurawski, S.M., and J.E. de Vries. 1994. Interleukin 13, an interleukin 4 like cytokine that acts on monocytes and B cells but not on T cells. Immunol. Today. 15:19.

29. van Epps, D.E. 1981. Suppression of human lymphocyte migration by PGE2. Inflammation. 5:81.