

This study compares the signal transduction pathway which leads to the upregulation of intercellular adhesion molecule-1 (ICAM-1) expression with that of the increase in the expression of inducible nitric oxide synthase (iNOS) protein and activity caused by endotoxin in cultured J774.2 macrophages. Treatment of J774.2 cells with lipopolysaccharide *E. coli* (LPS) induced a concentration-dependent increase in the expression of ICAM-1 on the cell surface within 4 h and an increase in iNOS protein and activity at 24 h. The upregulation of ICAM-1 expression on J774.2 macrophages caused by LPS was significantly inhibited by pretreatment of the cells with inhibitors of the activation of the nuclear transcription factor NF- κ B, such as L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK), pyrrolidine dithiocarbamate (PDTC), rotenone or calpain inhibitor I, but not by the tyrosine kinase inhibitors, tyrphostin AG126 or genistein. In contrast, genistein or tyrphostin AG126 also prevented the induction of iNOS protein and activity in J774.2 macrophages elicited by LPS. Thus, the increase in the expression of ICAM-1 on J774.2 macrophages by endotoxin involves the activation of NF κ B, but not of protein tyrosine kinase.

Key words: Inflammation, ICAM-1, iNOS, NF- κ B, Protein tyrosine kinase, Macrophages

Upregulation of ICAM-1 expression on J774.2 macrophages by endotoxin involves activation of NF- κ B but not protein tyrosine kinase: comparison to induction of iNOS

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Introduction

Intracellular adhesion molecule-1 (ICAM-1; CD54) is a 90-kDa inducible cell surface glycoprotein that promotes leukocyte adhesion in inflammatory conditions.^{1,2} Analysis of ICAM-1 complementary deoxyribonucleic acid sequence revealed it to be a member of the immunoglobulin gene superfamily.³ ICAM-1 is expressed basally at low levels on many cell types, including endothelial cells, macrophages, myocytes, and vascular smooth muscle cells,⁴⁻⁷ but can be induced to high levels by stimulation with lipopolysaccharide (LPS), phorbol ester, or inflammatory cytokines, such as tumour necrosis factor- α (TNF- α) or interleukin-1 β (IL-1 β). For that concerning cells of the mono-myelocytic lineage, ICAM-1 expression on the plasma membrane of macrophages is higher than that measured on circulating monocytes.⁸ Nonetheless, ICAM-1 is not only a marker of differentiation, and its expression can also be up-regulated following cell activation, such as after treatment with IL-1 β of U-937 cells or of murine macrophages.⁹ The second messenger pathways responsible for ICAM-1 induction are poorly understood, although there is some

evidence that activation of protein kinase C may be involved at least in the upregulation observed on the endothelial cell surface after treatment with TNF- α .¹⁰ In addition, a specific binding site for the nuclear transcription factor- κ B (NF- κ B) has been detected in the promotor region of the ICAM-1 gene.¹¹ From the functional point of view, endothelial ICAM-1 mediates the interaction between extravasating leukocyte and the endothelium of post-capillary venules by binding to the CD11/CD18 complex.¹² In monocyte/macrophages, surface ICAM-1 mediates the process of antigen presentation as well as the interaction with cell types other than endothelial cells, such as chondrocytes of the rheumatoid joint.¹³

Nitric oxide (NO) is a vasodilator autacoid which is produced by NO synthase from L-arginine in many mammalian cells.¹⁴ NO has many diverse biological functions in the cardiovascular, nervous and immune systems. Once formed, NO diffuses to adjacent cells where it activates soluble guanylate cyclase, resulting in the formation of cyclic guanosine monophosphate, which in turn mediates many, but not all, of the biological effects of NO. At least three isoforms of NO synthase have been cloned. The NO synthase in

endothelial cells and neuronal cells are expressed constitutively, while activation of macrophages and other cells with proinflammatory cytokines (e.g. TNF- α , IL-1 β) or endotoxin (LPS) results in the expression of an inducible isoform of NO synthase (iNOS), which is functionally independent of changes in intracellular calcium.¹⁵ The iNOS gene from murine macrophages has been cloned and characterized,¹⁶ and there is evidence that activation of protein tyrosine kinase and of the nuclear transcription factor NF- κ B is involved in the intracellular signal transduction pathway leading to the expression of this enzyme.¹⁷⁻¹⁹

An enhanced formation of NO following the induction of iNOS by LPS in macrophages and vascular smooth muscle cells contributes importantly to the circulatory failure (hypotension) and multiple organ dysfunction syndrome associated with septic shock.^{15,18,19} Moreover, during sepsis the infiltration of leukocytes plays a pivotal role in tissue damage. For instance, septic shock results in an early accumulation of polymorphonuclear leukocytes in the liver (after 3 h), which is followed by an infiltration of mononuclear phagocytes (after 30 h). There is evidence that the expression of ICAM-1 may contribute to the influx of leukocytes during septic shock and, therefore, play a role in tissue damage during septic shock.²⁰ Interestingly, circulating levels of ICAM-1 are higher in patients with septic shock than in patients with severe sepsis or sepsis without hypotension.²¹

In the present study, we used cultured J774.2 macrophages to study the signal transduction pathway, which leads to the upregulation of ICAM-1 expression by endotoxin and compared it with the one leading to an induction of iNOS.

Methods

Induction of nitrite formation by endotoxin in cultured macrophages

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum.¹⁸ Cells were cultured in 96-well plates with 200 μ l culture medium until they reached confluence. To induce iNOS in macrophages, fresh culture medium containing *Escherichia coli* lipopolysaccharide (LPS, 1 μ g/ml; serotype, 0127:B8) was added. Nitrite accumulation in the cell culture medium was measured after 24 h. The following drugs were added to cells 30 min before LPS: tyrphostin AG126 (30 μ M), genistein (100 μ M), rotenone (30 μ M), L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK; 30 μ M), pyrrolidine dithiocarbamate (PDTC; 25 μ M), calpain inhibitor I (30 μ M), anti-inflammatory cytokines such as interleukin-4 (IL-4; 100 ng/ml), interleukin-10 (IL-10; 100 ng/ml) or interleukin-13 (IL-13; 100 ng/ml). The concentrations of

compounds or cytokines were chosen from previous studies.^{18,19,26} The amount of nitrite, an indicator of NO synthesis, in the supernatant of J774.2 was measured by the Griess reaction²² by adding 100 μ l of Griess reagent to 100- μ l samples of unfiltered serum or supernatant. The optical density at 550 nm (OD550) was measured using a Molecular Devices microplate reader (Richmond, CA, USA). Nitrite concentrations were calculated by comparison with OD550 of standard solution of sodium nitrite prepared in control culture medium. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan.²³ Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg/ml for 60 min). Culture medium was removed by aspiration and cells solubilized in dimethylsulfoxide by measurement of OD550 using a Molecular Devices microplate reader. Formazan production was expressed as a percentage of the values obtained from untreated cells.

Western (immuno) blot analysis (iNOS)

J774.2 macrophages were cultured in six-well plates (37°C) and treated for 24 h with fresh medium alone (control) or medium containing LPS (1 μ g/ml) to induce iNOS. To assess the effects of tyrphostin AG126, genistein, rotenone, PDTC, TPCK or calpain inhibitor I on the expression of iNOS protein induced by immunostimulants, compounds were added to the cells 30 min prior to LPS. After 24 h, cells were washed with phosphate-buffered saline (PBS; pH 7.4) and incubated (5 min) with 1 ml of extraction buffer (pH 7.4) consisting of 50 mM Tris-HCl, 10 mM EDTA, 1% (v/v) Triton X-100, and the protease inhibitors pepstatin A 50 μ M, leupeptin 0.2 mM and phenylmethylsulphonylfluoride 1 mM. The cell extract was boiled for 10 min with gel-loading buffer (Tris, 20 mM; EDTA, 2 mM; SDS, 2% (w/v); glycerol, 20% (v/v); 2-mercaptoethanol, 10% (v/v); bromophenol blue, 2 mg/ml; pH 6.8) in a ratio of 1:1 (v/v). Samples were centrifuged at 10 000 g for 2 min before being loaded onto gradient gels (7.5% SDS gel) and subjected to electrophoresis (1 h at 150 V). The separated proteins were transferred to nitrocellulose (Bio-Rad; 1 h at 200 V). After transfer to nitrocellulose by electrophoresis, the membranes were primed over night at 4°C with a polyclonal antibody raised to macrophage iNOS developed in rabbits (a generous gift from Dr Claire Bryant, William Harvey Research Institute, UK).²⁴ The blots were then incubated with anti-rabbit IgG linked to horseradish peroxidase. All antibodies were used at a 1:5000 dilution. Horseradish peroxidase-conjugated secondary antibody was then added and blots developed using an enhanced horseradish peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents, Amersham

International, Buckinghamshire, UK) and exposed to X-ray film for 30–60 s.

Measurement of ICAM-1 and CD11b levels by fluorescence-activated cell sorter (FACS) analysis

J774.2 macrophages were cultured in six-well plates as described above and, after they reached confluence, were washed and incubated with or without LPS (0.01–10 mg/ml) for 4 or 24 h. At the end of the incubation period, cells were washed and removed with non-enzymatic dissociation medium and pelleted by centrifugation at 400g for 15 min at 4°C. Subsequently, cells were seeded in 96-well flat-bottom plates in 20 µl of PBS supplemented with 0.2% bovine serum albumin (BSA); non-specific sites were blocked by adding 20 µl of human IgG (15 mg/ml) prior to the addition of 20 µl of a rat anti-mouse ICAM-1 (16 µg/ml) or rat anti-mouse CD11b (16 µg/ml) monoclonal antibody. Control wells received an equal amount of rat IgG. After 1 h at 4°C, cells were washed twice with PBS/+0.2%BSA and incubated with 40 µl of diluted (1:40) F(ab')₂ fragment of goat anti-rat IgG antibody conjugated to fluorescein isothiocyanate (FITC). After a further 45 min at 4°C, cells were washed, resuspended in 200 µl PBS/+0.2% BSA and fixed with an equal volume of 2% paraformaldehyde. FACS analysis was performed within 5 days using a FACScan II analyser (Becton-Dickinson, Mountain View, CA) with an air-cooled 100 mW argon ion laser tuned to 488 nm and Consort 32 computer running Lysis II software (Becton-Dickinson). At least 10 000 events were analysed for each labelling. Data were analysed as units of fluorescence measured in the FL1 channel, corrected for control binding (rat IgG), and converted to the number of mAb molecules bound per cell with reference to microbeads labelled with standard molecules of FITC (Flow Cytometry Standards Corp., Research Triangle Park, NC).²⁵

Materials

Bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium, foetal calf serum, L-glutamine, MTT, pepstatin A, leupeptin, glycerol, 2-mercaptophanol, phenylmethylsulphonyl fluoride (PMSF), Bradford reagent, bovine serum albumine, rotenone, L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK), pyrrolidine dithiocarbamate (PDTC), human IgG, Triton X-100, Trizma base, EDTA, bromophenol blue, sodium dodecyl sulphate (SDS), sulphani-lamide, naphthylethylenediamide, phosphoric acid, non-enzymatic dissociation medium, and anti-rabbit IgG antibody were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Pure nitrocellulose membrane (0.45 µm) and filter paper were purchased from Bio-

Rad (Hertfordshire, UK). The following antibodies (AB) were purchased from Serotec (Oxford, UK): rat anti-mouse ICAM-1, monoclonal rat anti-mouse CD11b and monoclonal FITC-conjugated donkey anti-rat IgG. Tyrphostin AG126 [(3-hydroxy-4-nitrobenzylidene) malononitrile or a-cyano-(3-hydroxynitro)cinnamoni-trile], genistein [4,5,7-trihydroxy-isoflavone] and cal-pain inhibitor I (*N*-acetyl-leu-leu-norleucinal) were from Calbiochem (Nottingham, UK). Murine recombinant IL-4 and human recombinant IL-10 and IL-13 were a generous gift of Dr P. Grint (Schering-Plough, USA).

Statistical evaluation

Results shown are mean±S.E.M. from triplicate determinations (wells) from three separate experiments. One-way or two-way analysis of variance (ANOVA) followed by, if appropriate, Bonferroni multiple range test was used to compare means between groups. A *P* value less than 0.05 was considered to be statistically significant.

Results

LPS-induced ICAM-1 and iNOS expression in J774.2 macrophages

A constitutive expression of ICAM-1 and CD11b was found on the plasma membrane of cultured J774.2 macrophages (Fig. 1a,b). Incubation of J774.2 macrophages with 1 µg/ml LPS resulted, within 4 h, in an upregulation of ICAM-1 expression without further increase at the 24-h time-point (Fig. 1a). LPS-induced 4-h ICAM-1 expression was concentration dependent (Fig. 1c). In contrast, treatment of macrophages with different concentrations of LPS did not affect the expression of CD11b (Fig. 1b,d). Activation of J774.2 macrophages with endotoxin resulted within 24 h in a significant increase in nitrite in the cell supernatant (Table 1). This was mirrored by appearance of a 130-kDa protein which was recognized by a specific antibody to iNOS (Fig. 3, lane B).

In subsequent experiments, designed to elucidate similarities or differences in the LPS-induced signal transduction pathways which lead to the expression of iNOS protein and activity, and to the upregulation of ICAM-1 in J774.2 macrophages, only 1 µg/ml of LPS for 4 h (ICAM-1 expression) or 24 h (expression of iNOS protein and activity) was used.

Involvement of tyrosine kinase

Pretreatment of J774.2 macrophages with the protein tyrosine kinase inhibitors genistein (100 µM) or tyrphostin AG126 (30 µM) significantly attenuated the expression of iNOS protein and the formation of nitrite elicited by LPS (Table 1, Fig. 3). In contrast, neither inhibitor prevented LPS upregulation of the membrane-bound ICAM-1 (Fig. 2a).

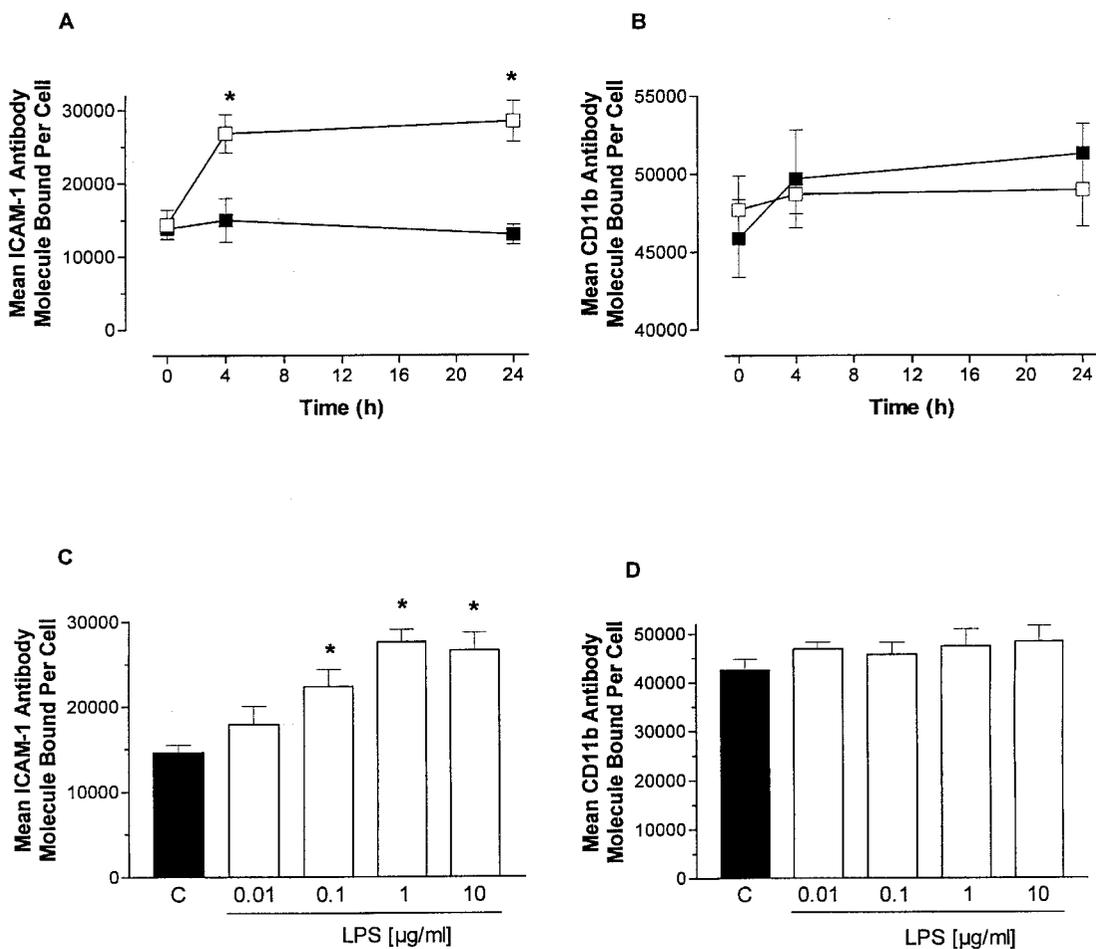


FIG. 1. Time course of the expression of (a) ICAM-1 and (b) of CD11b by J774.2 macrophages activated with endotoxin. J774.2 cells were incubated in the absence (solid squares) or presence (open squares) of LPS (1 µg/ml) for 0, 4 or 24 h, respectively. Endotoxin caused a concentration-dependent increase in the expression of (c) ICAM-1 but not (d) of CD11b by J774.2 cells. Macrophages were treated with vehicle (C, saline; solid columns) or LPS (0.01–10 µg/ml; open columns) for 4 h. At the end of experiments, adhesion molecule expression on the plasma membrane was quantified by FACS analysis. Data are expressed as mean±S.E.M. from triplicate determinations (wells) from three separate experimental days (n=9). *P<0.05 represents significant difference when compared to control values.

Table 1. Effect of tyrphostin AG126, genistein, rotenone, L-tosylamido-2-phenylethylchloromethyl ketone (TPCK), pyrrolidine dithiocarbamate (PDTC) or calpain inhibitor I on the increase in the formation of nitrite in supernatant of J774.2 macrophages activated with LPS (1 µg/ml) for 24 h

Compound	Concentration (µM)	Nitrite (µM)
Control	–	1.81 ± 0.4
LPS (1 µg/ml)	–	48.4 ± 3.6
+ tyrphostin AG126	30	17.5 ± 2.8*
+ genistein	100	13.4 ± 4.6*
+ rotenone	30	10.2 ± 3.8*
+ PDTC	25	8.6 ± 3.7*
+ TPCK	30	16.5 ± 2.3*
+ calpain inhibitor I	30	14.3 ± 3.1*

Data are expressed as mean±S.E.M. from triplicate determinations (well) from three separate experimental days (n=9). *P<0.05 represents significant difference when compared to LPS-control.

Involvement of NF-κB

Activation of NF-κB by LPS leads to the induction of iNOS in macrophages, and involves the formation of reactive oxygen intermediates.¹⁸ Accordingly, pre-treatment of J774.2 cells with PDTC (25 µM) or the antioxidant rotenone (30 µM) prevented the increase in the formation of nitrite and the expression on iNOS protein caused by endotoxin (Table 1). Both PDTC and rotenone also attenuated the expression of ICAM-1 in a concentration-dependent manner (Fig. 2b). Treatment of J774.2 cells with TPCK, a cysteine and serine protease inhibitor, or calpain inhibitor I prior to endotoxin, significantly attenuated the increase in the formation of nitrite and the expression of iNOS protein caused by LPS (Table 1, Fig. 3). In addition, the increase in the expression of ICAM-1 was also concentration-dependently attenuated by both TPCK or calpain inhibitor I (Fig. 2c). It is notable that none

of the drugs used modified the constitutive expression of ICAM-1 (Fig. 2a-c).

Incubation of J774.2 macrophages with LPS alone caused a small (~20%) reduction in cell viability at 24 h, as measured by formazan formation, with no

effect at the 4-h time-point. Incubation of J774.2 cells with the drugs used did not result in a significant reduction in cell viability. Similarly, none of the drugs used attenuated the reduction in cell viability caused by LPS at 24 h (Table 2).

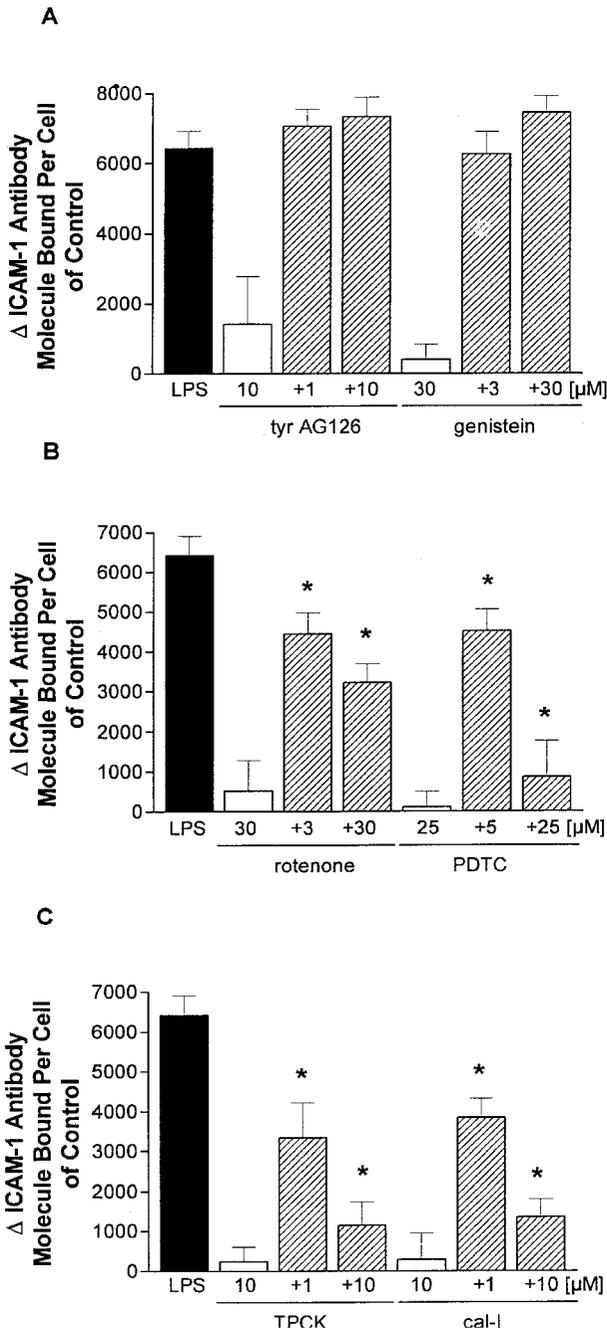


FIG. 2. Effect of (a) tyrphostin AG126 (tyr AG126) or genistein, (b) rotenone or pyrrolidine dithiocarbamate (PDTC), (c) L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK) or calpain inhibitor I (cal-I) on the increase in the expression of ICAM-1 on the cell plasma membrane of J774.2 macrophages activated with LPS (1 μg/ml) for 4 h. J774.2 cells were incubated with the drugs in the absence (open columns) or presence (hatched columns) of LPS. Data are expressed as mean ± S.E.M. from triplicate determinations (wells) from three separate experimental days (n=9) and are expressed as ΔICAM-1, the basal value (without LPS or drugs) being subtracted. *P<0.05 represents significant difference when compared to LPS-controls (solid column).

Anti-inflammatory cytokines

To test whether anti-inflammatory cytokines prevented the upregulation of membrane-bound ICAM-1 on macrophages, J774.2 cells were treated with IL-4, IL-10 or IL-13 (all 100 ng/ml) prior to LPS. None of these cytokines affected either basal or LPS-induced increase in the expression of ICAM-1 on the cell surface of J774.2 macrophages. In contrast, and confirming previous studies,^{26,27} IL-4, IL-10 or IL-13 significantly attenuated the increase in the formation of nitrite in the supernatant caused by LPS at 24 h (Fig. 4, Table 2).

Discussion

ICAM-1 expression at the plasma membrane of several cell types is related not only as marker of cell differentiation, but also to the degree of cell activation. Few studies have investigated the signalling pathways leading to the upregulation of ICAM-1 in mono-myelocytic cells. Using J774.2 macrophages which closely mimic primary macrophages in many aspects,²⁶ we have compared the signal transduction pathway leading to the upregulation of ICAM-1 expression with that of the increase in the expression of iNOS protein and activity caused by endotoxin.

The induction of iNOS protein and activity caused by wall fragments of Gram-negative (endotoxin), Gram-positive (lipoteichoic acid, peptidoglycan) bacteria or pro-inflammatory cytokines, such as IL-1β in cultured cells (e.g. macrophages), involves the phosphorylation of tyrosine residues in proteins, and is therefore prevented by the tyrosine kinase inhibitors genistein, erbstatin and tyrphostin AG126.²⁸⁻³⁰ We confirm here that two structurally distinct tyrosine kinase inhibitors, genistein (competitive inhibitor at

Table 2. Effect of interleukin-4, interleukin-10 or interleukin-13 on the increase in the formation of nitrite in supernatant of J774.2 macrophages activated with LPS (1 μg/ml) for 24 h

Compound	Dose (μg/ml)	Nitrite (μM)
Control	-	1.8 ± 0.4
LPS	1	52.4 ± 2.3
+ interleukin-4	0.1	23.5 ± 4.6*
+ interleukin-13	0.1	26.8 ± 3.3*
+ interleukin-10	0.1	43.4 ± 3.8*

Data are expressed as mean ± S.E.M. from triplicate determinations (well) from three separate experimental days (n=9). *P<0.05 represents significant difference when compared to LPS control.

130 kDa

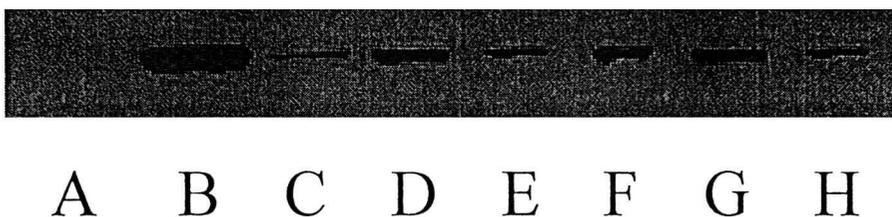


FIG. 3. Effect of tyrphostin AG126 (30 μ M; lane H) or genistein (100 μ M; lane G), rotenone (30 μ M; lane F), pyrrolidine dithiocarbamate (25 μ M; lane E), calpain inhibitor I (30 μ M; lane D) or L-1-tosylamido-2-phenylethylchloromethyl ketone (30 μ M; lane C) on the expression of iNOS protein in J774.2 macrophages caused by LPS (1 μ g/ml; lane B) within 24 h, as determined by Western (immuno)blot analysis. There was no iNOS protein detectable by the specific antibody against iNOS protein in unstimulated control macrophages (lane A). This immunoblot is representative for three separate experiments.

the ATP-binding site) or tyrphostin AG126 (competitive inhibitor at the substrate binding site), used at selected and validated concentrations,^{31,32} inhibit the expression of iNOS caused by LPS, clearly showing that tyrosine phosphorylation plays an important role in the signal transduction pathway leading to the expression of iNOS by LPS in J774.2 macrophages. In contrast, treatment of J774.2 macrophages with genistein or tyrphostin AG126 did not prevent the LPS-induced upregulation of the expression of membrane-bound ICAM-1. A similar finding has been described in endothelial cells. The tyrosine kinase inhibitors, genistein and tyrphostin AG1288 had no effect on the TNF- α -induced increase in ICAM-1 expression on cultured EA.hy926 endothelial cells at 4 h after TNF- α , but significantly increased the expression of ICAM-1 at 24 h.³³ In contrast, genistein and AG 1288 significantly inhibited TNF- α -induced upregulation of ICAM-1 expression on A549 epithelial cells at 4 h after TNF- α , but had no effect on ICAM-1 expression at 24 h.³⁴ These results suggest that the effects (increase, decrease or no effect) of tyrosine

kinase inhibitors on the TNF- α - or LPS-induced upregulation of ICAM-1 expression on the plasma membrane depend on the cell type and the time of exposure to immunostimulants. Our data demonstrated that this pathway is unlikely to be activated in J774.2 cells to induce this adhesion molecule.

The expression of inducible genes including iNOS are largely controlled by proteins, such as NF- κ B, which activate transcription.^{17,35} NF- κ B is itself activated by the exposure of cells to endotoxin or TNF- α , IL-1, IL-2 or phorbol 12-myristate 13-acetate (PMA).³⁶⁻³⁹ The most frequent form of NF- κ B is a heterodimer composed of two DNA-binding proteins, namely NF- κ B1 (or p50) and RelA (or p65), although other dimeric combinations also exist.⁴⁰ Under physiological conditions, NF- κ B is held (in an inactive form) in the cytoplasm by the inhibitory protein I κ B- α , which avidly binds to most heterodimers including the NF- κ B1/RelA heterodimer. Activation of NF- κ B involves the release of the inhibitory subunit I κ B- α from a cytoplasmic complex, which I κ B forms together with the DNA-binding subunit RelA and NF- κ B141,⁴² and, hence, activation of NF- κ B allows NF- κ B to translocate to the nucleus and to induce the expression of specific genes.^{43,44} The activation of iNOS in macrophages caused by LPS or LTA is diminished by inhibitors of the activation of NF- κ B, such as PDTC, TPCK, or calpain inhibitor I.^{18,30,46,47} However, the proteolytic degradation of I κ B- α is inhibited with some selectivity by the cysteine protease inhibitor calpain inhibitor I, but not by other inhibitors of serine and cysteine proteases, such as chymostatin or leupeptin.⁴⁸ There is evidence that activation of the promotor of ICAM-1 by cytokines on endothelial cells critically relies on the transcription factor NF- κ B.⁴⁹ Six potential NF- κ B sites have been identified in the ICAM-1 5'-flanking sequence and first intron.^{50,51} We show that PDTC which inhibits NF- κ B activation,⁵² not only prevented the expression of iNOS protein and activity (nitrite), but also the upregulation of ICAM-1 expression caused by LPS in J774.2 cells. In addition, the cysteine and serine protease inhibitors TPCK or calpain inhibitor I, which inhibit I κ B protease,⁴⁸ prevented the LPS-induced expression of iNOS protein and activity as well as the

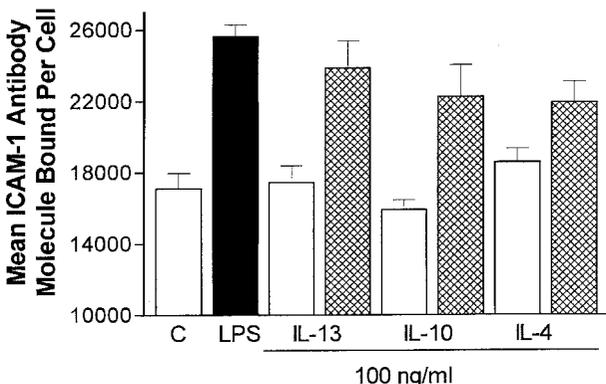


FIG. 4. Effect of interleukin-13 (IL-13), interleukin-10 (IL-10) or interleukin-4 (IL-4) on the increase in the expression of ICAM-1 on the cell plasma membrane of J774.2 macrophages caused by endotoxin. J774.2 cells were incubated in the absence (open columns) or presence (hatched columns) of LPS (1 mg/ml) with IL-13, IL-10 or IL-4 (all 100 ng/ml) for 4 h. At 4 h after LPS ICAM-1 expression was monitored by FACS analysis. Data are expressed as mean \pm S.E.M. from triplicate determinations (wells) from three separate experimental days ($n=9$). * $P<0.05$ represents significant difference when compared to LPS controls (solid column).

upregulation of ICAM-1 expression. This implies that activation of I κ B protease, known to be involved in the induction of iNOS, is also important in the upregulation of ICAM-1 expression caused by LPS on J774.2 macrophages.

One of the determinants of NF- κ B activation is the redox status of the cell,⁵³ which is determined by the concentration of reactive oxygen species, including superoxide, hydrogen peroxide or hydroxyl radicals.⁵² There is evidence that cytokines and LPS increase the formation of cellular reactive oxygen intermediates by causing an alteration of electron flow in the mitochondria.⁵³ We show that the antioxidant rotenone prevented the induction of iNOS protein and activity as well as the upregulation of ICAM-1 expression caused by LPS. A similar finding has been reported with the potent antioxidant *N*-acetyl cysteine which inhibits the induction of iNOS protein and activity caused by LPS in rat peritoneal macrophages: this effect was linked to a reduced activation of NF- κ B.⁵⁵ Moreover, *N*-acetyl cysteine also inhibits ICAM-1 mRNA expression in HeLa and HaCaT cells after exposure to ionizing radiation, known to produce reactive oxygen intermediates in mammalian tissues, by inhibition of the activation of NF- κ B.⁵⁶ Thus, reactive oxygen intermediates, presumably by their ability to activate NF- κ B, play an important role in the series of events leading to the expression of iNOS and ICAM-1 induced by LPS in these cells as well as in J774.2 cells, as described here.

The anti-inflammatory cytokines IL-4, IL-10 or IL-13 have been reported to inhibit the LPS-induced formation of NO by cultured macrophages by preventing the induction of iNOS protein expression.^{26,27} Indeed, we confirm in this study that all three cytokines, inhibited the induction of iNOS protein and activity caused by LPS in J774.2 macrophages. Interestingly, IL-4, IL-10 or IL-13 neither affected basal nor LPS-induced expression of ICAM-1 in these cells. Therefore, there is some cell specificity in IL-4 effects upon ICAM-1, since this cytokine directly increases ICAM-1 levels on other cell types including human dermal fibroblasts⁵⁷ and human umbilical vein endothelial cells.⁵⁸ Like IL-4, IL-13 also induces ICAM-1 expression in unstimulated human mast cells.⁴⁵ In contrast, IL-10 and IL-13 inhibit the IL-1- or IFN- κ -induced expression of ICAM-1 in human monocytes, human synovial fibroblasts and human endothelial cells.^{54,59,60} Thus, the effect of the anti-inflammatory cytokines IL-4, IL-10 or IL-13 on the expression of ICAM-1 depends on the cell system and the stimulus employed for activation.

In conclusion, this study demonstrated that increase in ICAM-1 and iNOS expression in J774.2 macrophages by endotoxin involves reactive oxygen intermediates, and the activation of NF- κ B. In contrast to the signal transduction pathway leading to the

induction of iNOS, activation of protein tyrosine kinase is not involved in the LPS-induced upregulation of ICAM-1 expression in J774.2 macrophages, and consequently this pathway is not sensitive to anti-inflammatory interleukins.

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