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Effect of interleukin-4 and interleukin-10 on leucocyte migration and nitric oxide production in the mouse

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1 The effect of systemic treatment of mice with murine recombinant interleukin-4 (IL-4) or interleukin-10 (IL-10) on neutrophil infiltration into a specific tissue site and nitric oxide (NO) production from peritoneal macrophages was investigated.

2 Intravenously (i.v.) administered IL-4 (0.01-10 μg per mouse, approximately 0.3-300 μg kg^{-1}, i.v.) and IL-10 (0.01-1 μg per mouse, approximately 0.3-30 μg kg^{-1}, i.v.) dose-dependently inhibited neutrophil accumulation into a 6-day-old murine air-pouch induced by local application of interleukin-1β (IL-1β, 5 ng), with approximate ED_{50}s of 0.35 and 0.90 μg, respectively. Neither IL-4 (1 μg, 30 μg kg^{-1}, i.v.) nor IL-10 (1 μg, 30 μg kg^{-1}, i.v.) prevented leucocyte accumulation in the mouse air-pouches when interleukin-8 (IL-8, 1 μg) was used as chemoattractant. Similarly, neither cytokine had any effect on the *in vitro* up-regulation of CD11b antigen on the surface of murine circulating neutrophils.

3 Treatment of mice with lipopolysaccharide (LPS, 0.3 mg kg^{-1}, i.p.) caused an increase in the formation of NO (measured as nitrite accumulation) in the supernatant of peritoneal macrophages *ex vivo*. Pretreatment of mice with IL-4 (0.01-1 μg i.v., 20 min before LPS), but not with IL-10 (1 μg i.v., 20 min before LPS), caused a dose-dependent reduction in this LPS-stimulated formation of nitrite by peritoneal macrophages *ex vivo*.

4 Activation of murine macrophages with LPS (1 μg ml^{-1} for 24 h) *in vitro* caused a significant increase in nitrite release in the supernatant of these cells. Pretreatment of either J774.2 or peritoneal macrophages with IL-4 (0.1-1 μg ml^{-1}, 20 min before LPS), but not with IL-10 (1 μg ml^{-1}, 20 min before LPS) caused a concentration-related attenuation of this LPS-stimulated nitrite formation.

5 Thus, both IL-4 and IL-10 inhibit the migration of leucocytes (stimulated by IL-1β) *in vivo*; IL-4 (but not IL-10) inhibits the induction of NO synthase caused by LPS in murine macrophages *in vitro* and *ex vivo*.

Keywords: Inflammation; neutrophils; CD11b; interleukin-1; interleukin-8; nitric oxide synthase

Introduction

Great interest has been generated by cytokines like interleukin-4 (IL-4) and interleukin-10 (IL-10), originally described for their effect on the immune system and, more recently, for their ability to depress the generation of a variety of mediators which are important in the control of local and systemic inflammatory responses (Hart *et al.*, 1989; De Waal Malefyt *et al.*, 1991).

IL-4 was originally described as a B cell-derived growth factor for T lymphocytes (Paul, 1987). IL-4 prevents the formation of tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and prostanoids by human monocytes activated with lipopolysaccharide (LPS, endotoxin) *in vitro* (De Wall Malefyt *et al.*, 1991). In addition, IL-4 prevents the release of interleukin-6 (IL-6) caused by LPS *in vitro* and *in vivo* (Donnelly *et al.*, 1993; Wong *et al.*, 1993) and enhances the expression of the endogenous IL-1 receptor antagonist, (IL-1ra) (Vannier *et al.*, 1992). Thus, it has been proposed that IL-4 is an endogenous anti-inflammatory cytokine. Indeed, intratracheal administration of IL-4 attenuates the degree of inflammation in the lung of rats challenged with immuno-complexes (Mulligan *et al.*, 1993).

IL-10 was first identified as a cytokine produced by a subclone of T helper lymphocytes (for a review, see Zlotnik & Moore, 1994). Pretreatment with IL-10 of human monocytes stimulated with LPS *in vitro* attenuates the formation of several pro-inflammatory cytokines including TNF-α, IL-1 and interferon-γ (IFN) (De Waal Malefyt *et al.*, 1991; Ralph *et al.*, 1992) and, as a consequence, attenuates the release of interleukin-8 (IL-8) (Cassatella *et al.*, 1993). Exogenous IL-10 also attenuates the inflammatory response in the lung of rats exposed to immuno-complexes (Mulligan *et al.*, 1993) and prevents experimental allergic encephalomyelitis in rats (Rott *et al.*, 1994). Moreover, the expression of mRNA and protein for IL-10 is enhanced in synovial membranes of patients with rheumatoid arthritis and osteoarthritis (Katsikis *et al.*, 1994). However, the mechanism(s) of the potent anti-inflammatory effects of IL-10 *in vivo* are not clear.

Nitric oxide (NO) is a recently described autacoid produced by a hitherto unrecognized enzymatic pathway in many mammalian cells. NO is produced from the guanidino nitrogen group of L-arginine by NO synthase (NOS) (for review, see Moncada & Higgs, 1993). Three different isoforms of NOS have been isolated, cloned, sequenced and expressed. The NOS in endothelial (eNOS) and neuronal cells (bNOS) are expressed constitutively, and both enzymes require an increase in intracellular calcium for activation. Under physiological conditions, the continuous release of NO following activation of eNOS dilates blood vessels and together with catecholamines regulates blood vessel diameter, organ blood flow and blood pressure (for review, see Moncada & Higgs, 1993). In addition, NO inhibits the adhesion of platelets and polymorphonuclear leucocytes (PMN) to the surface of the endothelium (Kubes *et al.*, 1993). Activation of macrophages with LPS or pro-inflammatory cytokines (TNF-α, IL-1, IFN) results in the expression of an 'inducible' isoform of NOS (iNOS), the activity of which is independent of changes in intracellular calcium. Once expressed, iNOS produces large amounts of NO (see Thiemermann, 1994). Interestingly, there is some evidence that

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'deactivating' cytokines such as IL-4 (Bogdan *et al.*, 1994) and IL-10 prevent the formation of NO by cultured cells activated by LPS. There is also evidence that local inflammatory responses are associated with the induction of iNOS (Kubes *et al.*, 1993; Kurose *et al.*, 1993; Vane *et al.*, 1994). However, the role of an enhanced production of NO by iNOS in inflammation is not clear.

To gain a better understanding of the mechanism by which IL-4 and IL-10 exert anti-inflammatory effects *in vivo*, we have investigated the effect of IL-4 or IL-10 administration on (i) the migration of polymorphonuclear leucocytes (PMN) into a specific tissue site in response to either IL-1β or IL-8; (ii) CD11b up-regulation caused by IL-8 in murine peripheral PMN *in vitro*; (iii) the induction of iNOS activity in murine macrophages obtained from mice challenged with LPS; and (iv) the expression of iNOS activity in primary and cultured macrophages stimulated with LPS *in vitro*.

Methods

Animals

Male Swiss Albino mice (28–32 g body weight; Tuck, Essex) were used for all experiments. Mice were maintained on a standard chow pellet diet with tap water *ad libitum*. In experiments designed to investigate the effects of IL-4 or IL-10 on neutrophil migration, animal weight was 22–24 g at day 0, but had reached 28–32 g by day 6 (see below).

Neutrophil migration

Migration and accumulation of PMNs into a 6 day-old murine air-pouch in response to murine IL-1β was evaluated as recently described (Perretti & Flower, 1993). Briefly, air-pouches were formed by s.c. injection of 2.5 ml of air on day 0 and day 3. On day 6, murine recombinant IL-1β (1, 5 or 20 ng) was dissolved in carboxymethylcellulose (0.5 ml CMC, 0.5% in phosphate-buffered solution, PBS) and injected into the air-pouches. Mice received systemic injections of IL-4 (0.01–10 μg per mouse i.v., approximately 0.3–300 μg kg^{-1}), IL-10 (0.01–1 μg i.v., approximately 0.3–30 μg kg^{-1}), IL-8 (1 μg i.v., approximately 30 μg kg^{-1}) or IL-6 (1–10 μg i.v., approximately 30–300 μg kg^{-1}) 10 min prior to the local challenge with IL-1β. Controls received PBS (0.1 ml i.v.). The effect of an inhibitor of NO synthesis, N^G-monomethyl-L-arginine (L-NMMA, 30–300 μg per pouch) was also studied by local injection together with IL-1β. In a separate set of experiments, IL-8 was used as a stimulus to cause PMN migration and the dose of 1 μg (in 0.5 ml CMC) was selected from a previous study (Perretti *et al.*, 1994). In all cases, air-pouches were thoroughly washed with sterile PBS (2 ml) containing ethylendiaminotetracetic acid (EDTA, 3 mM) and heparin (50 iu ml^{-1}) at 4 h after the local injection of IL-1β or IL-8. The PMNs recovered in the lavage fluids were stained in Turk's solution and counted in a Neubauer haematocytometer. The number of PMNs recovered from each pouch was then calculated. Unless otherwise indicated, results are expressed as net migration obtained by subtracting the mild migration caused by injection of vehicle alone from the accumulation observed with the respective pro-inflammatory cytokine (IL-1β or IL-8) and expressed as 10^6 of PMN recovered per mouse.

CD11b up-regulation on murine leucocytes

The expression of the CD11b antigen on the membrane of murine leucocytes was measured by flow cytometric analysis using a whole blood technique. Blood was pooled from at least 3–4 mice. Aliquots (200 μl) were preincubated with IL-4 or IL-10 (0.1 μg ml^{-1} for both cytokines) for 5 min prior to the addition of IL-8 (100 ng ml^{-1}) and incubated for a further 15 min at room temperature, before being washed twice with PBS and stained (at 4°C for 60 min) with a specific rat anti-murine CD11b monoclonal antibody (mAb) (clone 5C6) (Rosen & Gordon, 1987). After two washes, cells were stained (5 min) with fluoresceine-isothiocyanate (FITC)-conjugated anti-rat IgG mAb (Serotec, Oxford). After two washes in PBS, red blood cell lysis (for 1 min) was performed with Immuno-Lyse (Coulter Electronics Ltd, Luton). For storage purposes, cells were fixed with an equal volume of 2% paraformaldehyde in PBS. Flow cytometry was performed with a FACScan analyser (Becton Dickinson, Cowley, Oxford) with air-cooled 100 mW argon ion laser tuned to 488 nm and connected to a Consort 32 computer running Lysis II software. Three distinct cell populations (lymphocytes, monocytes and PMN) were discriminated using forward and side scatter characteristics. The number of molecules of endogenous CD11b per cell was estimated by median fluorescence intensity in the FL1 channel with reference to microbeads labelled with standard numbers of molecules of FITC (Flow Cytometry Standards Corp., NC) (Le Bouteiller *et al.*, 1983).

NO release from primary (peritoneal) and cultured (J774.2) murine macrophages

Ex vivo *experiments* Animals were pretreated with vehicle (sterile PBS; 100 μl, i.v.), IL-4(0.01–1μg, i.v. approximately 0.3–30 μg kg^{-1}) or IL-10 (1 μg i.v., approximately 30 μg kg^{-1}) at 10–15 min prior to challenge with LPS (0.3 mg kg^{-1} injected intraperitoneally in a total volume of 10 ml kg^{-1}; serotype 0111 : B4, Sigma Chem. Co., Poole, Dorset). At 3 h after injection of LPS, the peritoneal cavities were washed with sterile PBS (4 ml) containing EDTA (3 mM), heparin (50 iu ml^{-1}) and gentamycin (50 μg ml^{-1}). Lavage fluids were centrifuged and cells (≥80% macrophages, the remaining being mainly due to and a few PMNs) resuspended in Dulbecco modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), counted and seeded (0.5×10^6 Mø per well) in 96-well plates (Costar, Cambridge, MA, U.S.A.). After incubation for 3 h (37°C in 5% CO_2) non-adherent cells were removed and adherent cells (≥95% macrophages) were incubated for a further 20 h with fresh DMEM containing 10% FCS. NO synthesis was assessed by measuring the accumulation of nitrite, one of the breakdown products of NO in aqueous solutions, in cell supernatants. Nitrite was measured by adding 10 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of medium (Szabó *et al.*, 1993). Optical density at 550 nm (OD_{550}) was measured with an Anthos 1.21 microplate reader (Anthos Labtechnik, Salzburg, Austria). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium. The time-course (1, 3, 6, 24 h after LPS) and the dose-dependency (0.01–1 mg kg^{-1} of LPS) of the induction of nitrite formation by LPS in peritoneal macrophages *ex vivo* was studied in preliminary experiments, which demonstrated a maximal nitrite accumulation in the cell supernatant when macrophages were collected 3 h after treatment of animals treated with 0.3 mg kg^{-1} LPS.

In vitro *experiments* The effects of either IL-4 or IL-10 on the formation of nitrite by either primary or cultured (J774.2) macrophages activated with LPS was also investigated *in vitro*. Primary macrophages were obtained from untreated mice and seeded in 96-well plates as described above. The murine macrophages cell line, J744.2, was cultured in 96-well plates (6×10^4 cells per well) as previously described (Szabó *et al.*, 1993). Primary or cultured macrophages were incubated with vehicle (control cells) or LPS (1 μg ml^{-1}) and the formation of nitrite in the cell supernatant was measured after 20 h as described above. To elucidate the effects of cytokines on the formation of nitrite by macrophages activated with LPS, IL-4 (0.01–1 μg ml^{-1}), IL-6 (1 μg ml^{-1}) IL-8 (1 μg ml^{-1}) or IL-10 (1 μg ml^{-1}) were added to each well 10 min prior to LPS.

Cytokines

Murine recombinant IL-1β was supplied by Dr R.C. Newton (Du Pont-Merck, Wilmington, DE, U.S.A.); murine recombinant IL-4 and human recombinant IL-10 were a kind gift of Dr S. Narula (Schering-Plough Ltd, New Jersey, U.S.A.). Human recombinant IL-8 was obtained from Dr I. Lindley (Sandoz-Forschung-Institute, Wien, Austria), whereas human recombinant IL-6 was a gift of Dr G. Ciliberto (IRBM, Pomezia, Italy). All cytokines were kept at −80°C and fresh aliquots used for each experiments. Dilutions were made in sterile PBS.

Statistical analysis

Statistical differences between experimental groups were assessed by one-way analysis of variance followed, if significant, by the Bonferroni test for inter-group comparisons. A P value of less than 0.05 was taken as significant.

Results

Effect of IL-4 or IL-10 on PMN migration

Local application of IL-1β into a preformed air-pouch resulted in a significant and dose-related increase in PMN accumulation within 4 h. The dose of the cytokine used in this study was chosen on the basis of preliminary experiments, in which a PMN migration of $2.4\pm0.5\times10^6$, $4.3\pm0.6\times10^6$, $8.0\pm0.9\times10^6$ and $10.0\pm1.2\times10^6$ was measured with vehicle, 1, 5 or 20 ng of IL-1β, respectively ($n=6$). The submaximal dose of IL-1β of 5 ng per air-pouch was chosen for all subsequent studies aimed at elucidating the potential inhibitory effect of IL-4 or IL-10. The degree of PMN migration into the air-poch stimulated by IL-1β was not significantly affected by co-administration of the NOS inhibitor L-NMMA (Table 1). Pretreatment of mice with IL-4 (0.01–10 μg per mouse, i.v.) resulted in a dose-dependent reduction of accumulation of PMNs caused by IL-1β with an approximate ED_{50} of 0.9 μg, corresponding to 27 μg kg^{-1} (Figure 1). Like IL-4, pretreatment of mice with IL-10 (0.01–1 μg per mouse, i.v.) also caused a dose-dependent inhibition of accumulation of PMNs caused by IL-1β with an approximate ED_{50} of 0.35 μg per mouse (10 μg kg^{-1}). Treatment of mice with either IL-8 (1 μg per mouse, i.v.) or IL-6 (1–10 μg per mouse, i.v.) did not modify the PMN accumulation induced by IL-1β (Figure 1). Neither IL-4 nor IL-10, however, affected the mild migration of PMNs elicited by injection of the vehicle alone (Table 2). Like IL-1β, injection of IL-8 (1 μg) into the air-pouch induced a significant increase (5–10 fold) in PMN accumulation. However, pretreatment of mice with either IL-4 or IL-10 (both at 1 μg i.v. per mouse) did not cause a significant inhibition of the PMN migration induced by IL-8 (Table 2). It should be noted, however, that in these experiments IL-10, but not IL-4, attenuated IL-8-induced PMN migration by 30%, an effect that may have some biological relevance.

Effect of IL-4 or IL-10 on CD11b expression on murine PMN in vitro

In order to gain a better understanding of the mechanism(s) by which IL-4 or IL-10 attenuate the migration of PMNs, we have evaluated the effect of these cytokines on the expression of CD11b on the surface of murine PMNs activated with IL-8 (Figure 2). However, pretreatment of PMNs with either IL-4 or IL-10 (both at 0.1 μg ml^{-1}) did not affect the expression of CD11b in either control cells (cells incubated with the vehicle alone) or in cells stimulated with IL-8 (0.1 μg ml^{-1}).

Effect of IL-4 or IL-10 on nitrite formation by peritoneal macrophages obtained from mice treated with LPS

The concentration of nitrite in the supernatant of primary macrophages obtained from mice treated with vehicle (control) was 1.3 ± 0.2 μM. In contrast, the concentration of nitrite measured in the medium of macrophages obtained from mice treated with LPS *in vivo* (0.3 mg kg^{-1}, i.p., 3 h prior to harvesting the cells) was significantly enhanced (42 ± 8 μM, $n=8$) (Figure 3). Pretreatment of mice with IL-4 (0.01–1 μg per mouse, i.v.) prior to injection of LPS resulted in a dose-de-

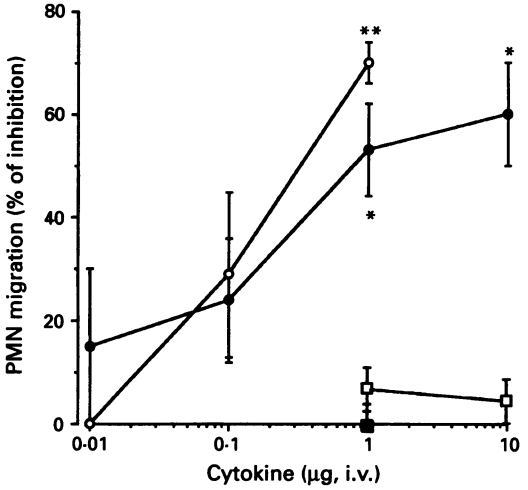

Figure 1 Interleukin-4 (IL-4) and IL-10 inhibit IL-1β-induced PMN migration into a mouse air-pouch. Mice received 100 ml i.v. of sterile PBS containing the reported doses of IL-4 (●), IL-10 (○), IL-6 (□) and IL-8 (■) 10 min before local challenge with IL-1β (5 ng in 0.5 ml CMC). Pouches were washed 4 h later and migrated PMN quantified following specific staining. Values (mean ± s.e.mean of 10–20 mice per group in the case of IL-4 and IL-10, and 6 mice per group for IL-6 and IL-8) are reported as % inhibition of net migration, with IL-1β alone giving a cell accumulation of $6.5\mp0.6\times10^6$ PMN per mouse, whereas CMC alone produced a mild accumulation of $1.2+0.2\times10^6$ PMN per mouse (mean ± s.e. of 9 experiments). *$P<0.05$ and **$P<0.01$ vs control group (vehicle-treated mice, calculated on original values).

Table 1 Lack of effect of NOS inhibition with N^G-monomethyl-L-arginine (L-NMMA) on IL-1-induced PMN migration

Treatment	*PMN* (10^6 per mouse)	*n*
Vehicle	1.15 ± 0.46	5
IL-8	7.88 ± 0.92*	5
+ L-NMMA 30 μg	9.32 ± 0.70	5
+ L-NMMA 100 μg	6.87 ± 0.69	6
+ L-NMMA 300 μg	5.93 ± 0.62	6

The doses of L-NMMA were given concomitantly with IL-1β (5 ng in 0.5 ml of vehicle) directly into the air-pouches. PMN migration was evaluated at the 4 h time-point. Values are mean ± s.e.mean of n animals per group. Note that IL-1β caused a significant increase in PMN migration when compared to vehicle. This response elicited by IL-1β, however, was not significantly affected by the NOS inhibitor L-NMMA. *$P<0.05$ vs vehicle.

Table 2 Lack of effect of IL-4 and IL-10 on IL-8-induced PMN migration

Pretreatment (100 µl i.v.)	*Stimulus*	*PMN* (10^6 per mouse)
PBS	CMC	1.50±0.5
IL-10 (µg)	CMC	1.90±0.2
PBS	IL-8 (1 µg)	8.60±1.0
IL-10 (1 µg)	IL-8 (1 µg)	6.04±1.2
PBS	CMC	0.36±0.11
IL-4 (1 µg)	CMC	0.33±0.11
PBS	IL-8 (1 µg)	4.47±1.05
IL-4 (1 µg)	IL-8 (1 µg)	4.93±1.16

Mice received 100 µl i.v. of either PBS alone or supplemented with the reported dose of IL-10 or IL-4 10 min before the local challenge into the air-pouches with carboxymethyl cellulose (CMC) alone or in combination with IL-8. PMN migration was measured 4 h later. Values (mean±s.e.mean) represent 2 distinct experiments with 6 mice per group.

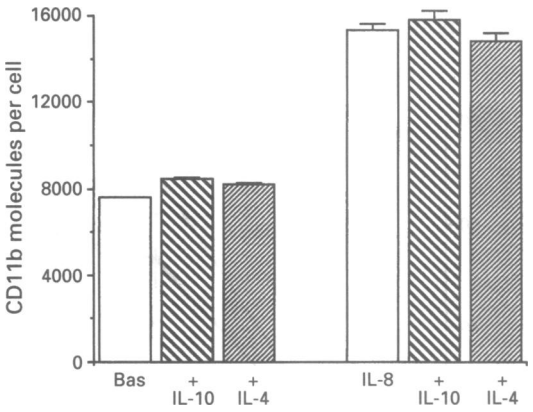

Figure 2 Lack of effect of interleukin-4 (IL-4) and IL-10 on IL-8-induced CD11b up-regulation on mouse peripheral blood PMNs. Blood was pooled from 3–4 mice and aliquots (200 ml) incubated with or without IL-4 or IL-10 (100 ng ml^{-1}) for 5 min at room temperature before the addition of either IL-8 (100 ng ml^{-1}) or its vehicle and incubated for further 15 min. A specific monoclonal anti-CD11b antibody was used to detect the antigen by flow cytometric analysis as described in the Methods section. Values are mean ±s.e.mean of three distinct measurements. Bas = basal release.

pendent reduction of the formation of nitrite by macrophages of these animals as assessed *ex vivo*. In contrast, pretreatment of mice with IL-10 (1 µg per mouse, i.v.) did not affect the increase in nitrite production by these cells collected from mice treated with LPS (Figure 3).

Effect of IL-4 or IL-10 on the formation of nitrite by primary and cultured murine macrophages activated by LPS in vitro

Activation of primary (peritoneal) macrophages with LPS (1 µg ml^{-1}) *in vitro* resulted in a significant increase in nitrite concentration in the cell supernatant from 10.1±0.8 µM (control cells treated with vehicle but not with LPS) to 58±2 µM at 24 h after addition of LPS. Pretreatment of cells with IL-4 (0.01–1 µg ml^{-1}) reduced the increase in nitrite formation caused by LPS in a dose-dependent manner (Figure 4a). In contrast, pretreatment of peritoneal macrophages with IL-10 (1 µg ml^{-1}) did not affect the rise in nitrite concentration in the supernatant of cells activated with LPS.

Activation of cultured J774.2 macrophages with LPS (1 µg ml^{-1}) also resulted in a significant increase in nitrite concentration in the supernatant from 4.5±1.2 µM to 37±1 µM at 24 h after LPS. Pretreatment of these cells with

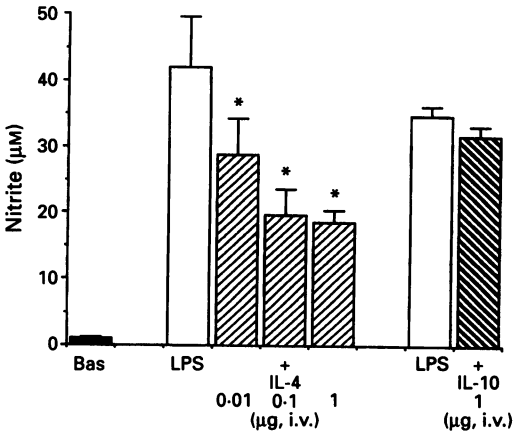

Figure 3 Distinct effect of interleukin-4 (IL-4) and IL-10 on lipopolysaccharide (LPS)-induced NO production from peritoneal Mø *ex vivo*. The reported doses of IL-4 or IL-10 were given to mice 10 min prior to challenge with LPS (0.3 mg kg^{-1}, i.p.). Nitrite production from peritoneal Mø cultured *ex vivo* for 24 h was measured with the Griess reaction as described in the Methods section. Basal release (Bas) represents the nitrite concentration in the supernatant of peritoneal cells taken from untreated mice. Values are mean±s.e.mean of 5–6 mice per group. *$P<0.05$ vs. respective LPS group.

IL-4, but not with IL-6, IL-8 or IL-10 (all cytokines tested at a concentration of 1 µg ml^{-1}), caused a significant reduction of nitrite accumulation induced by LPS (Figure 4b).

Discussion

This study demonstrates that the systemic administration of recombinant IL-4 or IL-10 to mice largely attenuates the migration of PMNs into a specific inflammatory site (air-pouch) caused by IL-1β. In contrast, systemic administration of either IL-6 or IL-8 did not inhibit the migration of PMNs caused by IL-1β *in vivo*.

Using this mouse air-pouch model, we have previously shown that the migration of PMNs caused by IL-1β *in vivo* is due to (i) activation by IL-1β of IL-1 type I receptors located on endothelial cells, (ii) a process which involves the *de novo* biosynthesis of proteins, (iii) activation of C fibres (sensory system), and (iv) the release of endogenous platelet activating factor (Perretti & Flower, 1993; Perretti *et al.*, 1993a). In addition, it is likely that the expression of the endogenous murine analogues of E-selectin and IL-8 plays an important role in the recruitment of PMNs afforded by IL-1β (Huber *et al.*, 1991). Moreover, inhibition of leucocyte rolling on the endothelium by fucoidin (Harris *et al.*, 1995) or of neutrophil adhesion to the endothelium by a specific antibody to the β_2-integrin CD11b (Perretti *et al.*, 1993b) abolishes the migration of PMNs into the air-pouch caused by either IL-1β or IL-8.

What, then, are the mechanisms by which IL-4 or IL-10 attenuate the migration of PMNs into a specific inflammatory site caused by IL-1β *in vivo*? Clearly, IL-4 or IL-10 do not cause a non-specific reduction in neutrophil function, for neither IL-4 nor IL-10 had any significant effect on (i) the mild degree of PMN infiltration caused by injection of vehicle alone, and (ii) the infiltration of PMNs caused by IL-8. The inhibition by IL-4 and IL-10 of the migration of PMNs observed in this study is also not due to a prevention by these cytokines of the up-regulation of the β_2-integrin CD11b on the membrane of murine PMNs, as (i) neither IL-4 nor IL-10 attenuated the up-regulation of CD11b caused by IL-8 *in vitro*, and (ii) the migration of PMNs induced by IL-8 was abolished by pretreatment of mice with a monoclonal antibody to CD11b (Perretti *et al.*, 1993b), but not by systemic administration of IL-4 or IL-10 (this study). Although the above results strongly suggest

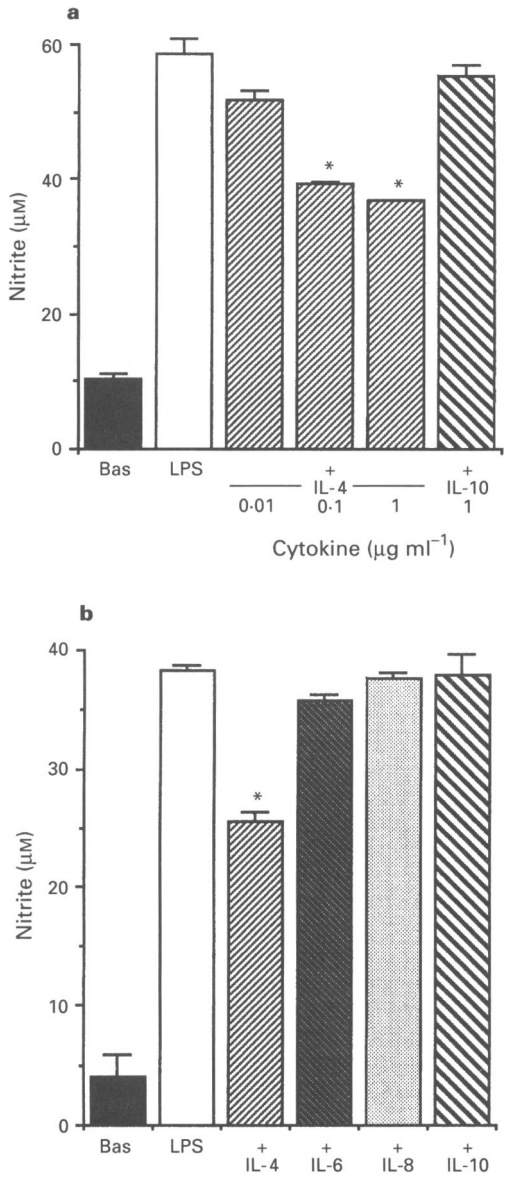

Figure 4 Different effect interleukin-4 (IL-4) and IL-10 effect on NO production from peritoneal Mø and J774.2 cell line *in vitro*. (a) IL-4 or IL-10 was added to 96-well plates containing peritoneal Mø at the reported concentrations 10 min before the addition of LPS (1 μg ml^{-1}). (b) Cytokines were added to J774.2 cells at the final concentration of 1 μg ml^{-1} 10 min before the addition of LPS (1 μg ml^{-1}). In both cases the concentration of nitrite in the supernatant was measured 20 h after LPS addition with the Griess reaction as described in the Methods section. Basal release (Bas) from unstimulated cells is also shown. Values are mean ± s.e.mean of quadruplicate measurements from 5–6 mice per group. $^*P < 0.05$ vs. respective LPS group.

that neither IL-4 nor IL-10 prevent the activation of PNMs by IL-8, one could argue that these cytokines may prevent the release of IL-8 *in vivo*. Indeed, IL-4 attenuates the release of IL-8 caused by endotoxin in porcine alveolar macrophages (Line *et al.*, 1994) and human neutrophils (Wertheim *et al.*, 1993) and prevents the LPS-induced increase in mRNA for IL-8 in alveolar macrophages (Roberts *et al.*, 1993). Similarly, IL-10 prevents the release of IL-8 caused by endotoxin in human monocytes (De Waal Malefyt *et al.*, 1991) as well as in human neutrophils stimulated with IL-1β (Cassatella *et al.*, 1993). As the formation of endogenous IL-8 is triggered by IL-1 (Huber *et al.*, 1991), it is indeed conceivable that the inhibition by IL-4 or IL-10 of the migration of PMNs caused by IL-1β in our model is secondary to the inhibition of the release of IL-8, or better, a murine analogue of this chemokine like macrophage-inflammatory protein-2, afforded by these anti-inflammatory cytokines. In addition, IL-4 or IL-10 may prevent the migration of PMNs caused by IL-1β by enhancing the formation of the endogenous IL-1 receptor antagonist (IL-1ra). Indeed, IL-4 increases the expression of IL-1ra mRNA and protein in human monocytes activated with LPS (Vannier *et al.*, 1992; Wong *et al.*, 1993). IL-10 also markedly potentiates the release of IL-1ra from PMNs stimulated with LPS by increasing the half life of IL-1ra mRNA (stabilisation of mRNA) (Cassatella *et al.*, 1994).

In contrast to IL-4 or IL-10, treatment of mice with IL-6 or IL-8 did not modify PMN accumulation into the air-pouches in response to IL-1β. Whereas the lack of effect of IL-6 was not entirely surprising (Dinarello, 1991), our demonstration that systemic administration of IL-8 did not affect the migration of PMNs elicited by IL-1β was unexpected. The inability of IL-8 to modify PMN accumulation in the mouse is not related to the species used, because human IL-8 attracts PMN in the mouse air-pouch (Perretti *et al.*, 1994) and systemic treatment of mice with this cytokine causes transient alteration in the number of circulating PMN (Harris *et al.*, 1995). Our hypothesis that treatment of mice with IL-8 would result in a reduction of IL-1-induced PMN migration was based on a study which demonstrated that the systemic treatment of rabbits with IL-8 reduces PMN infiltration in response to intradermal injection of several stimuli, including IL-1β (Hechtman *et al.*, 1991). It was proposed that this effect of IL-8 was due to shedding of L-selecting from the PMN plasma membrane resulting in the abolition of cell rolling onto the endothelium. However, a subsequent study has shown that intravenous administration of IL-8 in rabbits does not alter the expression of L-selectin on the surface of PMNs (Ley *et al.*, 1993), and a more recent study has shown that leucocyte rolling is mainly sustained by E-selectin during IL-1-induced inflammation (Olofsson *et al.*, 1994). Our data with the murine air-pouch model are consistent with the latter observations and support the view that IL-8 plays a pro-inflammatory role during the extravasation of PMNs in inflammation (Harada *et al.*, 1993; Broddus *et al.*, 1994).

As endothelium-derived NO inhibits the adhesion of leucocytes to the endothelium (Kubes *et al.*, 1993), we have investigated the role of NO in the migration of PMNs caused by IL-1β *in vivo*. We demonstrate that the formation of NO by neither the constitutive NOS (cNOS) in the endothelium nor by the inducible isoform of NOS (iNOS) influences the migration of PMNs caused by IL-1β *in vivo*, as (i) the degree of neutrophil migration caused by IL-1β was not affected by local administration of L-NMMA, an L-arginine analogue which inhibits both cNOS and iNOS activity (systemic treatment being hampered by the indirect effect that alterations in blood pressure would exert on PMN migration); and (ii) the neutrophil migration caused by IL-1β was reduced by treatment of mice with IL-10, but IL-10 did not affect the induction of iNOS caused by LPS in peritoneal murine macrophages *ex vivo*. Thus, we can exclude the possibility that the inhibition by IL-4 or IL-10 of the migration of PMNs induced by IL-1 is due to any effects of these cytokines on NO formation.

Nevertheless, the finding that pretreatment of mice with IL-4, but not with IL-10, attenuates the formation of nitrite and, hence, iNOS induction, from peritoneal macrophages obtained from mice treated with LPS is interesting in itself. The notion that IL-4, but not IL-10 inhibits iNOS induction, is strongly supported by our findings *in vitro*: short pretreatment of peritoneal macrophages *in vitro* with IL-4, but not with IL-10, prevented the increase in nitrite formation caused by LPS in these cells. The increase in nitrite formation caused by LPS in cultured J774.2 macrophages was also largely prevented by pretreatment of these cells with IL-4, but not by pretreatment with IL-10, IL-6 or IL-8. Thus, we demonstrate that IL-4 inhibits the induction of iNOS activity in macrophages of animals treated with LPS *in vivo*. The induction of iNOS caused by systemic administration of LPS to mice is mediated by the cytokines IL-1, interferon-γ and TNF-α (Green & Nacy, 1993; Cunha *et al.*, 1994). As IL-4 prevents the release of TNF-α and

up-regulates the expression of the endogenous IL-1 receptor antagonist (IL-1ra), it is conceivable that the inhibition of iNOS induction afforded by IL-4 is secondary to the prevention of iNOS induction caused by TNF-α and IL-1.

Our finding that the pretreatment of primary (peritoneal) and cultured murine macrophages (J774.2 cells) with IL-10 did not reduce the formation of nitrite caused by LPS was not expected, as pretreatment *in vitro* with IL-10 (i) causes a moderate reduction of the formation of iNOS mRNA caused by endotoxin in J774.2 macrophages (Chesrown *et al.*, 1994), (ii) attenuates the induction of iNOS in murine macrophages activated with interferon-γ, and (iii) reduces the induction of iNOS in murine endothelial cells (Schneemann *et al.*, 1993). On the other hand, IL-10 also enhances the expression of mRNA for iNOS as well as iNOS activity in murine macrophages activated with interferon-γ or TNF-α (Corradin *et al.*, 1993). Interestingly, in cultured microvascular endothelial cells, inhibition of iNOS induction by IL-10 is not seen when the cytokine is added simultaneously with this stimulus, but IL-10 attenuates the induction of iNOS when the cytokine is given 16 h before this stimulus to endothelial cells (Schneemann *et al.*, 1993). In accordance with this finding, we report here that administration of IL-10 immediately prior to LPS does not affect the formation of nitrite caused by endotoxin in either primary or cultured murine macrophages.

In conclusion, we demonstrate that the systemic administration of IL-4 or IL-10 to mice selectively attenuates the IL-1β-induced recruitment (migration) of PMNs into a specific inflammatory site *in vivo*. Ths mechanisms by which IL-4 or IL-10 inhibit IL-1-induced PMN migration *in vivo* are not entirely clear, but may include (i) an enhanced formation of IL-1ra or (ii) prevention of the release of an endogenous IL-8 analogue. We speculate that IL-4 or IL-10 may have beneficial effects in more complex inflammatory conditions by affecting the eliciting of PMN caused by IL-1. Furthermore, we show that IL-4, but not IL-10, prevents the LPS-stimulated formation of NO (probably by iNOS) *in vitro* and *in vivo* suggesting that IL-4 may be useful in the therapy of diseases associated with an overproduction of NO due to overexpression of iNOS.

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