



Differential inhibitory mechanism of cyclic AMP on TNF- α and IL-12 synthesis by macrophages exposed to microbial stimuli

¹Daniela O. Procópio, ²Mauro M. Teixeira, ^{1,3}Maristela M. Camargo, ⁴Luiz R. Travassos, ⁵Michael A.J. Ferguson, ⁵Igor C. Almeida & ^{*,1,3}Ricardo T. Gazzinelli

¹Department of Biochemistry and Immunology, Federal University of Minas Gerais, 31270-910 Belo Horizonte, MG, Brazil;

²Department of Pharmacology, Federal University of Minas Gerais, 31270-910 Belo Horizonte, MG, Brazil; ³Centro de Pesquisas René Rachou, FIOCRUZ, 30190-002 Belo Horizonte, MG, Brazil; ⁴Discipline of Cell Biology, Federal University of São Paulo, 04023-900 São Paulo, SP, Brazil and ⁵Department of Biochemistry, University of Dundee, Dundee, DD1 4HN, U.K.

1 Microbial stimuli such as bacterial lipopolysaccharide (LPS) or glycosylphosphatidylinositol-mucins derived from *Trypanosoma cruzi* trypomastigotes (tGPI-mucins) are effective stimulators of the synthesis of cytokines by macrophages. Here, we evaluated the ability of cyclic AMP mimetic or elevating agents to modulate TNF- α and IL-12 synthesis by murine inflammatory macrophages.

2 Cholera Toxin (ChTx) inhibited tGPI-mucins (2.5 nM) or LPS (100 ng ml⁻¹) induced TNF- α and IL-12(p40) synthesis in a concentration-dependent manner. Similarly, the cyclic AMP mimetics, 8-bromo cyclic AMP or dibutyryl cyclic AMP, or prostaglandin (PG) E₂ inhibited the synthesis of both cytokines by macrophages exposed to microbial stimuli.

3 The protein kinase A inhibitor H-89 partially reversed the inhibitory effects of dibutyryl cyclic AMP and PGE₂ on both IL-12(p40) and TNF- α synthesis.

4 Pretreatment of macrophages with dibutyryl cyclic AMP or ChTx augmented the synthesis of IL-10 triggered by microbial products. Elevation of cyclic AMP inhibited the synthesis of TNF- α , but not IL-12(p40), by inflammatory macrophages from IL-10 knockout mice.

5 Kinetic studies showed that synthesis of both TNF- α and IL-10 peaked at 8 h and IL-12 at 24 h after stimulation with microbial stimuli.

6 Together, our findings favour the hypothesis that the cyclic AMP inhibitory activity on IL-12(p40) but not on TNF- α synthesis is dependent on *de novo* protein synthesis, most likely involving IL-10, by macrophages stimulated with microbial products. Accordingly, dibutyryl cyclic AMP inhibited IL-12(p40) synthesis only when added before or at the same time of the stimuli. In contrast, the effect of this cyclic AMP analogue on TNF- α synthesis was protracted and observed even 2 h after the addition of the stimuli.

Keywords: Cyclic AMP; glycosylphosphatidylinositol-mucins; IL-12; IL-10; lipopolysaccharide; macrophage; microbial stimuli; prostaglandins; TNF- α ; *Trypanosoma cruzi*

Abbreviations: APCs, antigen presenting cells; cyclic AMP, cyclic adenosine monophosphate; ChTx, cholera toxin; DB, dibutyryl cyclic AMP; GPI, glycosylphosphatidylinositol; IL-12(p40), 40 kDa peptide of IL-12; IL-12(p35), 35 kDa peptide of IL-12; IL-12(p70), IL-12(p40)/IL-12(p35) heterodimer; LPS, *Escherichia coli* lipopolysaccharide; MacMed, DMEM supplemented with 40 μ g ml⁻¹ gentamicin and 5% heat-inactivated foetal calf serum; PGE₂, prostaglandin E₂; PKA, Protein Kinase A; tGPI-mucins, GPI-anchored mucin-like glycoproteins from *Trypanosoma cruzi* trypomastigotes

Introduction

Interleukin-12 (IL-12) is a heterodimeric cytokine which has two protein chains encoded by unrelated genes (Kobayashi *et al.*, 1989). The light chain of 35 kDa (p35) is synthesized constitutively by a broad range of cells, whereas the 40 kDa (p40) unit is tightly regulated and secreted mainly by macrophages and dendritic cells upon microbial stimulation (Trinchieri, 1995). A broad list of bacteria, viruses and intracellular parasites has been described as inducers of the IL-12 heterodimer by macrophages both *in vitro* and *in vivo* (Biron & Gazzinelli, 1995; Trinchieri, 1995; Trinchieri & Scott, 1995). Once released by professional phagocytic cells exposed to microbial products, IL-12 induces IFN- γ synthesis by natural killer (NK) cells as well as drives the differentiation of

naive Th precursor cells into Th1 lymphocytes. In this way, IL-12 is thought to play a primordial role on establishment of protective immunity against intracellular parasites (Biron & Gazzinelli, 1995; Scott, 1993; Trinchieri, 1995; Trinchieri & Scott, 1995). In agreement with this suggestion, animals treated with neutralizing doses of mAb against IL-12(p40) or lacking either IL-12(p40) or IL-12(p35) genes are highly susceptible to various intracellular pathogens (Biron & Gazzinelli, 1995; Trinchieri & Scott, 1995).

The synthesis of the IL-12(p40) chain is also regulated by a broad list of cytokines (D'Andrea *et al.*, 1993; 1995; Flesch *et al.*, 1995; Gazzinelli *et al.*, 1993; 1994; Kubin *et al.*, 1994; Ma *et al.*, 1996; Murphy *et al.*, 1995; Skeen *et al.*, 1996). In fact, it is widely accepted that for optimal production of IL-12 by macrophages stimulated with microbial products, co-stimulation with IFN- γ is required (Gazzinelli *et al.*, 1993; Ma *et al.*, 1996; Murphy *et al.*, 1995). Other cytokines such as GM-CSF, IL-1 and TNF- α (D'Andrea *et al.*, 1993; Kubin *et al.*, 1994), although not essential for maximal IL-12 synthesis, have also

*Author for correspondence at: Department of Biochemistry and Immunology, Federal University of Minas Gerais, Av. Antônio Carlos 6627, Pampulha, 31270-910 Belo Horizonte, MG, Brazil.
E-mail: ritoga@dedalus.lcc.ufmg.br

been shown to potentiate the synthesis and/or enhance IL-12 activity. Interestingly, IL-4 and IL-13 may play either negative or positive regulatory activity on IL-12 synthesis, depending on whether they are added to macrophage cultures before or after microbial stimuli, respectively (D'Andrea *et al.*, 1995). In contrast, IL-10 is a major negative regulator of IL-12 synthesis both *in vitro* and *in vivo* (D'Andrea *et al.*, 1993; Gazzinelli *et al.*, 1996; Hunter *et al.*, 1997).

We have recently shown that macrophages activated with glycosylphosphatidylinositol (GPI)-anchored mucin-like glycoproteins derived from *Trypanosoma cruzi* trypomastigotes (tGPI-mucins) have the ability to secrete several cytokines, including IL-12 and TNF- α (Camargo *et al.*, 1997a, b). Similarly to bacterial lipopolysaccharide (LPS), tGPI-mucins also activate macrophages to express IL-10. The precise role of tGPI-mucins in the pathophysiology of experimental and human infection by *T. cruzi* is not known, but tGPI-mucins induce several cytokines (such as IL-12, IL-10 and TNF- α) which are thought to be involved in protection and pathology in experimental Chagas' disease (Brenner & Gazzinelli, 1997). Thus, knowledge of the mechanisms underlying and controlling macrophage activation by tGPI-mucins may help to understand the interaction of *T. cruzi* and its host.

Recent evidence suggests that the intracellular levels of cyclic AMP play an important role in re-directing cytokine synthesis by LPS-activated macrophages (Benbernou *et al.*, 1997; Eigler *et al.*, 1998; Meisel *et al.*, 1996; van der Pouw Kraan *et al.*, 1995). Thus, PGE₂, a potent and physiological inducer of cyclic AMP synthesis, as well as cyclic AMP derivatives have been shown to inhibit both IL-12 and TNF- α synthesis by macrophages exposed to LPS (Eigler *et al.*, 1998; Kambayashi *et al.*, 1995a, b; Panina-Bordignon *et al.*, 1997; van der Pouw Kraan *et al.*, 1995). Although controversial, it is suggested that the release of IL-10 by macrophages upon activation with LPS may account, at least in part, for the inhibitory effects of cyclic AMP on TNF- α production both *in vitro* and *in vivo* (Eigler *et al.*, 1998; Kambayashi *et al.*, 1995a, b; Seldon *et al.*, 1998; Strassmann *et al.*, 1994; van der Pouw Kraan *et al.*, 1995). Much less is known about the ability of the cyclic AMP-induced IL-10 in modulating the release of IL-12.

In the present work we have assessed the effects of several cyclic AMP-elevating agents and cyclic AMP mimetic drugs on modulating both IL-12 and TNF- α production by macrophages activated with tGPI-mucins. Moreover, we addressed whether endogenous IL-10 played a role on the cyclic AMP-mediated inhibition of cytokine release. For comparison we studied the effects of these strategies on macrophages stimulated with LPS. Our results clearly indicate a role for cyclic AMP in modulating the synthesis of pro-inflammatory cytokines. We also provide strong evidence that cyclic AMP modulates tGPI-mucin-induced IL-12 production by macrophages indirectly, following the release of IL-10. In contrast, the effects of cyclic AMP regulation of TNF- α production are probably direct, and largely independent of IL-10 production.

Methods

Animals

Male C3H/HeJ, C57BL/6 and IL-10 knockout (KO) mice in the C57BL/6 genetic background, 6–7 week old, were obtained from the animal house of FIOCRUZ (Rio de Janeiro, RJ, Brazil) and from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD, U.S.A.) and used as source of inflammatory macrophages.

Reagents

ChTx, PGE₂ and H89 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); SQ-22536, dibutyl cyclic AMP (DB) and 8-bromo cyclic AMP were obtained from Calbiochem (La Jolla, CA, U.S.A.) and used according with the recommended specification. IFN- γ and LPS were purchased from Genzyme Corp. (Cambridge, MA, U.S.A.) and Sigma Chemical Co., respectively.

T. cruzi GPI-anchored mucins

tGPI-mucins were isolated from *T. cruzi* trypomastigotes as previously described (Almeida *et al.*, 1994a; Camargo *et al.*, 1997a) using sequential butanol:water partition and hydrophobic-interaction chromatography in an octyl-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) eluted with a propan-1-ol gradient (5–60%). In addition, we isolated the active moiety of the tGPI-mucins consisting of the GPI anchors (tGPI). A preparation of highly purified tGPI showed an activity identical with that of tGPI-mucins on a molar basis. The tGPI anchor showed no detectable contamination with any known lipopeptide from *Mycoplasma sp.* as indicated by matrix-assisted laser desorption ionization spectrometry (MALDI) as well as by amino acid sequence analysis, using an authentic synthetic lipopeptide as a standard (MALP-2; a kind gift from Peter Mühlradt, Germany; Mühlradt *et al.*, 1997) (data not shown).

Macrophage culture

Mice were inoculated intraperitoneally with 2 ml of 3% thioglycollate and, 4 days later, the elicited peritoneal exudate cells were harvested in cold serum-free DMEM. The medium used in the macrophage cultures (MacMed) consisted of DMEM (Gibco, Grand Island, NY, U.S.A.) supplemented with 40 $\mu\text{g ml}^{-1}$ gentamicin and 5% heat-inactivated foetal calf serum (FCS). Macrophages were resuspended in MacMed at $2 \times 10^6 \text{ ml}^{-1}$, and 100 μl aliquots dispensed into wells of a 96-well plate. Cells were allowed to adhere at 37°C and 5% CO₂ for 3 h, and were then washed once with serum-free DMEM and 100 μl of MacMed added to each well. When needed, the macrophages were primed with 50 U ml^{-1} IFN- γ . The plates were incubated overnight at 37°C and 5% CO₂. Different macrophage stimulating preparations were added to the macrophage cultures in a final volume of 200 μl well⁻¹. Aliquots of the supernatant (50 and 100 μl) were collected after 24 and 48 h of culture for TNF- α /IL-10 and IL-12(p40)/IL-12(p70) measurements, respectively (Camargo *et al.*, 1997a, b).

Determination of cytokine levels on supernatants of unstimulated and stimulated macrophages

TNF- α and IL-12(p70) were quantified employing ELISA kits (Genzyme, Duoset kit). IL-10 and IL-12(p40) were assayed by sandwich ELISA, using Maxsorp plates (Nunc, Denmark) as previously described (Gazzinelli *et al.*, 1994; Jankovic *et al.*, 1997). Capture (26571E) and detection (biotinylated 26572E) antibody (Pharmingen), and the enzyme reagent (streptavidin-horseradish peroxidase conjugated, 26167E), were used for quantifying IL-10. C17.1.5 (1510) rat mAb (5 $\mu\text{l ml}^{-1}$) and biotinylated C15.6 (676) rat mAb (1:750) were used for capture and detection of bound IL-12(p40), respectively. Recombinant murine IL-10 and IL-

12 were used as standards for the respective cytokine measurements. For TNF- α , IL-12(p40) and IL-12(p70) sandwich ELISAs were developed with 2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (Sigma) and read in a ELISA reader (SpectraMax Plus, Molecular Devices, U.S.A.) at 405 nm. For IL-10 ELISAs the reaction was developed with an ECL-PLUS chemiluminescent substrate, as previously described (Almeida *et al.*, 1994b). Briefly, plates (Fluoronunc Maxisorp - Nunc) were developed for 20 min using ECL-PLUS substrate (Amersham, Life Science, RPN-2132) and exposed for 10–20 min to Hyperfilm SL (Amersham, Life Science, RPN-3103H) placed at the top of ELISA plates. Developed films were attached to the top of unused plates and introduced in an ELISA reader (SpectraMax Plus, Molecular Devices) at 405 nm.

Inhibition of cytokine synthesis

Different signalling inhibitors were added to macrophage cultures in titration assays and incubated for 30 min, at 37°C and 5% CO₂. The cultures were incubated with tGPI-mucins or LPS at the concentrations indicated in presence or absence of IFN- γ . The cultures were maintained at 37°C and 5% CO₂, and 50 and 100 μ l of supernatant were collected after 24 and 48 h, respectively, for TNF- α and IL-12 measurements. By the end of 48 h of incubation, cells were washed in pre-warmed PBS and assayed for viability.

Cell viability assay

Cell viability was determined as previously described (Miller, 1994). Briefly, cells were incubated with 100 μ l well⁻¹ of supplemented medium containing 0.5 mg ml⁻¹ of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), overnight at 37°C and 5% CO₂. Cells were then washed and treated with 100 μ l well⁻¹ of 10% SDS in dimethylformamide:H₂O (1:1). Absorbency was read at 570 nm. Cell viability was calculated as a relative index of control cells (100% of viable cells).

IC₅₀ calculation

A dose-response curve was obtained by adding increasing concentrations of cholera toxin, cyclic AMP derivatives or PGE₂, 30 min before stimulation with microbial products, and evaluation of cytokine production after indicated times. Concentrations of effector compounds which rendered 50% inhibition of the maximum response were designated IC₅₀.

Statistical analysis

Differences between groups were determined using analysis of variance and *P* values assigned using Student-Newman-Keuls *post hoc* test. Results were considered significant when *P* < 0.05.

Table 1 Inhibitory concentrations (IC₅₀) of different inhibitors or activators of adenylate cyclase and/or Protein Kinase A on IL-12(p40) or TNF- α synthesis by macrophages stimulated with tGPI mucins or LPS*

Inhibitor/activator	tGPI mucins (1 pmol ml ⁻¹)		LPS (100 ng ml ⁻¹)	
	IL-12(p40)	TNF- α	IL-12(p40)	TNF- α
Cholera toxin (adenylate cyclase activator)	0.7 ng ml ⁻¹	0.3 ng ml ⁻¹	1.5 ng ml ⁻¹	0.7 ng ml ⁻¹
Dibutyl cyclic AMP (cyclic AMP derivative)	30 μ M	15 μ M	125 μ M	15 μ M
8-Bromo cyclic AMP (cyclic AMP derivative)	30 μ M	30 μ M	60 μ M	60 μ M
Prostaglandin E ₂ (PKA activator)	1 μ M	0.1 μ M	1 μ M	0.1 μ M
SQ-22536 (adenylate cyclase inhibitor)	†	–	–	–
H89 (PKA inhibitor)	–	–	–	–

*Macrophages were pre-treated with different concentrations of each compound for 30 min before stimulation. †No inhibitory effect on cytokine synthesis.

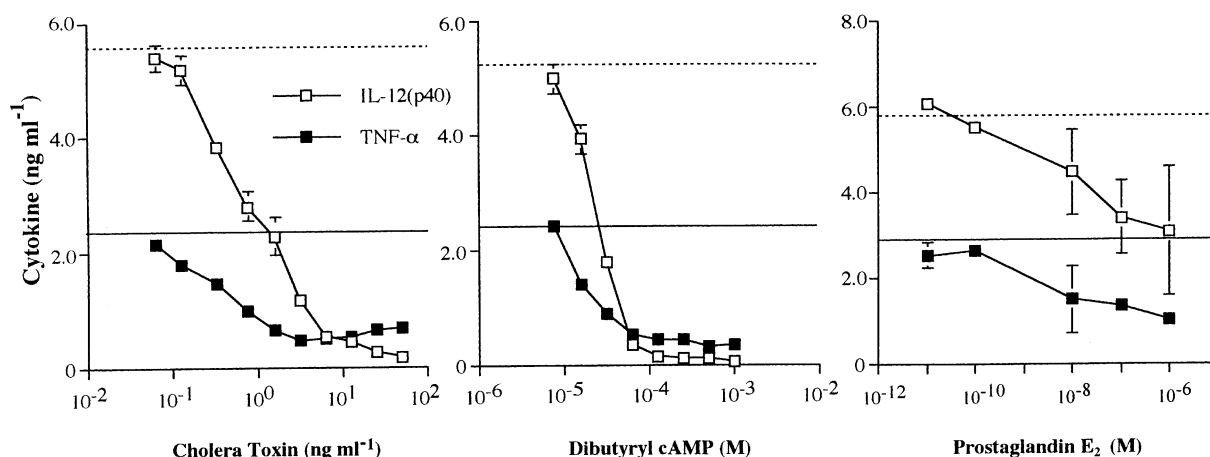


Figure 1 Inhibitory activity of cholera toxin, cyclic AMP derivatives and prostaglandin E₂ in the synthesis of TNF- α and IL-12(p40) by macrophages exposed to tGPI-mucins. Inflammatory macrophages were pre-treated for 30 min with range of concentrations of ChTx (left panel), DB (central panel) or PGE₂ (right panel) before stimulation with tGPI-mucins (2.5 nM) in the presence of 50 U ml⁻¹ of IFN- γ . TNF- α and IL-12(p40) were assayed in supernatants collected 24 and 48 h, respectively, after macrophage stimulation. Control cytokine levels in absence of inhibitors are shown by dashed (IL-12) and continuous (TNF- α) lines. Values are means \pm s.d. of triplicates. Similar results were obtained in three different experiments.

Results

Cyclic AMP is a negative regulator of IL-12 synthesis by inflammatory macrophage

Recent evidence suggests that the increase in intracellular levels of cyclic AMP is accompanied by a significant decrease of cytokine production by LPS-activated macrophages (Eigler *et al.*, 1998; Meisel *et al.*, 1996; van der Pouw Kraan *et al.*, 1995). We carried out preliminary studies with cholera toxin (ChTx), an effective stimulator of adenylate cyclase, in murine macrophages activated by LPS. Pretreatment with ng ml^{-1} concentrations of ChTx completely inhibited the production of both TNF- α and IL-12 from LPS-stimulated macrophages (Table 1). Similarly, ChTx totally inhibited the production of IL-12 and TNF- α by macrophages stimulated with tGPI-mucins in the presence or absence of IFN- γ (Figure 1 and Table 1). We also tested two cyclic AMP derivatives (DB and 8-bromo-cyclic AMP), that mimic the action of cyclic AMP, and PGE₂ in the regulation of IL-12 and TNF- α production by macrophages activated by microbial products. Both cyclic AMP derivatives effectively inhibited TNF- α and IL-12 synthesis induced by LPS or tGPI-mucins (Table 1 and Figure 1). PGE₂ also significantly blocked the production of cytokines by either LPS or tGPI-mucins but it was slightly less effective than the other modulators at the concentrations used (Table 1 and Figure 1). Overall, the inhibitory effects of cyclic AMP-based strategies were equally effective but more pronounced against cytokine synthesis induced by tGPI-mucins than by LPS (Table 1). In contrast to these inhibitory effects, blockade of endogenous cyclic AMP synthesis with SQ-22536 and of PKA with H89, had little effect on IL-12 or TNF- α production induced by either tGPI-mucins or LPS (Table 1). Overall the results demonstrate that cyclic AMP is an effective modulator of both IL-12 and TNF- α production by macrophages stimulated with LPS or tGPI-mucins.

The inhibitory effect of cyclic AMP on both IL-12(p40) and TNF- α synthesis involves PKA activation

Next, we examined the role of protein kinase A (PKA) in mediating the modulatory effects of cyclic AMP in macrophages. IFN- γ -primed macrophages were incubated in presence of the PKA inhibitor H89 (at $1 \mu\text{M}$) for 30 min before addition of PGE₂ or DB. The macrophages were then cultured in the presence or absence of LPS or tGPI-mucins and cytokines measured in the macrophage culture supernatants by ELISA. In these experiments, PGE₂ and DB caused a reduction of 50% or more of IL-12 and TNF- α production that was partially reversed by the presence of H89 (Figure 2). H89 alone had no significant effect on the IL-12 synthesis but caused a slight increase on TNF- α production. These results suggest that down-regulation of IL-12 and TNF- α synthesis caused by components that promote enhancement in the intracellular levels of cyclic AMP is, at least in part, mediated by PKA activation.

IL-10 is produced upon macrophage stimulation by tGPI-mucins and LPS in the presence of cyclic AMP

Since increased intracellular levels of cyclic AMP have been shown to augment the synthesis of IL-10 in both T lymphocytes (Benbernou *et al.*, 1997) and macrophages

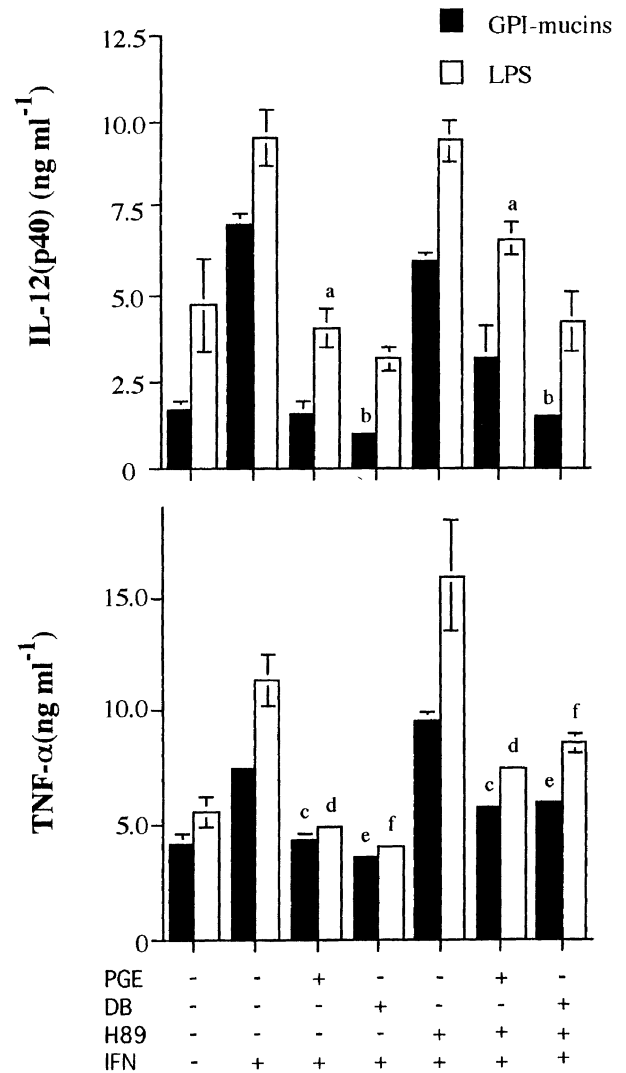


Figure 2 Protein Kinase A involvement in the inhibitory effect of DB and prostaglandin E₂ on the synthesis of TNF- α and IL-12(p40) by activated macrophages. Inflammatory macrophages were pre-treated with $1 \mu\text{M}$ H89 in the presence or absence of $10 \mu\text{M}$ PGE₂ or 0.3 mM DB, for 30 min before stimulation with tGPI-mucins (2.5 nM) or LPS (100 ng ml^{-1}) in the presence of 50 U ml^{-1} of IFN- γ . TNF- α and IL-12(p40) were assayed in supernatants collected 24 and 48 h, respectively, after macrophage stimulation. Bars indicated by the same letters represent values that are statistically different ($P < 0.05$). Values are means \pm s.d. of triplicates and are representative of two experiments.

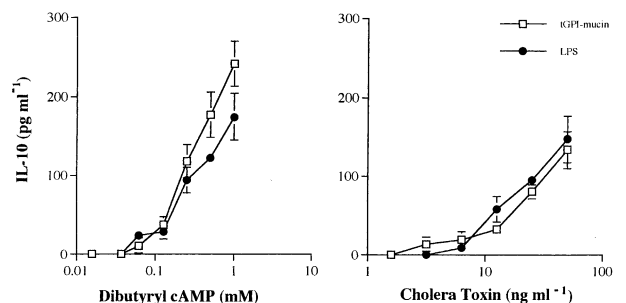


Figure 3 Cholera Toxin and DB enhance the synthesis of IL-10 by stimulated inflammatory macrophages. Inflammatory macrophages were stimulated with tGPI-mucins (2.5 nM) or LPS (100 ng ml^{-1}) in the absence or presence of increasing concentrations of ChTx or DB, as indicated. IL-10 was measured in the supernatants collected at 24 h post-stimulation. Values are means \pm s.d. of triplicates. Similar results were obtained in two different experiments.

(Eigler *et al.*, 1998; Meisel *et al.*, 1996; van der Pouw Kraan *et al.*, 1995) and since IL-10 modulates the synthesis of pro-inflammatory cytokines by macrophages, we investigated whether a similar phenomenon was involved in our system. Increasing concentrations of DB (Figure 3, left panel) or ChTx (Figure 3, right panel) added to IFN- γ -primed macrophages 30 min before stimulation with tGPI-mucins or LPS augmented the synthesis of IL-10 in a dose-dependent manner. Note that both DB and ChTx were considerably less potent at inducing IL-10 production than inhibiting IL 12 and/or TNF- α production (compare Figures 1 and 3). The latter results may, however, be explained by the lower sensitivity of our IL-10 ELISA.

Figure 4 demonstrates the effects of IL-10 on the synthesis of IL-12(p40) and TNF- α by IFN- γ primed macrophages stimulated with either LPS or tGPI-mucins. We observed that IL-10 inhibited both TNF- α and IL-12(p40) production in a dose dependent way, in either LPS or tGPI-mucin-stimulated macrophages (Figure 4).

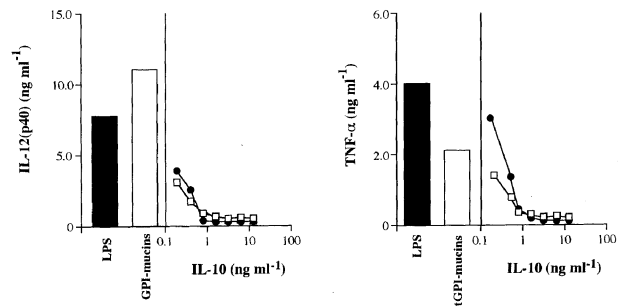


Figure 4 Inhibitory effect of IL-10 on the synthesis of TNF- α and IL-12(p40) by macrophages exposed to tGPI-mucins. IFN- γ -primed inflammatory macrophages were incubated for 30 min without (columns) or with (lines) different concentrations of rIL-10 before stimulation with LPS (100 ng ml⁻¹) or tGPI-mucins (2.5 nM). TNF- α and IL-12(p40) were measured on culture supernatants at 24 and 48 h, respectively, after stimulation with microbial products. Values are means \pm s.d. of triplicates and are representative of two different experiments. Standard deviations are too small and they don't appear in this figure.

Table 2 IL-12(p40) synthesis by inflammatory macrophages from wild type or IL-10^{-/-} KO mice treated with IFN- γ , and with either tGPI-mucins or LPS in the presence of cyclic AMP derivatives, cholera toxin or PGE₂*

Mouse genotype	Macrophage stimulation	Medium	IL-12(p40) secretion (ng ml ⁻¹)			
			Cholera toxin (5 ng ml ⁻¹)	PGE ₂ (1 μ M)	8-Bromo cyclic AMP (0.1 mM)	DB (0.1 mM)
Wild type	No addition	2.3 \pm 1.1	1.4 \pm 0.3	1.2 \pm 0.3	1.7 \pm 0.6	1.9 \pm 0.6
	IFN- γ	1.5 \pm 1.4	2.1 \pm 0.6	1.5 \pm 0.8	2.3 \pm 0.2	1.7 \pm 0.3
	LPS	5.7 \pm 1.1†	1.1 \pm 0.5#	2.2 \pm 0.3#	1.2 \pm 0.6#	1.6 \pm 0.6#
	LPS + IFN- γ	37.0 \pm 5.3†	6.1 \pm 2.1#	13.3 \pm 3.2#	5.2 \pm 1.2#	3.1 \pm 1.0#
	tGPI-mucins	4.6 \pm 1.1†	1.3 \pm 1.1#	1.7 \pm 1.0#	1.1 \pm 0.3#	1.3 \pm 1.2#
	tGPI-mucins + IFN- γ	25.0 \pm 6.2†	3.2 \pm 1.5#	10.3 \pm 1.9#	4.2 \pm 1.7#	2.9 \pm 1.1#
IL-10 KO	No addition	1.3 \pm 1.7	2.2 \pm 1.3	2.7 \pm 0.9	2.1 \pm 0.3	1.1 \pm 0.1
	IFN- γ	2.5 \pm 1.8	3.1 \pm 0.8	2.5 \pm 1.2	2.3 \pm 1.4	2.2 \pm 1.3
	LPS	7.8 \pm 1.2†	6.9 \pm 0.3†	7.2 \pm 1.3†	8.1 \pm 2.2†	6.3 \pm 1.1†
	LPS + IFN- γ	42.0 \pm 6.5†	43.5 \pm 5.6†	39.5 \pm 5.4†	36.2 \pm 2.3†	44.3 \pm 2.0†
	tGPI-mucins	5.3 \pm 1.4†	6.2 \pm 0.7†	4.9 \pm 1.1†	5.7 \pm 1.1†	6.1 \pm 1.3†
	tGPI-mucins + IFN- γ	33.0 \pm 5.5†	36.4 \pm 2.1†	36.8 \pm 2.1†	36.2 \pm 3.2†	35.7 \pm 3.4†

*IL-12(p40) was assayed in supernatants collected 48 h after macrophage stimulation. Values are means \pm s.d. of triplicates. Similar results were obtained in three different experiments. †, # Different symbols in the same line indicate that differences are statistically significant ($P < 0.01$).

Table 3 TNF- α synthesis by inflammatory macrophages from wild type or IL-10^{-/-} KO mice treated with IFN- γ and with either tGPI-mucins or LPS in the presence or absence of cyclic AMP derivatives, cholera toxin or PGE₂*

Mouse genotype	Macrophage stimulation	Medium	TNF- α secretion (ng ml ⁻¹)			
			Cholera toxin (5 ng ml ⁻¹)	PGE ₂ (1 μ M)	8-Bromo cyclic AMP (0.1 mM)	DB (0.1 mM)
Wild type	No addition	0.2 \pm 0.1	0.1 \pm 0.3	0.2 \pm 0.3	0.7 \pm 0.6	0.5 \pm 0.6
	IFN- γ	0.3 \pm 0.2	0.1 \pm 0.2	0.5 \pm 0.8	0.3 \pm 0.2	0.3 \pm 0.3
	LPS	4.2 \pm 0.5†	0.2 \pm 0.1#	0.5 \pm 0.3#	0.3 \pm 0.2#	0.2 \pm 0.1#
	LPS + IFN- γ	7.0 \pm 1.1†	0.3 \pm 0.1#	0.3 \pm 0.2#	0.2 \pm 0.2#	0.1 \pm 1.0#
	tGPI-mucin	3.2 \pm 0.7†	0.1 \pm 0.5#	0.7 \pm 0.3#	0.1 \pm 0.1#	0.2 \pm 0.1#
	tGPI-mucin + IFN- γ	5.0 \pm 1.2†	0.2 \pm 0.5#	0.3 \pm 0.4#	0.5 \pm 0.7#	0.6 \pm 1.1#
IK-10 KO	No addition	0.3 \pm 0.3	0.2 \pm 0.3	0.5 \pm 0.1	0.1 \pm 0.3	0.1 \pm 0.1
	IFN- γ	0.4 \pm 0.2	0.1 \pm 0.5	0.5 \pm 0.1	0.3 \pm 0.3	0.2 \pm 0.2
	LPS	5.7 \pm 0.6†	0.3 \pm 0.2#	1.2 \pm 0.5#	0.3 \pm 0.1#	0.2 \pm 0.2#
	LPS + IFN- γ	9.0 \pm 0.5†	0.5 \pm 0.4#	0.5 \pm 0.3#	0.2 \pm 0.2#	0.3 \pm 0.2#
	tGPI-mucin	3.9 \pm 0.2†	0.2 \pm 0.1#	1.3 \pm 0.3#	0.1 \pm 0.1#	0.2 \pm 0.1#
	tGPI-mucin + IFN- γ	6.0 \pm 0.5†	0.4 \pm 0.3#	0.8 \pm 0.1#	0.2 \pm 0.2#	0.7 \pm 0.4#

*TNF- α was assayed in supernatants collected 24 h after macrophage stimulation. Values are means \pm s.d. of triplicates. Similar results were obtained in three different experiments. †, # Different symbols in the same line indicate that differences are statistically significant ($P < 0.01$).

Endogenous IL-10 is necessary for the inhibitory effect of cyclic AMP on IL-12(p40) but not on TNF- α synthesis by stimulated macrophages

The inhibitory activity of endogenous IL-10 on IL-12 and TNF- α synthesis was further investigated using IL-10 KO mice (Tables 2 and 3). We observed that, although ChTx, PGE₂, 8-bromo-cyclic AMP and DB caused a major reduction of IL-12(p40) synthesis in wild type animals, these same compounds did not display any inhibitory activity on IL-12 synthesis in macrophages from IL-10^{-/-} KO mice exposed to either LPS or tGPI-mucins in the presence or absence of IFN- γ (Table 2). These results strongly suggest that the inhibitory activity on IL-12 production by compounds that cause an increase or mimic intracellular levels of cyclic AMP is in fact mediated by

IL-10. Interestingly, ChTx, PGE₂, 8-bromo cyclic AMP and DB inhibited TNF- α synthesis induced by tGPI-mucins or LPS even in macrophages from IL-10^{-/-} KO mice (Table 3). This indicates that the inhibitory activity of cyclic AMP on cytokine synthesis may involve more than one pathway, which may or may not be mediated by endogenous IL-10.

The kinetics of TNF- α and IL-12(p40) synthesis and inhibition by IL-10 and DB

Inasmuch as IL-10 is produced in response to high intracellular levels of cyclic AMP and it was capable of blocking the production of both IL-12 and TNF- α by macrophages, the contrasting results in macrophages from IL-10^{-/-} KO mice were at first unexpected. The next

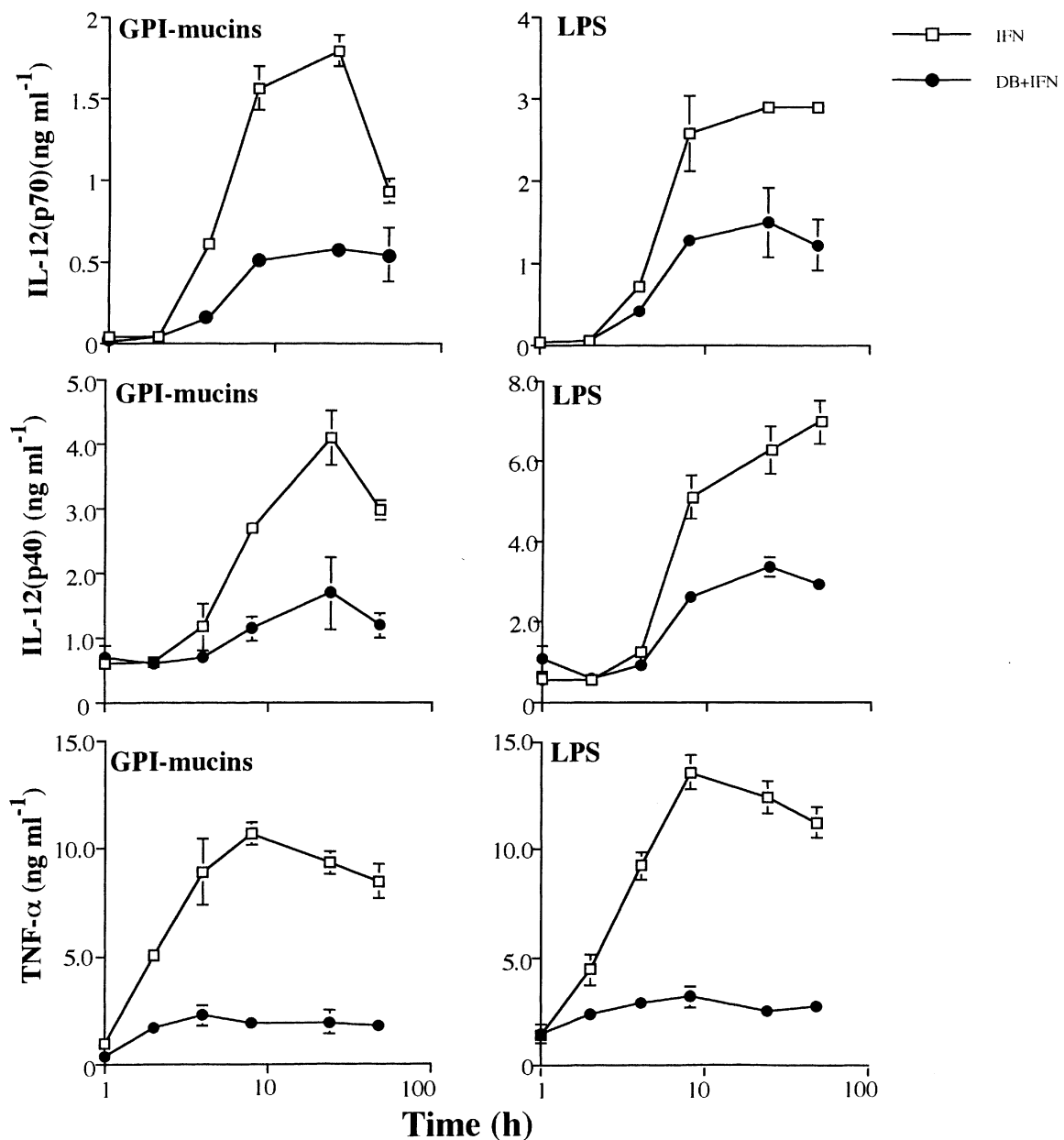


Figure 5 Kinetics of TNF- α , IL-12(p40) and IL-12(p70) synthesis by macrophages exposed to microbial stimuli. IFN- γ -primed or unprimed macrophages were stimulated with tGPI-mucins (2.5 nM) and LPS (100 ng ml⁻¹). IL-12 and TNF- α levels were determined at different time after stimulation of macrophage. Values are means \pm s.d. of triplicates. Similar results were obtained in two different experiments.

experiments were designed to investigate whether a kinetic difference in IL-12 and TNF- α production could explain the endogenous IL-10 dependency of the inhibitory activity of cyclic AMP. Macrophages primed or not with IFN- γ were exposed to either tGPI-mucins or LPS and supernatants collected at various times. Our results show that TNF- α

synthesis was already detectable at 2 h and peaked between 4–8 h after stimulation of macrophages (Figure 5). In contrast, synthesis of IL-12(p40) was delayed and only measurable after 8 h (peaking at 24 h or later) post-stimulation with either LPS or tGPI-mucins (Figure 5). Identical kinetics were observed for IL-12(p40) and IL-

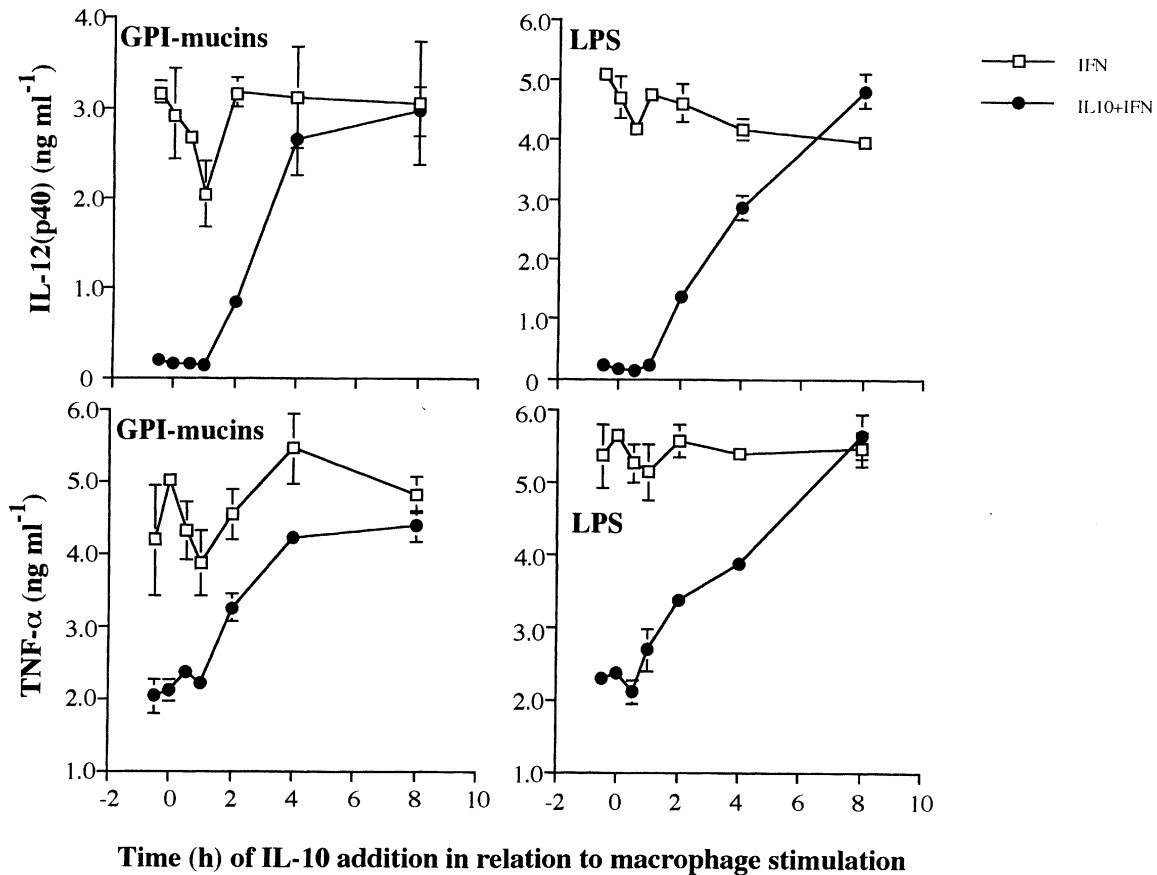


Figure 6 Time-dependence of the inhibitory effect of IL-10. IFN- γ -primed macrophages were stimulated with tGPI-mucins and LPS in the presence or absence of IL-10 (1.25 ng ml^{-1}) added at the indicated time points. IL-12 and TNF- α levels were determined after a 24 h culture. Values are means \pm s.d. of triplicates. Similar results were obtained in two different experiments.

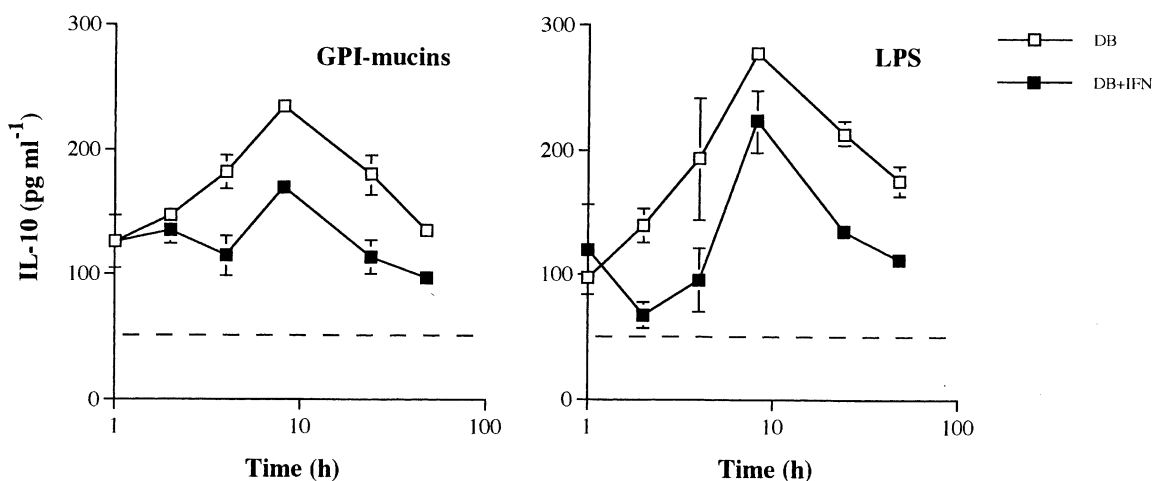


Figure 7 Kinetics of IL-10 synthesis by macrophages exposed to microbial stimuli in the presence of DB. IFN- γ -primed or unprimed macrophages were stimulated with tGPI-mucins (2.5 nM) and LPS (100 ng ml^{-1}) in the presence of 0.3 mM of DB. IL-10 levels were determined at different time after stimulation of macrophage. Horizontal dashed lines indicate the levels of IL-10 in culture supernatants from unstimulated macrophages. Values are means \pm s.d. of triplicates. Similar results were obtained in two different experiments.

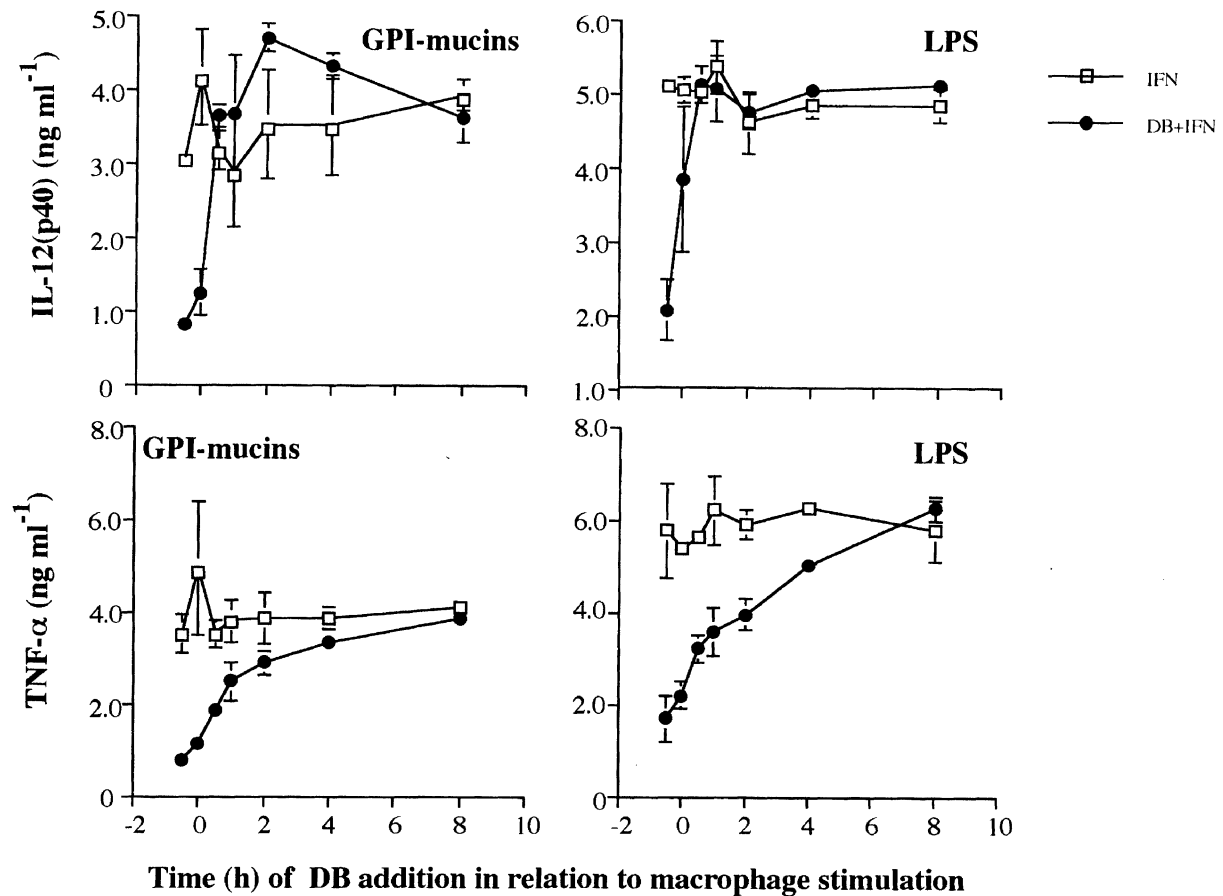


Figure 8 Time-dependence of the inhibitory effect of DB. IFN- γ -primed macrophages were stimulated with tGPI-mucins (2.5 nM) and LPS (100 ng ml⁻¹) in the presence or absence of DB (0.3 mM) added at the indicated time points. IL-12 and TNF- α levels were determined after a 24 h culture. Values are means \pm s.d. of triplicates. Similar results were obtained in two different experiments.

IL-12(p70) synthesis upon microbial stimuli in the presence or absence of DB (Figure 5).

Next, we studied the kinetics of the inhibitory effects of IL-10 on TNF- α and IL-12(p40) synthesis by activated macrophages. The cells were stimulated with either LPS or tGPI-mucins and IL-10 was added to the cells in culture at different times after the initial stimulation. Our results show that the inhibitory effect of IL-10 was immediate and protracted for both TNF- α and IL-12(p40) synthesis in stimulated macrophages (Figure 6). IL-10 inhibited the synthesis of either cytokines even when added at 2 or in some case 4 h after stimulation with tGPI-mucins or LPS. Interestingly, kinetic experiments showed that IL-10 synthesis by macrophages stimulated with either stimuli in the presence of DB peaked at 8 h post-stimulation, before IL-12(p40) and IL-12(p70) reach maximal levels in the culture supernatants, but after most TNF- α being produced (Figure 7). Therefore, our results indicate that upon microbial stimuli, IL-10 is produced and may play a role on macrophage deactivation, only after most of the TNF- α , but not IL-12(p40) and IL-12(p70), has been produced. In fact, published data suggest that the synthesis of IL-10 by LPS-stimulated macrophages occur after generation of TNF- α (Eigler *et al.*, 1998).

Consistent with the hypothesis that DB inhibitory effect on IL-12, but not TNF- α , production is dependent on synthesis of endogenous IL-10, DB was only inhibitory for IL-12(p40) synthesis if added prior to or at the same time of macrophage stimulation (Figure 8). In contrast, DB had a much more protracted action on TNF- α synthesis, and was inhibitory even

when added 2 h after macrophage stimulation with either LPS or tGPI-mucins (Figure 8). The delayed DB inhibitory effect on IL-12(p40), as compared to TNF- α synthesis, is even more dramatic if we consider that most TNF- α is produced in the first 8 h, whereas most IL-12(p40) is produced from 8–24 h post macrophage stimulation with microbial products.

Discussion

IL-12 is a key cytokine responsible for initiation of IFN- γ synthesis and establishment of cell mediated immunity (CMI), thus being an important determinant of resistance against a large number of pathogens (Biron & Gazzinelli, 1995; Scott, 1993; Trinchieri, 1995; Trinchieri & Scott, 1995). Consistent with this, the IL-12 heterodimer has been successfully used as vaccine adjuvant for selective induction of strong Th1 lymphocytes and resistance against some intra and extra-cellular pathogens (Afonso *et al.*, 1994; Miller *et al.*, 1995; Schijns *et al.*, 1995; Wynn *et al.*, 1995). Conversely, uncontrolled synthesis of this cytokine as in experimental infections with parasitic protozoa (Gazzinelli *et al.*, 1996; Hunter *et al.*, 1997), challenge with bacterial endotoxin (Heinzel *et al.*, 1994; Ozmen *et al.*, 1994; Wysoka *et al.*, 1995), or certain autoimmune diseases (Germann *et al.*, 1995; Leonard *et al.*, 1995), results in detrimental effects due to cytokine shock and/or excessive activation of the cellular compartment of the immune system. Moreover, in Chagas' disease, the IL-12-driven CMI may contribute to both resistance (Aliberti *et al.*, 1996; Hunter *et al.*, 1996) and

damage to vital organs such as the heart (Hunter *et al.*, 1997). Therefore, a better understanding of the biochemical signalling pathway(s) involved in activation and expression of IL-12 genes may lead to the development of new reagents and/or strategies that can be used either to induce desired, or inhibit detrimental, immune responses mediated by IFN- γ and CMI.

Recently, we have purified tGPI-mucins from *T. cruzi* trypomastigotes and determined that these components induce macrophage activation (Camargo *et al.*, 1997a, b). In the presence of IFN- γ , macrophages activated with tGPI-mucins produced significant amounts of nitric oxide (NO) and the pro-inflammatory cytokines IL-12 and TNF- α activation (Camargo *et al.*, 1997a, b). The precise role of tGPI-mucins in the pathophysiology of Chagas' disease is poorly understood, but the ability of these glycoconjugates to induce macrophages to secrete NO and cytokines such as IL-12 may underlie a fundamental host-parasite interaction (Aliberti *et al.*, 1996; Hunter *et al.*, 1996; 1997), akin to the importance of LPS in bacterial diseases.

In the present study we investigated the role of cyclic AMP-based strategies in modulating IL-12 and TNF- α synthesis in macrophages stimulated by tGPI-mucins and LPS. Because IL-12(p40) has been shown to be tightly regulated in cells from macrophage lineage (Ma *et al.*, 1996; Murphy *et al.*, 1995), whereas IL-12(p35) is expressed constitutively by different types of cell (Trinchieri, 1995), we focused on the expression of IL-12(p40) rather than on that of the IL-12 heterodimer. Moreover, we assessed whether the endogenous production of IL-10 was important for the inhibitory activity of cyclic AMP. Our results show that in IFN- γ primed macrophages the synthesis of TNF- α and IL-12 is favoured over that of IL-10. In fact, significant amounts of both TNF- α and IL-12 but not of IL-10 were detected upon activation with tGPI-mucins (or LPS). In conditions, however, which favour the induction of (or mimic) high levels of intracellular cyclic AMP, the synthesis of IL-10 is enhanced, whereas that of TNF- α , IL-12(p40) and IL-12(p70) heterodimer is mostly abolished. These results are in agreement with early studies performed elsewhere, which show that PGE₂, *via* its ability to raise intracellular cyclic AMP, is a potent regulator of IL-12 and TNF- α synthesis (van der Pouw Kraan *et al.*, 1995). Moreover, we show that the effects of cyclic AMP-based strategies were at least in part mediated by PKA, inasmuch as the PKA inhibitor H89 reversed the modulatory effects of elevated cyclic AMP. Thus, it is tempting to speculate that in situations where the cyclic AMP levels are enhanced in antigen presenting cells (APCs), T cell differentiation will be favoured towards the Th2 phenotype (Panina-Bordignon *et al.*, 1997). Conversely, priming with IFN- γ and low intracellular levels of cyclic AMP will produce high levels of IL-12 in APCs, which will favour the development of Th1 lymphocytes (Hsieh *et al.*, 1993; Seder *et al.*, 1993).

IL-10 has been shown to be a potent modulator of IL-12 and TNF- α expression (D'Andrea *et al.*, 1993; Fiorentino *et al.*, 1991; Gazzinelli *et al.*, 1996). In addition synthesis of IL-10 stimulated by LPS appears to be potentiated by cyclic AMP (Eigler *et al.*, 1998; van der Pouw Kraan *et al.*, 1995).

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Therefore, we investigated whether tGPI-mucins induced production of IL-10 protein in the presence of cyclic AMP and if the inhibitory effect of cyclic AMP on TNF- α and IL-12 synthesis occurred *via* IL-10. Our results clearly demonstrate that tGPI-mucins (and LPS) induce considerable amounts of IL-10 when activation occurs in the presence of elevated intracellular levels of cyclic AMP. Moreover, our results demonstrate that IL-10 is clearly capable of inhibiting the synthesis of both IL-12 and TNF- α induced by tGPI-mucins (or LPS) even when added up to 2 h after stimulation. However, cyclic AMP was still inhibitory for TNF- α , but not IL-12(p40), production by inflammatory macrophages lacking functional genes for IL-10. Thus, we suggest that, although IL-10 is capable of modulating both IL-12(p40) and TNF- α production, the regulatory activity of cyclic AMP on IL-12 but not on TNF- α synthesis is dependent on endogenous IL-10 activity.

The lack of inhibition of IL-12 production, but not of TNF- α , by cyclic AMP in macrophages from IL-10 knockout mice in response to tGPI mucins or LPS stimulation was intriguing. To examine this issue in more detail we performed kinetic studies to compare the onset of secretion of these cytokines in response to both stimulants in the presence and absence of cyclic AMP-elevating agents. Our results showed that whereas TNF- α production was first detected at 2 h and peaked around 4–8 h, IL-12 production began and peaked at much later time point. In addition, there is evidence showing that the production of IL-10 by activated macrophages has a late onset (Eigler *et al.*, 1998) similarly to that of IL-12, therefore later than the onset of TNF- α synthesis.

Together, our findings favour the hypothesis that the cyclic AMP inhibitory activity on IL-12(p40) or IL-12(p70) but not on TNF- α synthesis is dependent on *de novo* protein synthesis, most likely IL-10 synthesis, by macrophages stimulated with microbial products. In contrast, the IL-10 inhibitory effect on both IL-12(p40) and TNF- α expression was immediate and most likely independent of *de novo* protein synthesis. Thus, we can speculate that increased levels of cyclic AMP, which are normally transient, may be directly able to control genes expressed early (e.g. TNF- α) but not later after macrophage stimulation with microbial products. However, by inducing IL-10 gene expression, cyclic AMP may trigger anti-inflammatory activity (e.g. blockade of IL-12 production), which will persist even after the levels of cyclic AMP have decreased to normal.

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