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Methylprednisolone differentially regulates IL-10 and tumour necrosis factor (TNF) production during murine endotoxaemia

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SUMMARY

IL-10 is an endogenous antiinflammatory cytokine that inhibits TNF biosynthesis and protects mice from lipopolysaccharide (LPS)-induced lethality. As synthetic glucocorticoids are widely used as antiinflammatory agents, we analysed the effects of methylprednisolone administration on IL-10 biosynthesis during murine endotoxaemia. We found that low doses of methylprednisolone (2–10 mg/kg) markedly inhibited TNF production but did not affect serum levels of IL-10, while a high methylprednisolone dose (50 mg/kg) increased LPS-induced IL-10 levels. In parallel, we observed that LPS-induced IL-10 production is TNF-independent in this experimental setting. Experiments conducted *in vitro* indicated that methylprednisolone (from 0·01 to 100 μg/ml) also increased the biosynthesis of IL-10 by LPS-activated mouse peritoneal macrophages. We conclude that methylprednisolone differentially regulates IL-10 and TNF production induced by LPS both *in vivo* and *in vitro* at the macrophage level.

Keywords glucocorticoids IL-10 tumour necrosis factor lipopolysaccharide macrophage

INTRODUCTION

Glucocorticoids (GCs) are physiological inhibitors of inflammatory reactions and are used in the treatment of many inflammatory disorders [1]. GCs inhibit the production of inflammatory cytokines, including TNF, by lipopolysaccharide (LPS, endotoxin)-activated monocytes/macrophages and protect animals from LPS-induced lethality [2–5]. Endogenous GCs are produced during the course of inflammatory responses, including endotoxin shock [3,5,6]. Inhibition of the biosynthesis or effects of endogenously produced GCs in mice increases LPS-induced TNF production and lethality [4,6–8].

IL-10 is another endogenous mediator controlling inflammatory responses [9]. rIL-10 inhibits the production of inflammatory cytokines, including TNF, by LPS-activated monocytes/macrophages and protects mice from LPS-induced lethality [10,11]. Endogenous IL-10 is produced in patients with various inflammatory disorders, including septic shock [12–14], malaria [15], rheumatoid arthritis [16] and in animal models of septic shock including endotoxin shock and septic peritonitis [17–19]. Neutralization of endogenous IL-10 markedly increases the production of TNF and the lethality associated with endotoxin shock and septic peritonitis [18,19]. Monocytes and macrophages represent probably an important source of IL-10 in inflammatory disorders [20–22]. The mechanisms controlling IL-10 production by monocytes/macrophages remain poorly understood. TNF was shown to be involved in the production of IL-10 *in vitro* by LPS-activated human monocytes and *in vivo* in LPS-challenged primates [23,24]. However, agents increasing cytoplasmic concentrations of cAMP and inhibiting TNF biosynthesis, such as prostaglandin E_2 (PGE_2) or pentoxyfilline, increase LPS-induced IL-10 production by macrophages [25–27]. This effect probably involves the cAMP-responsive element in the promoter region [26] of the IL-10 gene and indicates that the production of IL-10 and TNF can be differentially regulated.

In this study we investigated the effects of a synthetic GC, methylprednisolone (MPDS), on the production of IL-10 during experimental endotoxaemia in mice. The differential regulation of TNF and IL-10 by MPDS led us to evaluate the role of TNF in the production of IL-10 in this experimental setting. Finally, *in vitro* experiments using mouse peritoneal cells indicated that TNF and IL-10 are differentially regulated by MPDS at the level of macrophages.

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MATERIALS AND METHODS

Reagents

LPS from *Escherichia coli* (serotype O55:B5) was purchased from Sigma Chemical Co. (St Louis, MO). MPDS was obtained from Upjohn (Puurs, Belgium). The JES5-2A5 MoAb, a rat IgG1 neutralizing mouse IL-10, was a kind gift from T. Mosmann (Department of Immunology, University of Alberta, Edmonton, Canada). The LO-DNP MoAb, a rat IgG1 MoAb used as control, was kindly given by H. Bazin (Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium). TN3, a hamster MoAb neutralizing mouse TNF MoAb [28] and the isotype-matched control MOPC21 MoAb were kindly provided by Celltech (Slough, UK). The endotoxin levels of MoAb preparations, as determined by the limulus assay, were < 50 pg/mg.

Animals

Eight to 12 week-old-female BALB/c mice were purchased from Bantin & Kingman (B & K Universal, Humberside, UK).

Experimental protocols

In vivo *experiments*. Mice were injected intraperitoneally with various doses of MPDS or medium alone 30 min before i.p. administration of 100 μg LPS. In some experiments, mice were injected with neutralizing anti-IL-10 (JES5-2A5, 2 mg) or isotype-matched control MoAb (LO-DNP) or with neutralizing anti-TNF (TN3, 0·5 mg) or isotype-matched control MoAb (mouse IgG1) 2 h before LPS challenge. Serum was collected after various periods of time for TNF and IL-10 level determinations.

In vitro *experiments*. Total peritoneal cells were obtained by washing the peritoneal cavity of BALB/c mice with 5 ml cold RPMI medium and were cultured in 24 well/plates in the presence of RPMI medium with 5% fetal calf serum (FCS). In some experiments, peritoneal macrophages were isolated by washing thoroughly non-adherent cells after 2 h incubation at 37°C. More than 75% of adherent cells were MAC-1$^+$ as determined by flow cytometry. Total peritoneal cells or peritoneal macrophages were incubated for 30 min in the presence of various concentrations of MPDS before addition of LPS (final concentration 1 μg/ml). Supernatants were collected after various periods of time for TNF and IL-10 level determinations.

Cytokine determinations

TNF levels were measured in sera and cell supernatants by sandwich ELISA using a polyclonal rabbit anti-mouse TNF antibody for coating and the same polyclonal antibody that was biotinylated for detection (Wim Buurman, Maastricht, The Netherlands). In some experiments, bioactive TNF levels were measured as previously described [29] using the actinomycin-D-treated WEHI-164 clone 13 cells. IL-10 levels were measured in sera and cell supernatants by ELISA using a commercially available immunoassay produced by Perceptive Diagnostics (Cambridge, MA). The sensitivities of the TNF and IL-10 ELISA were 15 and 100 pg/ml, respectively. The lower limit of detection of the TNF bioassay was 40 pg/ml.

Statistical analysis

Cytokine levels were compared using the two-tailed Wilcoxon rank sum test on unpaired samples.

RESULTS

High dose MPDS increases LPS-induced IL-10 release in vivo *in mice*

The effect of GCs on LPS-induced IL-10 production was first examined *in vivo* in mice. As previously described [18], injection of 100 μg LPS into mice resulted in the rapid release of TNF (Fig. 1a) and IL-10 (Fig. 1b) as measured at 90 min. Pretreatment of the mice with increasing doses of MPDS 30 min before LPS challenge markedly inhibited the release of TNF, more than 90% inhibition being observed with 50 mg/kg MPDS (Fig. 1a). In contrast, we found that 50 mg/kg MPDS induced a two- to three-fold increase in IL-10 serum levels, while lower doses (2 and 10 mg/kg) had no inhibitory effect (Fig. 1b). We then analysed the kinetics of IL-10 production in endotoxaemic mice pretreated with MPDS (Fig. 2). Although the serum levels of IL-10 observed in this experiment were lower than in Fig. 1, we confirmed that MPDS (10 and 50 mg/kg) increased IL-10 levels measured at 90 min. In contrast, MPDS (2–50 mg/kg) did not influence IL-10 serum levels measured at 3 h and 6 h after LPS injection (Fig. 2). The injection of MPDS alone (50 mg/kg) did not result in any detectable serum IL-10 (data not shown).

IL-10-independent mechanisms mediate most of the inhibitory effect of MPDS on TNF production during murine endotoxaemia

As IL-10 is a potent inhibitor of TNF biosynthesis, the suppressive effect of MPDS on TNF release might at least partly be related to its ability to increase IL-10 levels. This hypothesis was tested by injecting mice with neutralizing anti-IL-10 MoAb (JES5-2A5, 2 mg) before MPDS (50 mg/kg) and LPS administration. As previously reported, no immunoreactive IL-10 could be detected in the serum of anti-IL-10-treated mice ([18] and data not shown). Figure 3 shows that neutralization of endogenous IL-10 up-regulated TNF production in mice injected with LPS with or without MPDS (hatched bars). However, TNF levels in mice injected with the combination of anti-IL-10 MoAb and MPDS remained much lower than in mice receiving no MPDS. These data demonstrate that IL-10-independent mechanisms mediate most of the inhibitory effect of MPDS on TNF production during murine endotoxaemia.

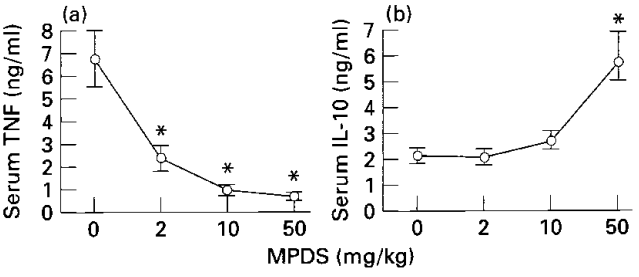

Fig. 1. Effect of methylprednisolone (MPDS) on lipopolysaccharide (LPS)-induced TNF and IL-10 production *in vivo* in mice. Mice were injected intraperitoneally with various doses of MPDS or medium alone 30 min before LPS challenge (100 μg). Serum was collected 90 min after LPS injection for TNF (a) and IL-10 (b) level determination. Cytokine levels were measured by ELISA. Data shown are mean ± s.e.m. of 10–16 mice studied in three independent experiments. *P < 0·05 compared with mice injected with LPS alone.

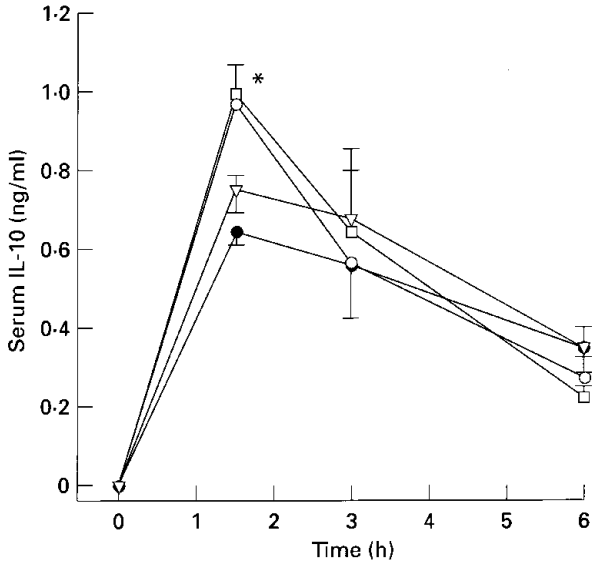

Fig. 2. Kinetics of lipopolysaccharide (LPS)-induced IL-10 production in mice pretreated with methylprednisolone (MPDS). Mice were injected intraperitoneally with various doses of MPDS (2 mg/kg, ▽; 10 mg/kg, □; 50 mg/kg, ○) or medium alone (●) 30 min before LPS challenge (100 μg). Serum was collected at various time points after LPS injection for immunoreactive IL-10 level determinations. Data shown are mean ± s.e.m. of five mice studied in one experiment. *$P < 0{\cdot}05$ compared with mice injected with LPS alone.

LPS-induced IL-10 production in vivo *in mice is not dependent on TNF*

Previous studies in primates indicated that TNF is involved in the production of IL-10 induced *in vivo* by LPS [23]. As our data demonstrate a differential regulation of TNF and IL-10, we examined whether the release of IL-10 in our model of experimental endotoxaemia is also dependent on TNF. Mice were injected intraperitoneally with 0·5 mg TN3 anti-TNF neutralizing

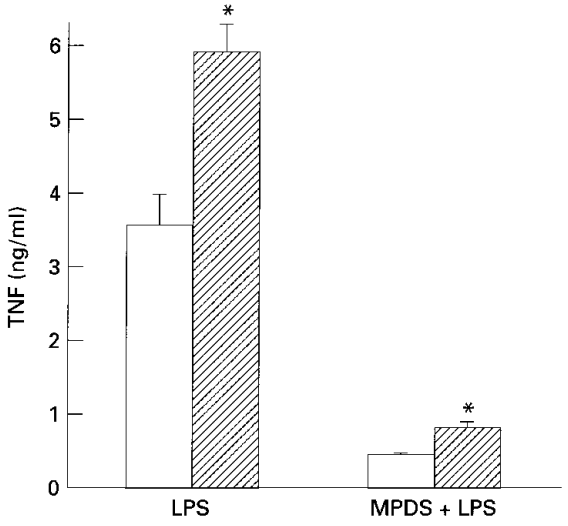

Fig. 3. Effect of IL-10 neutralization on TNF serum levels in lipopolysaccharide (LPS)-challenged mice pretreated with methylprednisolone (MPDS). Mice were injected intraperitoneally with LPS with or without pretreatment with MPDS (50 mg/kg) combined with pretreatment with either neutralizing anti-IL-10 (JES5-2A5, 2 mg, ▨) or isotype-matched control MoAb (□). Serum was collected 90 min after LPS challenge for immunoreactive TNF level determinations. Data shown are mean ± s.e.m. of seven mice studied in one experiment. *$P < 0{\cdot}05$ compared with mice injected with control MoAb.

Table 1. Lipopolysaccharide (LPS)-induced IL-10 production in mice is TNF-independent

	Bioactive TNF (ng/ml)*	IL-10 (ng/ml)*
IgGl + LPS	2·67 ± 0·45	2·34 ± 0·35
Anti-TNF + LPS	<0·04†	2·44 ± 0·33‡

*Eight to 12-week-old BALB/c mice were injected intraperitoneally with neutralizing anti-mouse TNF (YN3, 0·5 mg) or isotype-matched control MoAb 2 h before LPS challenge (100 μg). Serum samples were collected at 90 min for bioactive TNF and immunoreactive IL-10 level determinations. Data are given as mean ±s.e.m. of nine to 14 animals per group.

†$P < 0{\cdot}01$ compared with IgGl + LPS.

‡Not significantly different from IgGl + LPS.

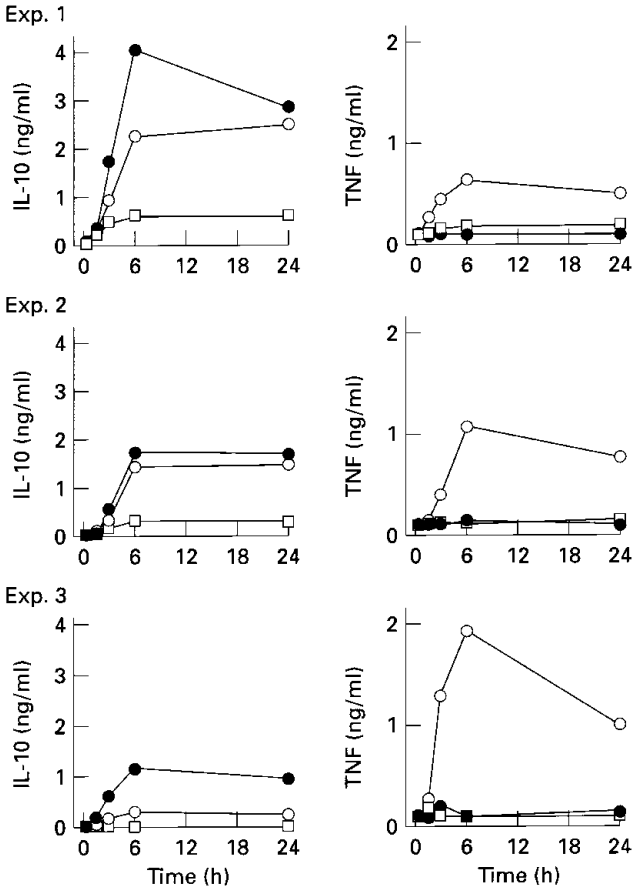

Fig. 4. Methylprednisolone (MPDS) increases IL-10 production by lipopolysaccharide (LPS)-activated mouse peritoneal cells. Total peritoneal cells from BALB/c mice (10^6 cells/ml) were cultured in medium alone (□), or in presence of LPS (1 μg/ml, ○) or in presence of LPS (1 μg/ml) after 30 min pretreatment with MPDS (100 μg/ml, ●). Immunoreactive IL-10 and TNF levels were measured in supernatants after various periods of time. Figure shows data from three independent experiments.

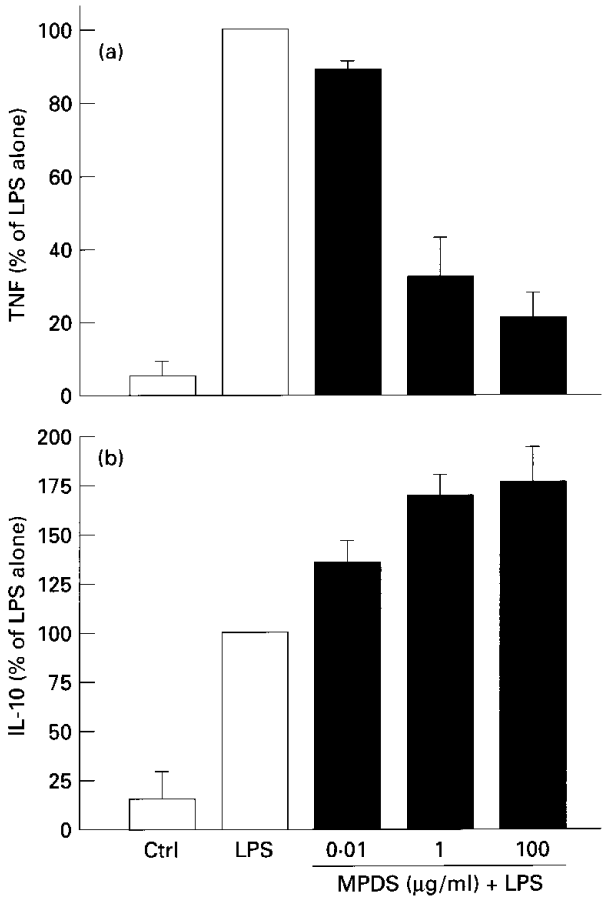

Fig. 5. Methylprednisolone (MPDS) increases IL-10 production by peritoneal macrophages activated by lipopolysaccharide (LPS). Adherent mouse peritoneal cells (more than 75% MAC-1^+, 10^6 cells/ml) were cultured in the presence of various concentrations of MPDS for 30 min before addition of LPS (1 μg/ml). Immunoreactive TNF and IL-10 levels were measured in supernatants after 6 h incubation. Data are expressed as percentage of TNF (a) and IL-10 levels (b) produced by macrophages activated by LPS alone. Figure shows mean $\pm$ s.e.m. of three independent experiments. IL-10 concentrations in supernatants from macrophages stimulated with LPS alone were 576 $\pm$ 298 pg/ml (mean $\pm$ s.e.m.).

MoAb or isotype-matched control MoAb (mouse IgG1) 2 h before LPS administration. We first verified that anti-TNF MoAb efficiently neutralized serum TNF bioactivity (Table 1). As shown in Table 1, we found that peak IL-10 levels were not affected by TNF neutralization.

MPDS increases LPS-induced IL-10 synthesis by mouse peritoneal macrophages in vitro

As macrophages probably represent an important source of IL-10 during experimental endotoxaemia [20–22], we evaluated whether the differential regulation of TNF and IL-10 observed *in vivo* also occurs at the level of macrophages *in vitro*. We first studied the effects of MPDS on the production of IL-10 by LPS-activated mouse peritoneal cells. Figure 4 shows that, in three independent experiments, incubation of total peritoneal cells in the presence of LPS (1 μg/ml) resulted in the production of IL-10 (left panels) and TNF (right panels), peak cytokine levels being observed after 6 h of culture. Incubation of peritoneal cells with MPDS (100 μg/ml) for 30 min before addition of LPS increased IL-10 production while TNF synthesis was almost completely abrogated. In order to evaluate more directly the influence of MPDS on macrophages, we conducted similar experiments using isolated adherent peritoneal cells (more than 75% MAC-1^+). As shown in Fig. 5, MPDS increased IL-10 biosynthesis by macrophages in a dose-dependent manner (Fig. 5b), whereas TNF production was suppressed (Fig 5a). No IL-10 was detected after incubation of macrophages with MPDS alone (data not shown). In the same series of experiments, we observed that neutralization of TNF had only a minor inhibitory effect (0–10%) on the production of IL-10 induced by LPS. Taken together, these data demonstrate that LPS-induced IL-10 production by peritoneal macrophages is largely TNF-independent and is up-regulated by MPDS.

DISCUSSION

In this study, we first show that MPDS differentially regulates IL-10 and TNF biosynthesis during experimental endotoxaemia in mice. Low doses of MPDS inhibited TNF production without affecting IL-10 release, while high doses of MPDS significantly increased serum levels of IL-10. Experiments conducted *in vitro* indicated that MPDS increases biosynthesis of IL-10 by LPS-activated peritoneal macrophages. As severe combined immunodeficient (SCID) mice challenged with LPS produce normal amounts of IL-10 [20], the increased levels of IL-10 induced by LPS *in vivo* are probably dependent on an increased IL-10 biosynthesis by macrophages. Several recent studies demonstrated that other molecules also differentially regulate the production of IL-10 and TNF induced by LPS. These molecules include agents increasing cytoplasmic cAMP levels [25–27], chlorpromazine [30], a sigma-ligand [20], desferrioxamine and transforming growth factor-beta (TGF-β) [31]. In the case of GCs, the enhancing effect on IL-10 production was also recently observed on macrophages stimulated *in vitro* with bacille Calmette–Guérin (BCG) (L. Sigola and G. J. Bancroft, personal communication).

The differential regulation of IL-10 and TNF biosynthesis by macrophages contrasts with previous studies showing that TNF is involved in the production of IL-10 by LPS-activated human monocytes *in vitro* [24] and during experimental endotoxaemia in primates [23]. We found that TNF plays no significant role in the production of IL-10 in our model of endotoxaemia and is marginally involved in the synthesis of IL-10 by LPS-activated peritoneal macrophages. As a matter of fact, our results are consistent with those reported by Barsig *et al.*, showing that the rapid release of IL-10 induced by LPS injection in mice does not require TNF [32]. Taken together, these observations suggest that the regulation of IL-10 production by glucocorticoids might depend on the species considered and on the cellular source of IL-10.

The inhibition of TNF biosynthesis by GCs is partly related to a decreased transcription of the TNF gene [33]. This lower transcription appears to be dependent on a reduced activity of NF-κB, a transcription factor playing a central role in the activation of TNF gene expression in LPS-activated macrophages [34–36]. Inhibition of NF-κB activity by GCs is considered to be the consequence of a transrepressive effect of the activated glucocorticoid receptor [34] and/or of the increased transcription of the inhibitor I-κBα gene [35,37,38]. Although mouse IL-10 promoter contains NF-κB-like binding sites [39], our results might suggest that NF-κB does not play a central role in the activation of IL-10 gene transcription. This hypothesis is supported by the fact that these NF-κB-like binding sites were not found in the promoter of the human IL-10

gene [26]. Interestingly, glucocorticoid-responsive elements have been described in the mouse and human IL-10 promoters [26,39]. Further studies should define their role in the up-regulation of IL-10 production by MPDS.

IL-10 is a potent antiinflammatory molecule which acts not only by inhibiting the synthesis of proinflammatory cytokines but also by stimulating the release of the IL-1 receptor antagonist [40] and of soluble TNF receptors [41,42]. We suggest that these antiinflammatory properties of endogenous IL-10 might contribute to the therapeutic effect of glucocorticoids in inflammatory disorders.

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