

Divergent mechanisms of action of the inflammatory cytokines interleukin 1- β and tumour necrosis factor- α in mouse cremasteric venules

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1 Protein synthesis dependency and the role of endogenously generated platelet activating factor (PAF) and leukotriene B₄ (LTB₄) in leukocyte migration through interleukin-1 β (IL-1 β)- and tumour necrosis factor- α (TNF α)-stimulated mouse cremasteric venules was investigated using established pharmacological interventions and the technique of intravital microscopy.

2 Based on previously obtained dose-response data, 30 ng rmIL-1 β and 300 ng rmTNF α were injected intrascrotally (4 h test period) to induce comparable levels of leukocyte firm adhesion and transmigration in mouse cremasteric venules.

3 Co-injection of the mRNA synthesis inhibitor, actinomycin D (0.2 mg kg⁻¹), with the cytokines significantly inhibited firm adhesion (49 ± 13.6%) and transmigration (67.2 ± 4.2%) induced by IL-1 β , but not TNF α .

4 *In vitro*, TNF α (1–100 ng ml⁻¹), but not IL-1 β , stimulated L-selectin shedding and increased β_2 integrin expression on mouse neutrophils, as quantified by flow cytometry.

5 The PAF receptor antagonist, UK-74,505 (modipafant, 0.5 mg kg⁻¹, i.v.), had no effect on adhesion induced by either cytokine, but significantly inhibited transmigration induced by IL-1 β (66.5 ± 4.5%).

6 The LTB₄ receptor antagonist, CP-105,696 (100 mg kg⁻¹, p.o.), significantly inhibited both IL-1 β induced adhesion (81.4 ± 15.2%) and transmigration (58.7 ± 7.2%), but had no effect on responses elicited by TNF α . Combined administration of the two antagonists had no enhanced inhibitory effects on responses induced by either cytokine.

7 The data indicate that firm adhesion and transmigration in mouse cremasteric venules stimulated by IL-1 β , but not TNF α , is protein synthesis dependent and mediated by endogenous generation of PAF and LTB₄. Additionally, TNF α but not IL-1 β , can directly stimulate mouse neutrophils *in vitro*. The findings provide further evidence to suggest divergent mechanisms of actions of IL-1 β and TNF α , two cytokines often considered to act *via* common molecular/cellular pathways.

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Abbreviations: IL-1 β , interleukin-1 β ; LTB₄, leukotriene B₄; mAb, monoclonal antibody; PAF, platelet activating factor; PECAM-1, platelet-endothelial cell adhesion molecule-1; TNF α , tumour necrosis factor α

Introduction

IL-1 β and TNF α are multifunctional cytokines with potent immunomodulatory and pro-inflammatory properties (Dinarello, 2000; Kunkel *et al.*, 1991; Larrick & Kunkel, 1988). These cytokines can be generated during both acute and chronic phases of an inflammatory response and as such have been implicated in the pathogenesis of a variety of inflammatory disease states, such as rheumatoid arthritis, atherosclerosis and inflammatory bowel disease (Brennan *et al.*, 1992; Cominelli *et al.*, 1990; Cominelli & Dinarello, 1989; Fiotti *et al.*, 1999; Haraoui *et al.*, 2000; Huang *et al.*, 2001; Kollias *et al.*, 1999; Maini *et al.*, 1995; Ross, 1993; Schiff, 2000). Both cytokines can enhance the adhesiveness of endothelial cells for leukocytes *in vitro* (Bevilacqua *et al.*,

1987; 1989; Bochner *et al.*, 1991; Dustin *et al.*, 1986; Osborn *et al.*, 1989; Pober *et al.*, 1986) and are potent inducers of leukocyte accumulation *in vivo* (Cybulsky *et al.*, 1989; Issekutz *et al.*, 1981; Nourshargh *et al.*, 1989; Perretti & Flower, 1994; Rampart *et al.*, 1989a; Rampart & Williams, 1988; Sanz *et al.*, 1995; 1997), responses that are largely attributed to the ability of the cytokines to induce the expression of endothelial cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 (Bevilacqua *et al.*, 1987; 1989; Bochner *et al.*, 1991; Dustin *et al.*, 1986; Osborn *et al.*, 1989; Pober *et al.*, 1986). The pro-inflammatory properties of IL-1 β and TNF α also includes their ability to stimulate the release of other inflammatory mediators, such as chemokines, PAF and eicosanoids (Barnes *et al.*, 1998; Brain & Williams, 1990; Bussolino *et al.*, 1988; Huber *et al.*, 1991; Strieter *et al.*, 1989). Given their overlapping properties, such as those

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exemplified above, IL-1 β and TNF α are frequently used in an interchangeable manner in both *in vitro* and *in vivo* inflammatory models and are commonly considered to exert their pro-inflammatory effects *via* similar molecular and cellular pathways.

There is, however, evidence for diverse modes of actions of IL-1 β and TNF α . For example, *in vitro* studies have shown that TNF α can induce human neutrophil degranulation and generation of superoxide anions from adherent leukocytes (Nathan & Sporn, 1991) and stimulate rapid adhesion of human and murine neutrophils to cultured endothelial cells or protein-coated plates, respectively (Gamble *et al.*, 1985; Thompson *et al.*, 2001). No such stimulatory effects have been reported for IL-1 β . *In vivo*, Rampart *et al.* (1989b) demonstrated that intradermal injection of IL-1 β induced a slow (maximum within 3–4 h) and protein synthesis dependent neutrophil accumulation response in rabbit skin, whilst neutrophil accumulation induced by TNF α was rapid (maximum within 30 min) and protein synthesis independent. Furthermore, we have recently found that neutrophil transmigration through mouse cremasteric venules induced by IL-1 β , but not TNF α , is suppressed in platelet endothelial cell adhesion molecule-1 (PECAM-1) deficient mice (Thompson *et al.*, 2001). The aim of the present study was to extend these observations by investigating the existence of further differences in the mechanisms by which these cytokines elicit leukocyte migration from the vascular lumen to the extravascular tissue. For this purpose, we have used well-characterized pharmacological interventions and the technique of intravital microscopy to investigate the protein synthesis dependency and the role of endogenously generated PAF and LTB $_4$ in the different stages of leukocyte migration through mouse cremasteric venules stimulated by IL-1 β and TNF α . The results provide further evidence for the divergent mechanisms of action of these cytokines and demonstrate a clear involvement for endogenously generated LTB $_4$ and PAF in IL-1 β -induced, but not TNF α -induced, leukocyte firm adhesion and transmigration, respectively.

Methods

Animals

Male C57BL/6 mice (20–25 g, Harlan-Olac, Bicester, U.K.) were used for all experiments.

Intravital microscopy

Intravital microscopy was used to observe cytokine elicited leukocyte responses within mouse cremasteric venules, following intrascrotal (*i.s.*) administration of IL-1 β (30 ng in 400 μ l) or TNF α (300 ng in 400 μ l), as compared to responses in animals injected with *i.s.* saline (400 μ l). To assess the role of protein synthesis, the RNA transcription inhibitor, actinomycin D (Act D, 0.2 mg kg $^{-1}$), was co-administered *i.s.* with saline or cytokines. The roles of endogenously generated PAF and LTB $_4$ in cytokine-induced responses were investigated by using the selective PAF and LTB $_4$ receptor antagonists, UK-74,505 (modipafant; Alabaster *et al.*, 1991) and CP-105,696 (Koch *et al.*, 1994), respectively. UK-74,505, initially made up at 15 mg ml $^{-1}$ in

0.1 M hydrochloric acid and then diluted with saline, was administered intravenously (*i.v.*) at the dose of 0.5 mg kg $^{-1}$ 10 min before *i.s.* injection of cytokines or saline control. CP-105,696 was administered as an oral suspension in 0.5% carboxymethyl cellulose at the dose of 100 mg kg $^{-1}$ 19 h prior to the *i.s.* injection of cytokine or saline. Some groups of mice were pre-treated with both UK-74,505 and CP-105,696 or their corresponding vehicles.

Four hours after cytokine injection, the mice were prepared for intravital microscopy as previously described (Ley *et al.*, 1995; Thompson *et al.*, 2001). After induction of anaesthesia with ketamine (100 mg kg $^{-1}$) and xylazine (10 mg kg $^{-1}$) by intraperitoneal (*i.p.*) injection, animals were maintained at 37°C on a custom-built heated Perspex microscope stage. The cremaster muscle was exteriorized through an incision in the scrotum and one testis gently drawn out to allow the cremaster muscle to be opened and pinned out flat over the optical window within the microscope stage. The tissue was kept warm and moist throughout each experiment by superfusion of warmed Tyrode's balanced salt solution. Leukocyte-endothelial cell interactions were observed on an upright fixed-stage microscope (Axioscop FS, Carl Zeiss, Welwyn Garden City, U.K.) fitted with water immersion objectives. Video recordings were made with a chilled colour video camera (C5810-01, Hamamatsu Photonics, Enfield, U.K.) and S-VHS videocassette recorder (AG-MD830E, Panasonic, Bracknell, U.K.). Leukocyte responses of rolling, firm adhesion and transmigration in postcapillary venules of 20–40 μ m diameter were quantified as previously detailed (Nourshargh *et al.*, 1995). Briefly, leukocyte rolling flux was quantified as the number of rolling cells moving past a fixed point on the venular wall per minute, averaged over 5 min. Firmly adherent leukocytes were considered as those remaining stationary for at least 30 s within a given 100 μ m vessel segment. Transmigrated leukocytes were quantified as those in the extravascular tissue within 50 μ m of the 100 μ m vessel segments quantified. In each animal, responses in several vessel segments (3–5), from multiple vessels (3–5), were studied and averaged.

In selected experiments, microvascular centreline erythrocyte velocity and peripheral leukocyte counts were determined. With respect to the former, centreline velocity was quantified using a Doppler velocimeter (Microvessel Velocity OD-RT, CircuSoft Instrumentation LLC, Hockessin, DE, U.S.A.) and in each animal, values from at least three vessels were determined. With respect to leukocyte counts, blood samples were collected from tail veins into acid citrate dextrose (ACD) and total and differential leukocyte counts were carried out using a haemocytometer following the staining of blood samples with Kimura.

Flow cytometry

Whole blood was taken by cardiac puncture from donor animals and diluted 50:50 with anti-coagulant (20 mM EDTA in 1 \times PBS). The blood was incubated for 20 min at room temperature with a range of concentrations (1–100 ng ml $^{-1}$) of IL-1 β and TNF α , PMA (100 ng ml $^{-1}$) as a positive control and with PBS (supplemented with 1 mM Ca $^{2+}$ and Mg $^{2+}$) used as vehicle control. After washing, samples were incubated with primary monoclonal antibody (mAb) directed against either L-selectin (MEL-14, IgG2a,

PharMingen, Oxford, U.K.) or β_2 integrin (GAME-46, IgG1, PharMingen, Oxford, U.K.) on ice and the binding of test mAbs was detected with a F(ab')₂ FITC-conjugated goat anti-rat IgG (Serotec Ltd, Oxford, U.K.) antibody. Isotype-matched controls (PharMingen, Oxford, U.K.) were also used in all experiments. Erythrocytes were lysed by treating the samples with a FACS lysing reagent (Becton Dickinson, Oxford, U.K.) before analysing the samples on an EPICS XL flow cytometer (Beckman Coulter, High Wycombe, U.K.). Gating on neutrophils was based on characteristic forward and side scatter parameters as well as the binding of the mAb, Gr-1 (PharMingen, Oxford, U.K.). The ratio of fluorescence intensities associated with the binding of primary mAbs and isotype-matched control mAbs was used to express specific binding of test mAbs in terms of relative fluorescence intensity (RFI).

Drugs and reagents

The following reagents were purchased: recombinant murine IL-1 β and TNF α (Serotec, Oxford, U.K.); ketamine (Ketalar, Parke-Davis, Eastleigh, U.K.); xylazine (Rompun, Bayer, Bury St. Edmunds, U.K.); LTB₄ (Calbiochem, U.K.); ACD (Baxter Healthcare Ltd, Newbury, U.K.); MEL-14, GAME-46, Gr-1 and isotype matched controls (PharMingen, Oxford, U.K.); FACS lysing reagent (Becton Dickinson, Oxford, U.K.); F(ab')₂ FITC-conjugated goat anti-rat IgG (Serotec Ltd, Oxford, U.K.). UK-74,505 [4-(2-chlorophenyl)-1,4-dihydro-3-(ethoxycarbonyl)-6-methyl-2-[4-(2-methylamidazo[4,5-c-pyrid-1-yl]-5-[N-(2-pyridyl)carbamoyl]pyridine)] (modipafant; Alabaster *et al.*, 1991; Cooper *et al.*, 1992; Pons *et al.*, 1993) and CP-105,696 [(+)-1-(3S,4R)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]-cyclopentane carboxylic acid] (Koch *et al.*, 1994; Showell *et al.*, 1996) were gifts from Pfizer, Sandwich, U.K., and Pfizer, Groton, CT, U.S.A., respectively. All other reagents were purchased from Sigma-Aldrich (Poole, U.K.).

Statistical analysis

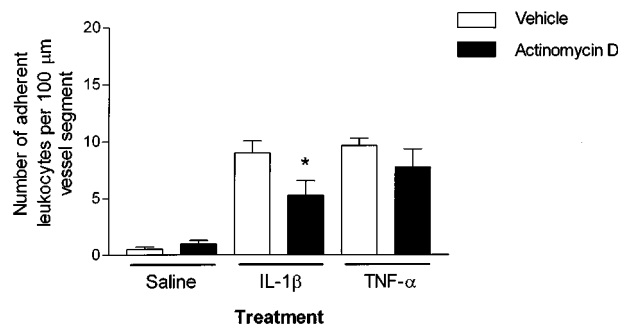
Results are given as mean \pm s.e.mean, unless otherwise stated, and analysed using either an unpaired *t*-test or one-way ANOVA and Newman-Keuls post test with $P < 0.05$ taken as significant (Prism, GraphPad Software, San Diego, CA, U.S.A.).

Results

Effect of actinomycin D on leukocyte responses elicited by IL-1 β and TNF α

Based on previous dose-response data (Thompson *et al.*, 2001), IL-1 β and TNF α were administered intrascrotally at the doses of 30 ng and 300 ng respectively and 4 h later leukocyte responses within mouse cremasteric venules were observed and quantified by intravital microscopy. Figure 1 shows that at these doses the cytokines elicited comparable and significant levels of leukocyte firm adhesion and transmigration as compared to animals injected with intrascrotal saline. No significant change in leukocyte rolling flux was observed with IL-1 β or TNF α as compared to

a) Adhesion



b) Transmigration

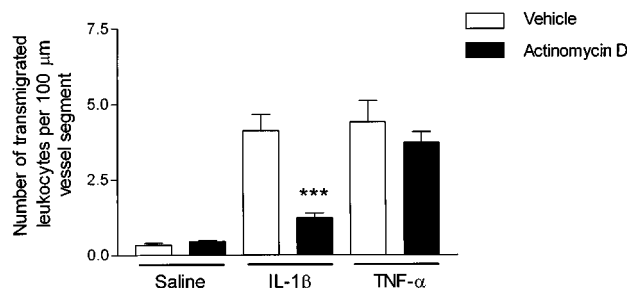


Figure 1 Effect of the protein synthesis inhibitor, actinomycin D, on IL-1 β - or TNF α -induced leukocyte adhesion and transmigration in mouse cremasteric venules. Mice were injected intrascrotally with saline (400 μ l), IL-1 β (30 ng in 400 μ l saline) or TNF α (300 ng in 400 μ l saline) with or without actinomycin D (0.2 mg kg⁻¹) and leukocyte firm adhesion (a) and transmigration (b), per 100 μ m vessel segment, were quantified by intravital microscopy 4 h later, as detailed in Methods. Results are presented as mean \pm s.e.mean for $n = 5$ mice per group. Statistically significant differences (one-way ANOVA and Newman-Keuls post-test) between control and drug-treated groups are shown by asterisks, * $P < 0.05$ and *** $P < 0.001$.

saline-injected mice (data not shown). The role of local protein synthesis in leukocyte responses induced by the cytokines was investigated using the transcription inhibitor, actinomycin D. When co-administered with IL-1 β , actinomycin D (0.2 mg kg⁻¹), significantly suppressed leukocyte firm adhesion ($49.0 \pm 13.6\%$; $P < 0.05$) and transmigration ($67.2 \pm 4.2\%$; $P < 0.001$) as compared to responses elicited by the cytokine alone (Figure 1a,b). In contrast, actinomycin D had no effect on TNF α -induced leukocyte adhesion and transmigration (Figure 1a,b). Local administration of actinomycin D had no effect on blood flow within the cremaster muscle venules or on peripheral blood leukocyte counts (Table 1).

Effect of IL-1 β and TNF α on cell surface expression of L-selectin and β_2 integrins on mouse neutrophils

Since a potential explanation for the findings of the above experiments is that TNF α may be directly stimulating mouse neutrophils, the aim of the following studies was to address this possibility by investigating the effect of TNF α on regulation of expression of the adhesion molecules L-selectin and β_2 integrin on mouse neutrophils. For this purpose, mouse whole blood was stimulated with IL-1 β and TNF α

Table 1 Venular blood flow measurements and total blood leukocyte counts in mice treated with actinomycin D and the PAF and LTB $_4$ receptor antagonists, when used alone or in combination

	(1) Control		(2) Act. D		(3) CP-105,696		(4) UK-74,505		(5) CP+UK	
	IL-1 β	TNF α	IL-1 β	TNF α	IL-1 β	TNF α	IL-1 β	TNF α	IL-1 β	TNF α
Venular blood flow (mm s $^{-1}$)	1.6 \pm 0.7	2.0 \pm 0.7	2.6 \pm 0.5	2.4 \pm 0.5	1.8 \pm 0.4	2.2 \pm 0.4	2.1 \pm 0.4	1.7 \pm 0.3	1.4 \pm 0.2	1.4 \pm 0.3
Total blood leukocytes ($\times 10^6$ ml $^{-1}$)	3.8 \pm 0.1	3.7 \pm 0.1	3.8 \pm 0.0	3.3 \pm 0.1	3.9 \pm 0.9	4.8 \pm 0.8	3.7 \pm 0.5	3.6 \pm 0.3	3.7 \pm 0.3	4.5 \pm 0.9

Five groups of mice, all injected intrascrotally with either IL-1 β (30 ng) or TNF α (300 ng), were analysed. The five groups were treated with (1) vehicle, (2) actinomycin D (Act. D, 0.2 mg kg $^{-1}$ i.s.), (3) the LTB $_4$ receptor antagonist CP-105,696 (100 mg kg $^{-1}$ p.o.), (4) the PAF receptor antagonist UK-74,505 (0.5 mg kg $^{-1}$ i.v.), or (5) both antagonists. Venular blood flow and total leukocyte counts were determined as detailed in Methods. The results are mean \pm s.e.mean from $n=5$ mice and expressed as mm s $^{-1}$ and $\times 10^6$ leukocytes ml $^{-1}$ for venular blood flow and total blood leukocyte counts, respectively.

(1–100 ng ml $^{-1}$), for 20 min, and cell surface expression of L-selectin and β_2 quantified by indirect immunofluorescent staining and flow cytometry, as detailed in Methods. The concentrations of the cytokines employed were based on previous investigations from our own group (Thompson *et al.*, 2001) and other researchers (Bochner *et al.*, 1991; Brandt *et al.*, 1992; Christofidou-Solomidou *et al.*, 1997; Takahashi *et al.*, 2001). The results in Table 2 and Figure 2 clearly demonstrate that TNF α dose-dependently enhanced cell surface expression of β_2 integrin (significant at 10 ng ml $^{-1}$) and the shedding of L-selectin (significant for all concentrations investigated). The potent leukocyte stimulant, PMA, gave a similar profile of responses to that observed with TNF α . In contrast, IL-1 β -stimulated samples failed to show any significant change in cell surface expression of molecules as compared to unstimulated samples.

Effect of the PAF receptor antagonist UK-74,505 on leukocyte responses elicited by IL-1 β and TNF α

To further investigate potential differences in mechanisms of actions of the two cytokines, the role of endogenously generated PAF in leukocyte responses elicited by IL-1 β and TNF α was investigated using the selective PAF receptor antagonist UK-74,505 (Alabaster *et al.*, 1991; Cooper *et al.*, 1992). Based on previous studies in mice (Miotla *et al.*, 1998), rats (Sanz *et al.*, 1995) and rabbits (Pons *et al.*, 1993), animals were injected intravenously with this antagonist at the dose of 0.5 mg kg $^{-1}$. In mice treated with UK-74,505, no significant effects on leukocyte firm adhesion induced by either cytokine were observed (Figure 3a). However, leukocyte transmigration elicited by IL-1 β was significantly inhibited (66.5 \pm 4.5%, $P<0.05$), while TNF α -induced transmigration was unaffected (Figure 3b).

Effect of the LTB $_4$ receptor antagonist CP-105,696 on leukocyte responses elicited by IL-1 β and TNF α

The LTB $_4$ receptor antagonist, CP-105,696 (Koch *et al.*, 1994; Showell *et al.*, 1996), was used to investigate the role of endogenously generated LTB $_4$ in IL-1 β - and TNF α -induced leukocyte responses. In mice treated with this antagonist (100 mg kg $^{-1}$, p.o., 19 h prior to intrascrotal injection of cytokines), leukocyte firm adhesion and transmigration induced by IL-1 β was significantly inhibited by 81.4 \pm 15.2% ($P<0.05$) and 58.7 \pm 7.2% ($P<0.05$), respectively (Figure 4a,b). In contrast, CP-105,696 had no effect on

Table 2 Expression of L-selectin and β_2 integrin on cytokine-stimulated mouse neutrophils

		Relative fluorescence intensity	
		L-selectin	β_2
Unstimulated		202.3 \pm 44.3	83.7 \pm 13.0
TNF α	1 ng ml $^{-1}$	42.7 \pm 36.6*	125.9 \pm 18.3
	10	33.6 \pm 26.3*	134.1 \pm 13.9*
	100	33.7 \pm 27.3*	147.1 \pm 35.7
IL-1 β	100 ng ml $^{-1}$	227.2 \pm 79.4	102.1 \pm 16.8
PMA	10 ng ml $^{-1}$	31.9 \pm 27.1*	287.1 \pm 73.2*

Whole blood was collected by cardiac puncture from wild-type mice and treated with PBS (supplemented with Ca $^{2+}$ and Mg $^{2+}$), IL-1 β , TNF α or the positive control, PMA, for 20 min at room temperature. Samples were then labelled with mAbs directed against mouse L-selectin and β_2 integrin and analysed by flow cytometry, as detailed in Methods. Neutrophils were identified by their characteristic forward and side scatter parameters as well as the binding of the mAb, Gr-1. The ratio of fluorescence intensities associated with the binding of primary mAbs and isotype-matched control mAbs was used to express specific binding of test mAbs in terms of relative fluorescence intensity (RFI). The results are mean \pm s.e.mean from $n=3-4$ separate experiments and analysed using an unpaired t -test ($*P<0.05$).

leukocyte responses induced by TNF α (Figure 4a,b). Additional analysis of the data for IL-1 β , involving calculation of the ratio of adherent to transmigrated leukocytes as a measure of the fraction of adherent leukocytes that transmigrated, suggested that the inhibitory effect of the CP-105,696 compound was most likely at the stage of adhesion and not transmigration (a ratio of 1.76 for CP-105,696-treated animals compared with 2.00 for control-treated animals). Of importance, in cremasteric venules stimulated with IL-1 β , topical LTB $_4$ could induce a further increase in leukocyte responses in control mice but not CP-105,696-treated animals, demonstrating that the dosing regime employed for the administration of the antagonist was effective at abrogating LTB $_4$ elicited responses in this model (data not shown).

Effect of combined PAF and LTB $_4$ receptor antagonist treatment on cytokine-induced responses

In a final series of experiments, the effect of combined treatment of mice with both the PAF and LTB $_4$ receptor antagonist, using the dosing regimes detailed above, was

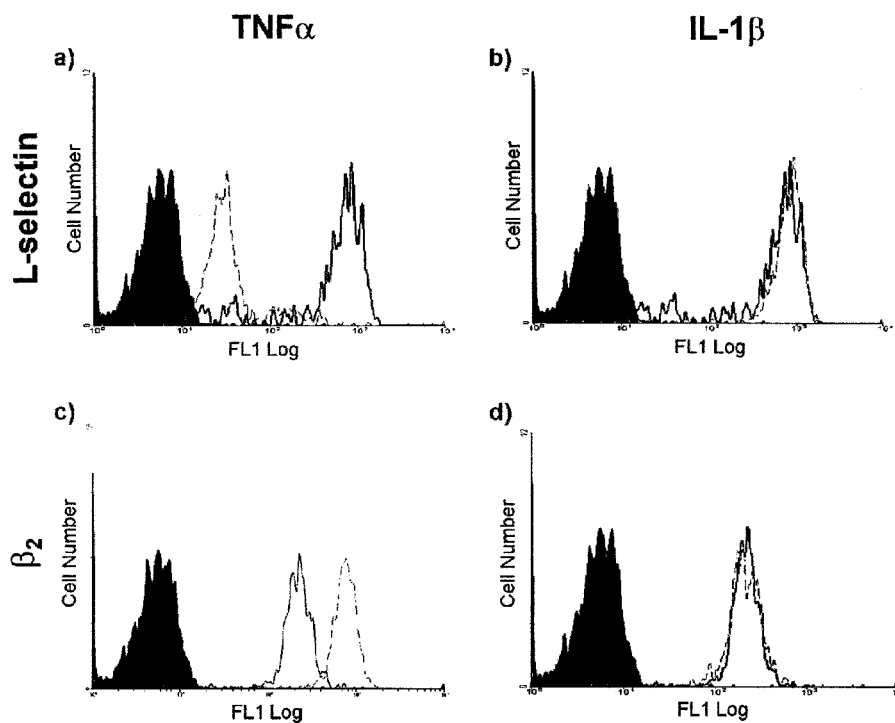


Figure 2 Effect of TNF α and IL-1 β on expression of L-selectin and β_2 integrin on mouse neutrophils. Figure shows representative fluorescence histograms comparing cell surface expressions of L-selectin and β_2 integrins on unstimulated and cytokine stimulated mouse neutrophils. Briefly, blood samples were collected by cardiac puncture and stimulated with the cytokines (both at 100 ng ml $^{-1}$) at room temperature for 20 min prior to labelling with isotype-matched control mAbs or mAbs directed against L-selection (a/TNF α and b/IL-1 β) and β_2 (c/TNF α and d/IL-1 β), followed by incubation with a FITC-conjugated F(ab') $_2$ mAb. Samples were analysed by flow cytometry as described in Methods. The filled tracings are from blood samples incubated with an isotype-matched control mAb, solid lines represent unstimulated cells and dashed lines represent stimulated cells. The histograms represent the cell number relating to the expression of the molecule examined and are representative of four separate experiments.

investigated. For clarity, the data from these experiments are compared with data obtained in parallel experiments when each antagonist was used alone, and expressed as percentage inhibition of cytokine-induced responses. Hence, as previously found, Figure 5a shows that IL-1 β -induced leukocyte firm adhesion was significantly inhibited by the LTB $_4$ receptor antagonist, CP-105,696, but not the PAF receptor antagonist, UK-74,505. Furthermore, treatment of mice with both antagonists resulted in a similar inhibition to that observed with the LTB $_4$ receptor antagonist alone. The combined treatment of mice with the two antagonists also led to a similar level of inhibition of IL-1 β -induced transmigration as that observed with either antagonist alone (Figure 5b). The combined administration of the PAF and LTB $_4$ receptor antagonists, as found when the antagonists were administered alone, had no effect on responses induced by TNF α (data not shown). Of relevance to the whole study, treatment of mice with the antagonists, when administered alone or in combination, had no effect on blood flow or leukocyte blood counts as compared with vehicle-treated control mice (Table 1).

Discussion

The pro-inflammatory cytokines, IL-1 β and TNF α , have many overlapping properties and as such are often used

within experimental inflammatory models interchangeably. However, a number of studies have also demonstrated key differences in the mechanisms of action of these cytokines suggesting divergence in their molecular and cellular targets (Rampart *et al.*, 1989b; Nathan & Sporn, 1991; Thompson *et al.*, 2001). The aim of the present study was to further investigate differences in the mechanisms of action of these cytokines by specifically examining the contribution of protein synthesis and the endogenously generated phospholipid mediators, PAF and LTB $_4$, in different stages of leukocyte migration elicited by IL-1 β and TNF α . For this purpose the effect of the transcription inhibitor actinomycin D and PAF or LTB $_4$ receptor antagonists on leukocyte migration through cytokine-stimulated cremasteric venules, as observed by intravital microscopy, was investigated. In addition, the study provides data demonstrating that TNF α , but not IL-1 β , can regulate the cell surface expression of the adhesion molecules L-selectin (down-regulation) and β_2 integrins (up-regulation) on mouse neutrophils. Collectively, the findings suggest that leukocyte adhesion and/or transmigration induced by IL-1 β , but not TNF α , is mediated by endogenously generated proteins and the lipid mediators, PAF and LTB $_4$, whilst TNF α -induced responses may be at least partly mediated *via* direct stimulation of mouse neutrophils. The present findings add to the growing list of important mechanistic differences in the pro-inflammatory actions of these cytokines.

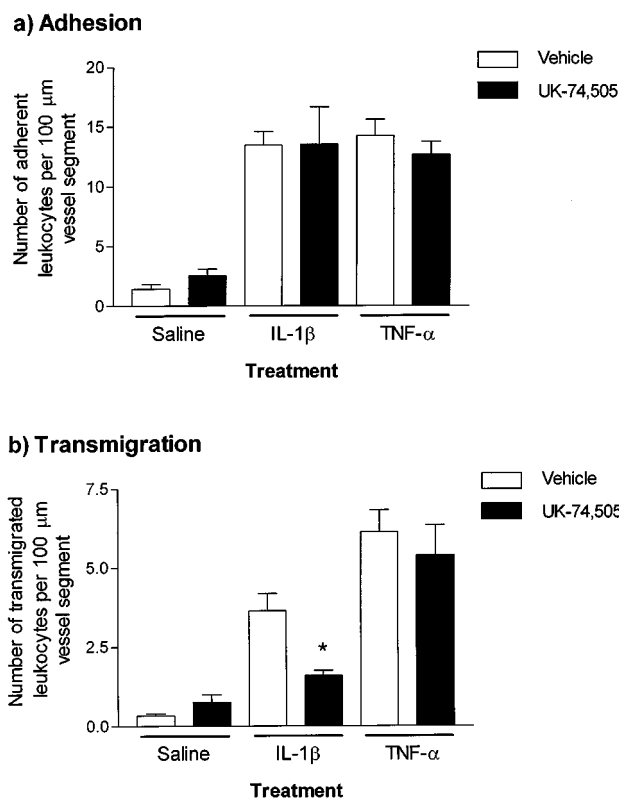


Figure 3 Effect of the PAF receptor antagonist, UK-74,505, on IL-1 β - or TNF α -induced leukocyte adhesion and transmigration in mouse cremasteric venules. Mice were pretreated with vehicle or UK-74,505 (0.5 mg kg⁻¹, i.v.) 10 min before the intrascrotal administration of saline (400 μ l), IL-1 β (30 ng in 400 μ l saline) or TNF α (300 ng in 400 μ l saline). Four hours later, leukocyte responses of adhesion (a) and transmigration (b), per 100 μ m vessel segment, were quantified by intravital microscopy, as detailed in Methods. Values represent mean \pm s.e.mean of $n=5$ mice per group. Statistically significant differences (one-way ANOVA and Newman-Keuls post-test) between vehicle and drug-treated groups are shown by asterisks, * $P<0.05$.

Local administration of IL-1 β or TNF α into the mouse cremaster muscle induced significant leukocyte adhesion and transmigration responses as compared to the local injection of saline, as previously reported (Thompson *et al.*, 2001). The involvement of locally generated proteins in these responses was investigated by using the RNA synthesis inhibitor, actinomycin D. Co-administration of actinomycin D with the cytokines markedly suppressed leukocyte adhesion (49%) and transmigration (67%) induced by IL-1 β , but had no effect on responses elicited by TNF α . The present observations are in agreement with the findings of Rampart and colleagues who reported that the accumulation of ¹¹¹In-neutrophils in rabbit skin in response to IL-1 β , but not TNF α , was protein synthesis dependent (Rampart & Williams, 1988; Rampart *et al.*, 1989b). Interestingly, using a very similar model, Cybulsky *et al.* (1989) found that neutrophil accumulation into rabbit skin induced by both IL-1 β and TNF α was blocked by local administration of protein synthesis inhibitors. The reason for the difference between our findings and those of Rampart and colleagues as compared to the results reported by Cybulsky *et al.* is

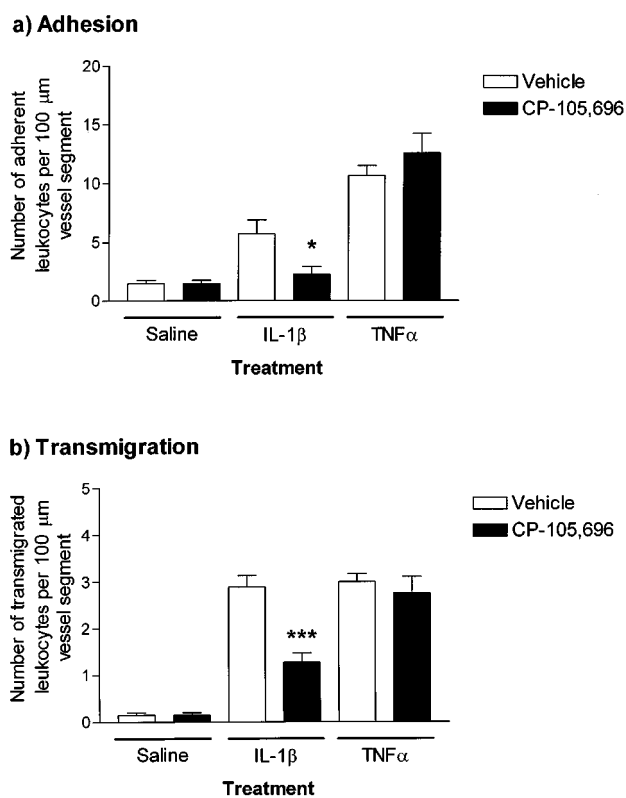


Figure 4 The effect of the LTB₄ receptor antagonist, CP-105,696, on IL-1 β - and TNF α -induced leukocyte adhesion and transmigration in mouse cremasteric venules. Mice were pretreated with vehicle or CP-105,696 (100 mg kg⁻¹, p.o.) 19 h prior to intrascrotal administration of saline (400 μ l), IL-1 β (30 ng in 400 μ l saline) or TNF α (300 ng in 400 μ l saline). Four hours later, leukocyte responses of adhesion (a) and transmigration (b), per 100 μ m vessel segment, were quantified by intravital microscopy, as detailed in Methods. Values represent mean \pm s.e.mean of $n=5$ mice per group. Statistically significant differences (one-way ANOVA and Newman-Keuls post-test) between vehicle and drug-treated groups are shown by asterisks, * $P<0.05$ and *** $P<0.001$.

unclear, but may be related to the species and/or doses of cytokines and inhibitors employed in the different studies.

Since one potential explanation for the above findings is that TNF α , but not IL-1 β , may be directly stimulating mouse neutrophils, as part of the present investigation the effect of the cytokines on regulation of expression of the adhesion molecules L-selectin and β_2 integrins was investigated. The results clearly demonstrated the ability of TNF α to induce rapid shedding of L-selectin and upregulation of β_2 integrins on mouse neutrophils in a dose-dependent manner. In contrast, IL-1 β was found to be without any such effects. Of direct relevance, we have previously found that TNF α , but not IL-1 β , can stimulate the adhesion of murine neutrophils to protein-coated plates (Thompson *et al.*, 2001). Furthermore, Gamble *et al.* (1985) demonstrated that TNF α could stimulate neutrophil adhesion to cultured endothelial cells through effects on both cell types, though the effect on neutrophils was rapid (5 min) and protein synthesis independent whilst the effect on endothelial cells was slow (4 h) and dependent on *de novo* protein generation. Evidence for the ability of TNF α to directly stimulate mouse neutrophils

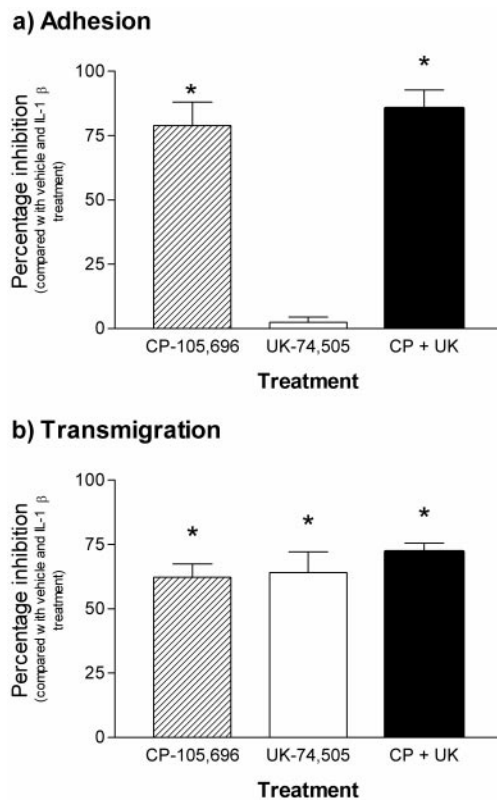


Figure 5 Effect of combined administrations of PAF (UK-74,505) and LTB₄ (CP-105,696) receptor antagonists on IL-1 β -induced leukocyte adhesion and transmigration in mouse cremasteric venules. Mice were pretreated with CP-105,696 (100 mg kg⁻¹, p.o., 19 h prior to the intrascrotal injection of saline or 30 ng IL-1 β), UK-74,505 (0.5 mg kg⁻¹ i.v., 10 min prior to intrascrotal injection of saline or 30 ng IL-1 β), or both antagonists. The results are presented as percentage inhibition of leukocyte adhesion (a) and transmigration (b) as compared to responses obtained in animals treated with appropriate vehicles and intrascrotal IL-1 β , following subtraction of responses detected in mice treated with intrascrotal saline. Values represent mean \pm s.e. mean from $n=5$ mice per group. Statistically significant difference (one-way ANOVA and Newman-Keuls post-test) from vehicle treated groups is shown by asterisks, * $P < 0.05$.

leading to firm adhesion is also suggested by the *in vivo* observations of Thorlacius *et al.* (2000), where it was found that topical application of TNF α to the mouse cremaster muscle induced an LFA-1-dependent increase in leukocyte firm adhesion within a 30 min test-period.

Collectively, the above *in vitro* and *in vivo* findings strongly indicate that in IL-1 β -stimulated cremasteric venules, locally generated proteins are involved in mediating leukocyte adhesion to and migration through endothelial cells. In contrast however, the results suggest that leukocyte responses elicited by TNF α occur independently of local protein synthesis and that in eliciting acute neutrophil migration, the principal target cell of TNF α may be the neutrophil itself or cells resident within the tissue (e.g. mast cells). This property of TNF α may be associated with direct neutrophil stimulatory effects of the cytokine leading to cellular responses such as enhanced adhesion, and/or with the ability of TNF α to stimulate the rapid release of preformed protein

or lipid mediators, such as IL-8 or LTB₄, from adherent neutrophils.

Proteins mediating IL-1 β -induced leukocyte responses may include recognition and/or activation structures such as adhesion molecules (e.g. ICAM-1) (Oppenheimer-Marks *et al.*, 1991) and inflammatory mediators (e.g. the chemokine, IL-8) (McCull & Clark-Lewis, 1999), respectively. In addition, IL-1 β may be inducing the expression of enzymes, such as phospholipase A₂, lyso-PAF acetyl transferase or 5-lipoxygenase, that are responsible for the synthesis of inflammatory mediators, such as PAF and LTB₄, as suggested by *in vitro* studies (Bussolino *et al.*, 1988; Jackson *et al.*, 1993; Nassar *et al.*, 1997; Schwemmer *et al.*, 2001). To investigate the roles of endogenously generated PAF and LTB₄ in leukocyte migration through IL-1 β -stimulated venules, in comparison with responses elicited by TNF α , the selective PAF and LTB₄ receptor antagonists, UK-74,505 (modipafant; Alabaster *et al.*, 1991) and CP-105,696 (Koch *et al.*, 1994), respectively, were used. Pretreatment of mice with the PAF antagonist had no effect on IL-1 β -induced leukocyte adhesion but markedly inhibited leukocyte transmigration elicited by this cytokine (66.5%). These results are in agreement with our previous findings investigating leukocyte migration through rat mesenteric venules as observed by intravital microscopy (Nourshargh *et al.*, 1995) and the *in vitro* findings of Kuijpers *et al.* (1992). Interestingly, this suppression appeared to occur at the level of the endothelium, as determined by transmission electron microscopy (R.D. Thompson and S. Nourshargh, unpublished observations), indicating a role for locally generated PAF in leukocyte migration through endothelial cells as opposed to leukocyte migration through the perivascular basement membrane, induced by IL-1 β . The precise mechanism by which IL-1 β -induced PAF mediates this response is unclear. However, since the majority of the PAF generated by endothelial cells remains cell bound (typically 70–80%) (Bussolino *et al.*, 1986; 1988; Camussi *et al.*, 1987), endothelial cell associated PAF may (1) act in a haptotactic manner to guide the firmly adherent leukocytes through endothelial cell junctions, or (2) may stimulate the adherent leukocytes so that the expression profile of their cell surface molecules changes in favour of leukocyte migration as opposed to firm adhesion. Finally, (3) PAF may regulate the function and/or ligand binding profile of junctional endothelial cell adhesion molecules, and hence facilitate transmigration. In this context, PAF receptor antagonists have been shown to abolish both PECAM-1 phosphorylation induced by endothelial cell activation, and subsequent monocyte transmigration *in vitro* (Kalra *et al.*, 1996). Despite the reported ability of TNF α to stimulate PAF generation in different cell systems (Alloatti *et al.*, 2000; Bussolino *et al.*, 1990; Myers *et al.*, 1990), in our studies, leukocyte transmigration in response to TNF α was unaffected by PAF receptor blockade. These findings suggest that in the present model, TNF α -induced leukocyte migration occurred independently of endogenously generated PAF, illustrating yet another important mechanistic difference between the responses elicited by IL-1 β and TNF α . Interestingly, PAF antagonists have no inhibitory effects on leukocyte transmigration elicited by the direct neutrophil chemoattractant FMLP both *in vitro* (Kuijpers *et al.*, 1992) and *in vivo* (Nourshargh *et al.*, 1995), yet again suggesting that TNF α may be acting in a similar manner to direct neutrophil

chemoattractants and eliciting neutrophil transmigration *via* direct neutrophil stimulation.

In mice pre-treated with the LTB₄ receptor antagonist, IL-1 β -induced leukocyte adhesion and transmigration were significantly suppressed (81 and 59%, respectively). On analysing the ratio of transmigrated leukocytes to adherent leukocytes, a similar ratio was obtained in mice treated with vehicle and mice treated with the LTB₄ antagonist, suggesting that the observed inhibition of leukocyte transmigration was directly associated with the inhibition of leukocyte adhesion. Hence, collectively, the present results suggest that whilst IL-1 β -induced firm adhesion is mediated by endogenously generated LTB₄, transmigration through IL-1 β -stimulated venules is mediated by endogenously generated PAF. Alternatively, protein mediators such as IL-8 induced in response to IL-1 β (from endothelial cells or other tissue cells) may in turn stimulate adherent leukocytes to generate additional inflammatory mediators, such as LTB₄, that may act in an autocrine manner to further stimulate the activation of leukocyte integrins, hence contributing to the adhesive response (Marleau *et al.*, 1999). The adherent leukocytes can then become activated and/or guided through endothelial cell junctions *via* endothelial cell associated PAF, as discussed above. As found with actinomycin D and the PAF receptor antagonist, the LTB₄ antagonist had no effect on leukocyte responses induced by TNF α . However, since IL-1 β and TNF α can reportedly stimulate the generation of both LTB₄ and PAF (Alloatti *et al.*, 2000; Borish *et al.*, 1990; Bussolino *et al.*, 1988; 1990; Camussi *et al.*, 1989), to investigate the potential additive or synergistic effects of these mediators in leukocyte migration elicited by the two cytokines, in a final series of experiments, mice were pretreated with both receptor antagonists. The results indicated that co-administration of the blockers did not lead to a greater inhibitory effect than

that observed when the antagonists were administered alone, suggesting that the two lipid mediators did not act in a cooperative manner.

In summary, the results of the present study strongly suggest that TNF α can stimulate leukocyte responses of firm adhesion and transmigration within the mouse cremaster microcirculation without the need for local protein synthesis or the generation of the pro-inflammatory lipid mediators PAF and LTB₄. In conjunction with previously published results (discussed above), the present findings support the hypothesis that TNF α can induce leukocyte migration *via* direct effects on leukocytes and/or tissue inflammatory cells, such as mast cells, capable of releasing pre-formed protein mediators in response to TNF α (van Overveld *et al.*, 1991). In contrast, leukocyte firm adhesion and transmigration through IL-1 β -stimulated cremasteric venules was dependent on local generation of proteins and indicated that in the present model, IL-1 β -induced leukocyte firm adhesion was dependent on endogenously generated LTB₄, whilst transmigration was strongly mediated by endogenously generated PAF. As well as identifying components of the leukocyte migration response elicited by IL-1 β , the present study has also directly compared the effects of IL-1 β and TNF α in a commonly used inflammatory model and identified key differences in their mechanisms of action. A better understanding of the mechanisms of action of IL-1 β and TNF α may aid the development of more specific anti-inflammatory therapies for disease states in which these cytokines have been implicated.

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