

Tumour necrosis factor- α -induced expression of intercellular adhesion molecule-1 on human eosinophilic leukaemia EoL-1 cells is mediated by the activation of nuclear factor- κ B pathway

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Summary

Background Intercellular adhesion molecule-1 (ICAM-1) has been shown to mediate the adhesion and migration of eosinophils to the site of allergic inflammation. However, molecular mechanisms regulating the expression of ICAM-1 in eosinophils are still being elucidated. We investigated the effect of tumour necrosis factor- α (TNF- α) on ICAM-1 expression of eosinophils.

Methods The surface expression of ICAM-1 on a human eosinophilic leukaemic cell line, EoL-1, was assessed by immunocytochemical staining. The phosphorylation of inhibitor kappa B- α (I κ B- α) and p38 mitogen-activated protein kinase (MAPK) was detected by Western blot. Nuclear factor kappa-B (NF- κ B) pathway-related genes were evaluated by the cDNA expression array system, whereas the activity of NF- κ B was measured by electrophoretic mobility shift assay (EMSA).

Results TNF- α was found to induce the cell surface expression of ICAM-1. A specific proteasome inhibitor N-cbz-Leu-Leu-leucinal (MG-132), but not a p38 MAPK inhibitor (SB 203580), was found to suppress the TNF- α -induced expression of ICAM-1 on EoL-1 cells. The gene expressions of ICAM-1, NF- κ B and I κ B- α were up-regulated after the stimulation with TNF- α . Further, TNF- α was shown to induce I κ B- α phosphorylation and degradation, thereby indicating the activation of NF- κ B. In EMSA, there was a shifted NF- κ B band on TNF- α -treated cells with or without SB 203580, but no shifted band was observed on MG-132-treated cells.

Conclusion *In vitro* studies of EoL-1 cells, an eosinophilic leukaemic cell line, confirmed that NF- κ B plays an important role in the expression of ICAM-1 and recruitment of eosinophils in allergic inflammation.

Keywords EoL-1 cells, intercellular adhesion molecule-1, nuclear factor- κ B, p38 mitogen-activated protein kinase, tumour necrosis factor- α

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Introduction

Eosinophils are the major inflammatory effector cells accumulating at the site of allergic inflammation, e.g., airway submucosa [1]. The activated eosinophils release cytotoxic molecules, such as major basic protein, eosinophil peroxidase, eosinophilic cationic protein, lipid mediators and cytokines, that cause tissue damage and consequently the manifestation of allergic disease, e.g. asthma [2]. The recruitment and migration of eosinophils through the vascular endothelial cells therefore play an important role in the pathogenesis of allergy. These processes can be mediated and enhanced by several cytokines, including interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1) [3–5], and chemotactic factors, including regulated upon activation normal T cell expressed and

secreted (RANTES), eotaxin, IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1) [6, 7]. These inflammatory mediators activate intracellular signalling cascades, including mitogen-activated protein kinase (MAPK) and transcription factor nuclear factor- κ B (NF- κ B) signal transduction pathways [8–10]. In recent years, adhesion molecules have been reported to have an essential function in inflammatory processes in allergy and asthma [2, 11]. Intracellular adhesion molecule-1 (ICAM-1) belongs to the immunoglobulin (Ig) superfamily cellular adhesion molecules (CAMs), which are 80 to 114 kDa calcium-independent transmembrane glycoproteins [12–14]. ICAM-1 has five Ig-like domains, a single transmembrane region, and a short cytoplasmic domain [15]. ICAM-1 can bind to lymphocyte function associated antigen-1 (LFA-1), macrophage antigen-1 (Mac-1), fibrinogen, hyaluronan and CD43 on leucocytes [16]. Although freshly purified peripheral blood eosinophils do not express ICAM-1, the expression can be transcriptionally induced by various inflammatory cytokines including TNF- α , IFN- γ and IL-1 β [17–19]. The interactions of ICAM-1 and

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integrins have been shown to be critical for the recruitment and migration of eosinophils [20].

Recent studies have reported that activation of NF- κ B mediated the transcription of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-8 in leucocytes [8, 21, 22]. NF- κ B comprises inducible transcription factors that serve as important regulators in allergic inflammation [8, 9]. In quiescent cells, NF- κ B is bound to an inhibitory protein known as I κ B. Upon stimulation, I κ B kinase is activated to phosphorylate I κ B, which is then degraded by the proteasome [23]. This results in the translocation of NF- κ B from cytoplasm to the nucleus, where it binds to DNA response elements and activates the transcription of specific genes [24]. The p38 MAPK is a serine/threonine protein kinase that phosphorylates both cytoplasmic and nuclear targets and activates downstream protein kinase cascades in response to cell proliferation, stress and inflammation [3]. It has been shown that TNF- α , IL-1 and PAF can induce RANTES and GM-CSF release in human bronchial epithelial cells through the regulation of p38 MAPK pathway [25]. p38 MAPK can be strongly activated by pro-inflammatory cytokines. However, the activation of signal transduction mechanisms of p38 MAPK and NF- κ B on the adhesion and migration of eosinophils are not fully understood.

In the present study, we used a human eosinophilic leukaemia cell line, EoL-1, to investigate the effect of TNF- α on the expression of ICAM-1, and elucidate the NF- κ B and p38 MAPK mediated signalling mechanisms regulating the expression of ICAM-1.

Materials and methods

Reagents and antibodies

Human recombinant TNF- α was obtained from PeproTech Inc., Rocky Hill, NJ, USA. FITC-conjugated mouse anti-human ICAM-1 monoclonal antibody and fluorescein-conjugated mouse IgG₁ isotype were purchased from R & D systems Inc., Minneapolis, MN, USA. Rabbit antiphospho-p38 MAPK, anti-I κ B- α and antiphospho-I κ B- α polyclonal antibodies were purchased from Cell Signalling Technology Inc., Beverly, MA, USA. NF- κ B proteasome inhibitor N-cbz-Leu-Leu-Leucinal, MG-132, and specific p38 MAPK inhibitor, SB 203580, were purchased from Calbiochem Corp., San Diego, CA, USA.

Cell culture

The human eosinophilic leukaemic cell line, EoL-1 (Riken Cell Bank, Tsukuba Science City, Japan) was maintained in RPMI 1640 medium with 25 mM HEPES (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco) in 5% CO₂ and 95% humidified air at 37 °C. For the study of the effect of MG-132 (Calbiochem), MG-132 was dissolved in dimethyl sulfoxide (DMSO) and added to EoL-1 culture at a concentration of 5 μ M for 1 h. The concentration of DMSO was no more than 0.05% (v/v). Human peripheral blood eosinophils were purified from the fresh buffy coat fraction obtained from the Hong Kong Red Cross Blood Transfusion Service using anti-human CD16 microbead magnetic cell sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Endotoxin-free solutions

Cell culture medium was purchased from Gibco, free of detectable lipopolysaccharide (LPS) (<0.1 EU/mL). All other solutions were prepared using pyrogen-free water and sterile polypropylene plasticware. No solution contained detectable LPS, as determined by the Limulus amoebocyte lyase assay (sensitivity limit 12 pg/mL; Associate of Cape Cod, Wooks Hole, MA, USA).

Flow cytometric analysis

EoL-1 cells (5×10^5 /mL) were treated with or without MG-132 or SB 203580 for 1 h before stimulation with TNF- α for different time points. Cells were washed and resuspended in cold phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA). After blocking with 2% human pooled serum for 20 min at 4 °C and washing with PBS supplemented with 0.5% BSA, the cells were incubated either with FITC-conjugated mouse anti-human ICAM-1 monoclonal antibody or fluorescein-conjugated mouse IgG₁ isotype for 30 min at 4 °C in dark. After washing, the cells were finally resuspended in PBS supplemented with 0.5% BSA. Surface ICAM-1 expression was then analysed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA). The expression of surface ICAM-1 was measured as mean fluorescence intensity (MFI).

cDNA expression array analysis

Total RNA was extracted from EoL-1 cells (1×10^7) treated with or without TNF- α (20 ng/mL) for 4 h using Tri-reagent (Molecular Research Center Inc., Cincinnati, OH, USA). A Pathway-specific Human NF- κ B Pathway-1 GEArrayTM Kit (SuperArray Inc., Bethesda, MD, USA) was used to analyse the gene expression profile of the NF- κ B pathway. Briefly, 5 μ g total RNA was used as template for biotinylated cDNA probe synthesis. RNA was reverse-transcribed by gene-specific primers with biotin-16-dUTP. Biotinylated cDNA probes were denatured and hybridized to NF- κ B pathway gene-specific cDNA fragments spotted on the membranes. The GEArray membranes were then washed and blocked with GEA blocking solution, and incubated with alkaline phosphatase conjugated streptavidin. The hybridized biotinylated probes were detected by a chemiluminescent method using the alkaline phosphatase substrate, CDP-Star[®]. The results were analysed using Bio-Rad Quantity OneTM software (Bio-Rad Laboratories, Hercules, CA, USA). Each array membrane comprised 23 marker genes in duplicate spots, two positive controls including β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and a negative control, bacterial plasmid pUC18. The relative expression levels of different genes were estimated by comparing their signal intensity with that of internal control GAPDH. A more than twofold increase in signal intensity was considered to be significant.

Western blot analysis

EoL-1 cells (1×10^7) with different treatments were washed with PBS and lysed in 0.1 mL lysis buffer (20 mM Tris-HCL, pH 8.0, 120 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM EGTA, 0.05% 2-mercaptoethanol, 1X protease inhibitors). Cell debris was removed by centrifugation at 10 000 g for 10 min and the supernatants were boiled in Laemmli sample

buffer (Bio-Rad) for 5 min. An equal amount of proteins was subjected to sodium dodecyl sulphate-10% polyacrylamide gel electrophoresis, and the proteins were blotted onto a PVDF membrane (Amersham and Pharmacia Biotech, Amersham, UK). Membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20, pH 7.6 overnight at 4 °C and probed with different primary rabbit anti-human antibodies for 1 h at room temperature. After washing, membranes were incubated with secondary donkey anti-rabbit antibody coupled to horseradish peroxidase (Amersham and Pharmacia Biotech) for 1 h at room temperature. Antibody-antigen complexes were then detected using the ECL chemiluminescent detection system according to the manufacturer's instructions (Amersham and Pharmacia Biotech).

Electrophoretic mobility shift assay (EMSA)

After EoL-1 cells (1×10^7) were harvested and washed, nuclear proteins were extracted with NE-PERTM nuclear and cytoplasmic extraction reagents (Pierce Chemical Co., New York, NY, USA) according to the manufacturer's instructions. Equal amounts of nuclear extracts were subjected to a test for NF- κ B protein/DNA binding using LightShiftTM chemiluminescent EMSA kit (Pierce Chemical Co.) with a biotin end-labelled NF- κ B oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') (Research Genetics, Huntsville, AL, USA). Briefly, nuclear extracts were incubated with biotin end-labelled NF- κ B oligonucleotide for 20 min at room temperature to allow DNA/protein binding. The DNA/protein complexes were then resolved by a 6% native polyacrylamide gel electrophoresis and transferred to a Hybond-N⁺ membrane (Amersham and Pharmacia Biotech). The biotin end-labelled DNA was detected using a streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate according to the manufacturer's instructions.

Statistical analysis

All data are expressed as mean $\pm$ SEM. Differences between groups were assessed by the non-parametric Kruskal-Wallis test. A probability $P \leq 0.05$ was considered significantly different. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, version 10.1.4 (SPSS Inc., Chicago, IL, USA).

Results

Effect of TNF- α on the expression of cell surface ICAM-1 on EoL-1 cells and blood eosinophils

As shown in Fig. 1, normal EoL-1 cells expressed low levels of surface ICAM-1 molecules (MFI about 10). Treatment with TNF- α (20 ng/mL) for 1 h did not cause any significant increase in ICAM-1 expression. However, TNF- α could significantly induce ICAM-1 expression on EoL-1 cells in a time-dependent manner from 2 to 4 h with two- to threefold increases compared with the control ($P = 0.0273$). We have also performed the same experiment on normal peripheral blood eosinophils, and found overnight incubation with TNF- α (20 ng/mL) could significantly induce ICAM-1 expression. (mean $\pm$ SEM MFI: isotypic control 3.3 ± 0.24 ; medium control 3.8 ± 0.24 ; TNF- α treatment 6.6 ± 0.57 ; TNF- α treatment vs. medium control, $P = 0.0025$).

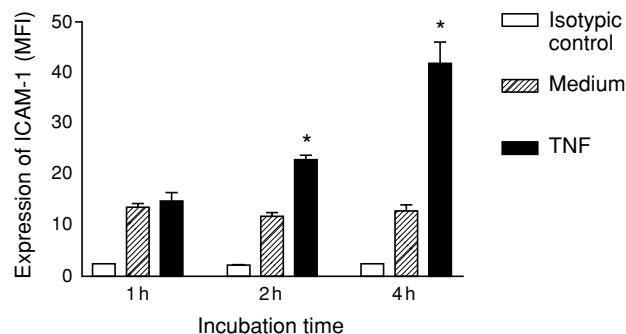


Fig. 1. Effect of TNF- α on the expression of surface ICAM-1 on EoL-1 cells. EoL-1 cells (5×10^5 /mL) were treated with or without TNF- α (20 ng/mL) from 1 h to 4 h followed by incubating with mouse anti-ICAM-1 antibody. Results are expressed as the arithmetic mean $\pm$ SEM of triplicate experiments. *MFI of TNF- α treated group for 2 and 4 h vs. 1 h, $P = 0.027$.

Effect of MG-132 and SB 203580 on surface ICAM-1 expression of EoL-1 cells

As shown in Fig. 2, treatment of TNF- α alone (20 ng/mL) for 4 h could significantly increase surface ICAM-1 expression on EoL-1 cells. There was no significant difference in ICAM-1 expression between control and treatment with MG-132 (5 μ M) or SB 203580 (20 μ M) alone. Figure 2(a) shows that 1 h pre-incubation of cells with MG-132 (5 μ M) could significantly suppress the TNF- α -induced ICAM-1 expression ($P = 0.01$). However, it was found that 1 h pre-treatment with SB 203580 (20 μ M) could not suppress surface ICAM-1 expression on EoL-1 cells (Fig. 2b). There was no significant change in TNF- α -induced ICAM-1 expression when the cells were pre-incubated with DMSO solvent (Fig. 2a).

Effect of TNF- α on the expression profile of NF- κ B pathway-related genes in EoL-1 cells

Figure 3 shows that TNF- α modulated the expression profile of genes involved in NF- κ B signal transduction pathway in EoL-1 cells. The locations of each duplicated gene are shown in Table 1. The expression of positive controls β -actin (3G, 4G) and GAPDH (5G, 6G, 7G, 8G, 8E and 8F) remained constant in both the presence and absence of TNF- α . As also shown in Table 1, the mRNAs of NF- κ B (p105) and its inhibitor I κ B- α were up-regulated in TNF- α -treated EoL-1 cells. The ICAM-1 mRNA level in EoL-1 cells was markedly elevated by TNF- α . The mRNA level of several pro-inflammatory cytokines and chemokines such as TNF- α , GM-CSF, IL-8 and MCP-1 were also significantly increased with TNF- α stimulation.

Effect of TNF- α on p38 MAPK phosphorylation in EoL-1 cells and blood eosinophils

It was found that TNF- α (20 ng/mL) could also activate the p38 MAPK signalling pathways in both EoL-1 cells (Fig. 4a) and blood eosinophils (Fig. 4b) by inducing a rapid phosphorylation of p38 MAPK at 10 min.

Effect of TNF- α , MG-132 and SB 203580 on I κ B- α expression and phosphorylation in EoL-1 cells

As shown in Fig. 5(a), I κ B- α was degraded quickly in EoL-1 cells after 10 min treatment with 20 ng/mL TNF- α . Compared

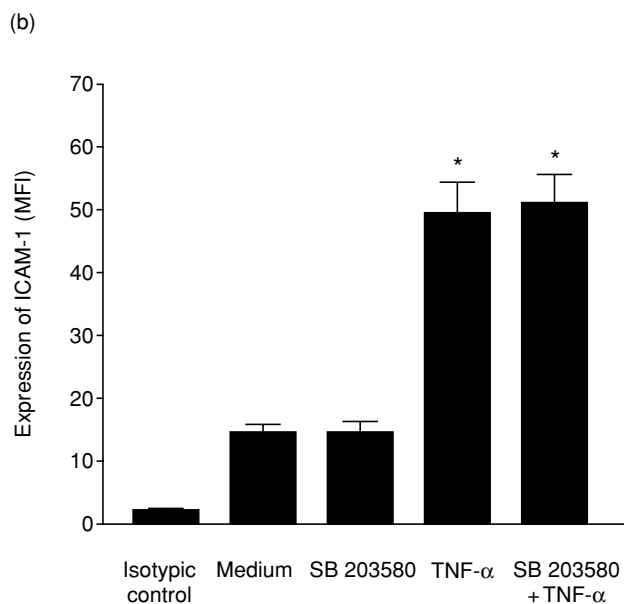
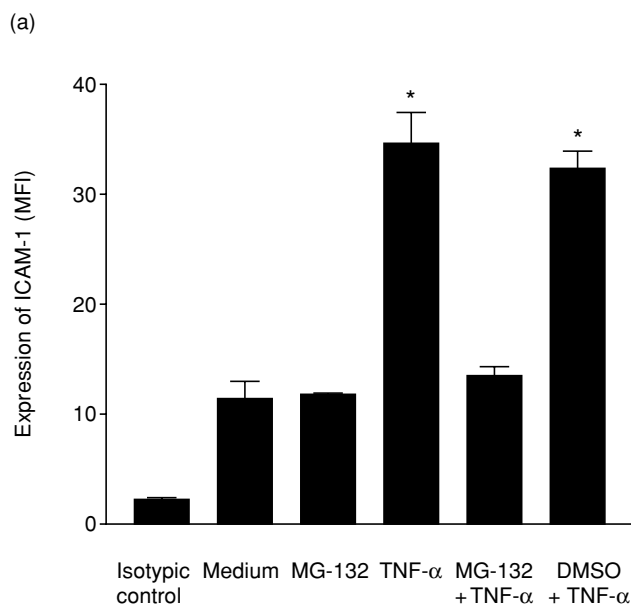


Fig. 2. Effect of MG-132 and SB 203580 on the TNF- α -induced ICAM-1 expression on EoL-1 cells. EoL-1 cells (5×10^5 /mL) were treated with or without (a) MG-132 (5 μ M) and (b) SB 203580 (20 μ M) for 1 h followed by stimulation with or without TNF- α (20 ng/mL) for a further 4 h. Results are expressed as the arithmetic mean $\pm$ SEM of triplicate experiments. * (a) MFI of TNF- α treatment $\pm$ DMSO vs. medium control, $P = 0.01$ and (b) MFI of TNF- α treatment $\pm$ SB 203580 vs. medium control, $P = 0.02$.

with the control, EoL-1 cells pre-treated with MG-132 (5 μ M) for 1 h before TNF- α stimulation could greatly protect I κ B- α from being degraded. However, pre-incubating EoL-1 cells for 1 h with SB 203580 (20 μ M) did not protect I κ B- α and I κ B- α expression was similar to that of TNF- α -treated cells. Figure 5 (b) shows that TNF- α (20 ng/mL) could rapidly induce phosphorylation of I κ B- α in EoL-1 cells. It was also demonstrated that 1 h pre-incubation with either MG-132 (5 μ M) or SB 203580 (20 μ M) in EoL-1 cells did not show any effect on I κ B- α phosphorylation.

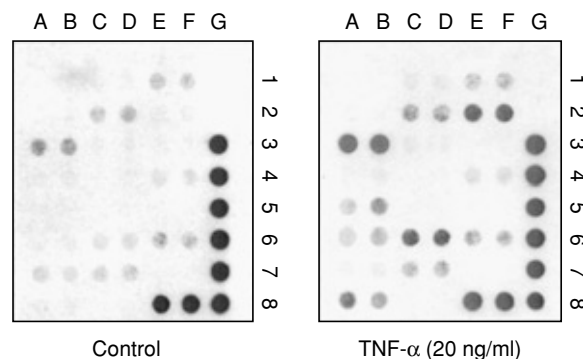


Fig. 3. The expression profile of NF- κ B pathway-related genes in EoL-1 cells treated with or without TNF- α (20 ng/mL). Each gene is presented in duplicate, and the locations in the arrays are as shown. Negative control: pUC18 DNA (1G, 2G). Positive control: β -actin (3G, 4G) and glyceraldehyde-3-phosphate dehydrogenase GAPDH (5G, 6G, 7G, 8G, 8E and 8F). Experiments were performed in triplicate with essentially identical results, and representative blots are shown.

Table 1. Differential expression profile of genes related to the human NF- κ B pathway in control or TNF- α -treated EoL-1 cells

Array location	Gene names	Ratio in gene expression level (TNF- α /control)
6C (6D)	NF- κ B (p105)	10.4
3A (3B)	I κ B- α	7.7
4A (4B)	I κ B kinase gamma subunit	3.3
2E (2F)	ICAM-1	23.2
7C (7D)	TNF- α	2.1
8A (8B)	Tumor necrosis factor, alpha-induced protein 1	18.0
2C (2D)	GM-CSF	2.3
6A (6B)	MCP-1	10.2
5A (5B)	IL-8	19.8
6E (6F)	Mitogen-activated protein kinase kinase kinase14 (MAP3K14)	
3C (3D)	I κ B kinase alpha subunit	NS
1C (1D)	c-rel	NS
2A (2B)	G-CSF	NS
4E (4F)	IL-6	NS
1E (1F)	E-selectin	NS
7A (7B)	P-selectin	NS
1A (1B)	c-myc	ND
3E (3F)	I κ B kinase beta subunit	ND
5C (5D)	Inducible nitric oxide synthase	ND
4C (4D)	IL-2	ND
7E (7F)	TNF- β	ND
5E (5F)	IRF-1	ND
8C (8D)	VCAM-1	ND

NS = no significance when compared with control; ND = non-detectable in both TNF- α stimulated and control cells.

Effect of MG-132 and SB 203580 on TNF- α -induced NF- κ B activity

Figure 6 illustrates that untreated EoL-1 cells did not show a shifted band in lane 1, indicating a low activity of NF- κ B. In lane 2, TNF- α (20 ng/mL) was shown to activate NF- κ B for subsequent genes transcription as there was a band shift observed. MG-132 (lane 3) was found to inhibit TNF- α -induced

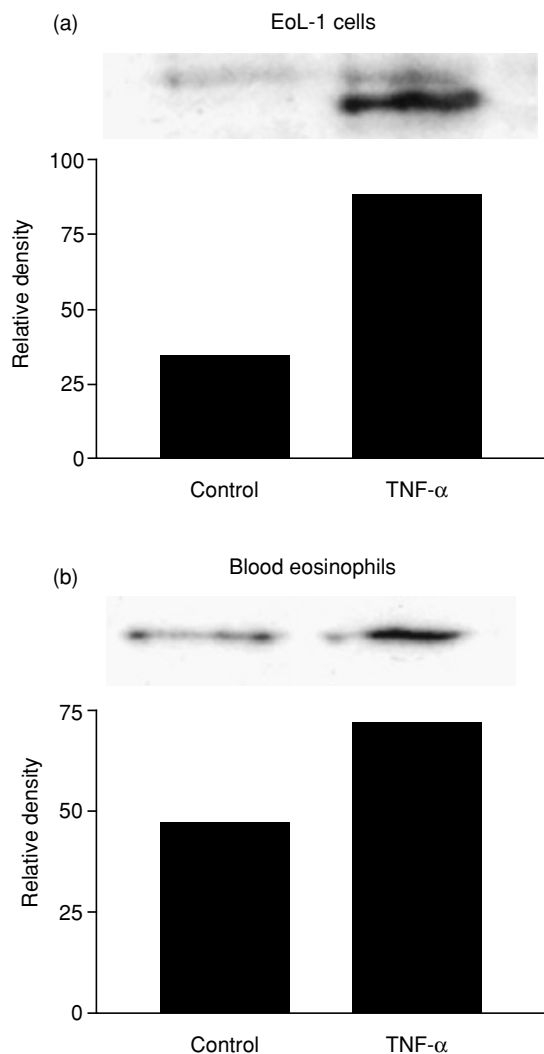


Fig. 4. Effects of TNF- α on the activation of p38 MAPK. Cells (5×10^6) were stimulated with or without TNF- α (20 ng/mL) for 10 min. Total cellular proteins were extracted for the detection of phosphorylated p38 MAPK by Western blot analysis in (a) EoL-1 cells and (b) blood eosinophils. Experiments were performed in triplicate with essentially identical results, and representative blots and densitometric analysis are shown.

NF- κ B activation. However, addition of SB 203580 (lane 4) did not show inhibiting effect. Lane 5 and lane 6 acted, respectively, as negative control and competitive control to confirm the specificity of the NF- κ B band shift.

Discussion

The transcription factor NF- κ B and the protein kinase p38 MAPK have been shown to serve as important regulators in allergic inflammation [8, 10]. NF- κ B was found to regulate adhesion molecules including ICAM-1 and VCAM-1, and a number of proinflammatory cytokines including IL-1, IL-2, IL-6, IL-8, GM-CSF and RANTES at transcription level in many inflammatory diseases [8, 9]. p38 MAPK could regulate the expression of RANTES and GM-CSF [25, 26], adhesion molecules [27], cellular degranulation and chemotaxis [28].

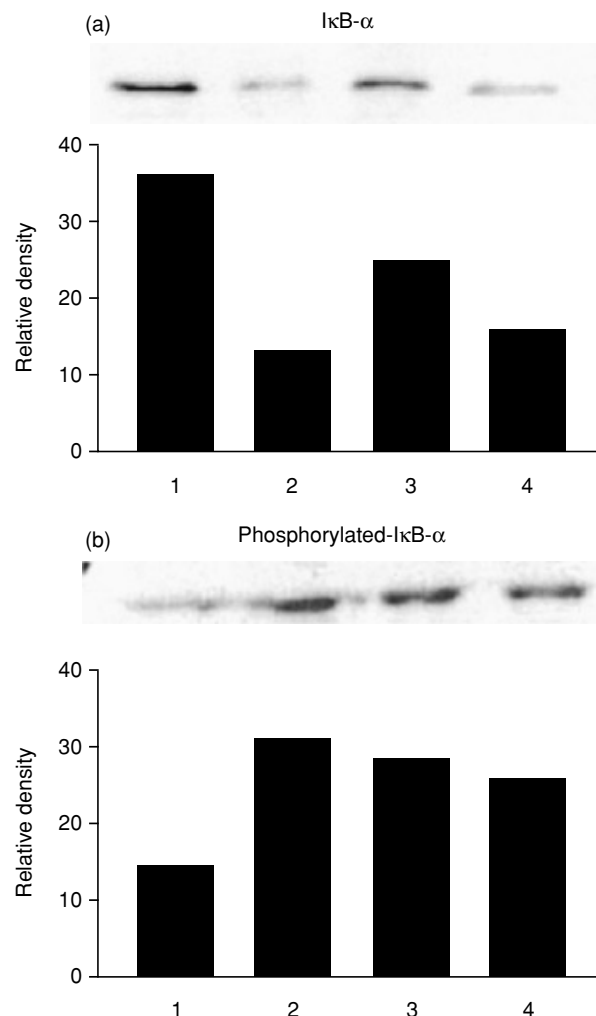


Fig. 5. Effects of MG-132 and SB 203580 on I κ B- α and its phosphorylation. Cells (5×10^6) were treated with or without MG-132 (5 μ M) or SB 203580 (20 μ M) for 1 h, followed by stimulation with TNF- α (20 ng/mL) for 10 min. Total cellular proteins were extracted for the detection of (a) I κ B- α and (b) phosphorylated I κ B- α by Western blot analysis. Experiments were performed in triplicate with essentially identical results, and representative blots and densitometric analysis are shown. Lane 1: control. Lane 2: TNF- α (20 ng/mL). Lane 3: MG-132 (5 μ M) + TNF- α (20 ng/mL). Lane 4: SB 203580 (20 μ M) + TNF- α (20 ng/mL).

Because human blood eosinophils have limited life-span and the purification of sufficient numbers of eosinophils is difficult and time consuming, a human eosinophilic leukaemic cell line, EoL-1, has been considered to be a useful *in vitro* model for studying the functions and regulation of human eosinophils [29]. These cells have been demonstrated to possess many characteristics of peripheral blood eosinophils including the expression of eosinophilic cationic protein (ECP), CD 95 (Fas), surface receptors of IL-3, IL-5, and GM-CSF [29]. They also express adhesion molecules including CD49d, CD11b, LFA-1, ICAM-1, Mac-1 and integrin α 4 [30]. Another study has reported that the expression of ICAM-1 on another human eosinophilic leukaemia, EoL-3, could be enhanced by platelet activating factor and platelet factor 4 [31]. Eosinophils have been shown to express TNF receptor I and receptor II [32]. TNF- α was found to stimulate I κ B degradation accompanied with subsequent NF- κ B translocation in many leucocytes

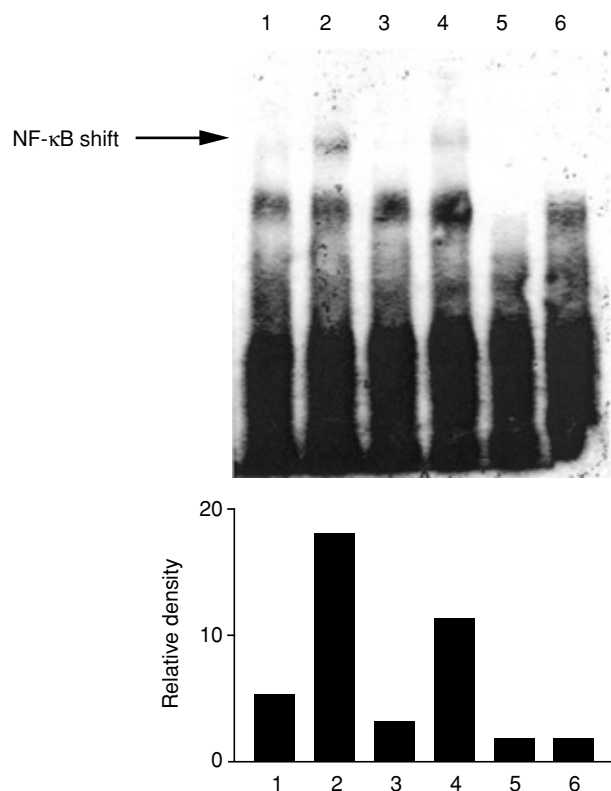


Fig. 6. Effects of MG 132 and SB 203580 on TNF- α -induced NF- κ B translocation and DNA binding activities. EoL-1 cells (1×10^7) were treated with or without MG-132 (5 μ M) or SB 203580 (20 μ M) for 1 h followed by stimulation with TNF- α (20 ng/mL) for a further 4 h. Nuclear protein was extracted and 5 μ g was subjected to EMSA. Experiments were performed in triplicate with essentially identical results, and representative blots and densitometric analysis are shown. Lane 1: control. Lane 2: TNF- α (20 ng/mL). Lane 3: MG 132 (5 μ M) + TNF- α (20 ng/mL). Lane 4: SB 203580 (20 μ M) + TNF- α (20 ng/mL). Lane 5: biotin-labelled NF- κ B oligos only. Lane 6: TNF- α (20 ng/mL) + biotin-labelled NF- κ B oligos and excess unlabelled NF- κ B oligos.

[9, 23]. Peripheral blood eosinophils have been reported to express LFA-1 and be able to express little ICAM-1 on their surface [33]. ICAM-1 interacted with LFA-1 molecules, which is important for the migration of eosinophils from blood to inflammatory sites. The expression of ICAM-1 on eosinophils can be induced following activation both *in vitro* [19] and *in vivo* [34]. A study has reported that TNF- α could induce the ICAM-1 expression on blood eosinophils [19] and we also demonstrated such a phenomenon. However, the detailed intracellular mechanism of p38 and NF- κ B regulating ICAM-1 expression on eosinophils has not yet been fully elucidated.

In the present study, untreated EoL-1 cells expressed a low level of ICAM-1 (Figs 1 and 2), but TNF- α could significantly enhance cell surface ICAM-1 expression on EoL-1 cells in a time-dependent manner (Fig. 1). The proteasome inhibitor MG-132 is a substrate analogue for inhibiting proteasome activity on the degradation of phosphorylated I κ B protein, thereby inhibiting the dissociation and activation of NF- κ B [35]. Inhibition of NF- κ B with MG-132 could almost completely inhibit the surface expression of TNF- α -induced ICAM-1 in EoL-1 cells (Fig. 2a). MG-132 alone did not have any significant effect on the surface expression of ICAM-1. We used the optimal concentration of MG-132 (5 μ M) and SB

203580 (20 μ M) with the highest inhibitory effect without any cell toxicity. Moreover, MG-132 (> 5 μ M) did not significantly increase the inhibitory effect on TNF- α -induced ICAM-1 expression (data not shown).

In our cDNA expression array analysis of NF- κ B pathway-related genes, the expression of ICAM-1 gene was greatly up-regulated by TNF- α at transcription level. TNF- α was found to enhance the mRNA expression of NF- κ B, its inhibitor I κ B- α , and the gamma subunit of I κ B protein kinase that phosphorylates and causes subsequent degradation of I κ B in EoL-1 cells. The above results imply that TNF- α not only triggers but also maintains its effect on NF- κ B mediated signal transduction in eosinophils. In addition, the gene expression of a group of pro-inflammatory cytokines and chemokines including TNF- α , TNF- α -induced protein-1, GM-CSF, IL-8 and MCP-1 were also up-regulated by TNF- α treatment. These results further indicate the critical role of NF- κ B in the regulation of pro-inflammatory cytokine expression and the gene transcriptions in activated eosinophils.

A recent report has demonstrated that p38 MAPK is required for NF- κ B-dependent expression of cytokine genes by the modulation of DNA binding of TATA-binding protein (TBP) to the TATA box [36]. Inhibition of p38 MAPK could potentially attenuate NF- κ B-dependent transcription [37]. Our recent results have also shown that the activation of both p38 MAPK and NF- κ B are required for TNF- α -induced eotaxin release in peripheral blood eosinophils [38]. Using Western blot analysis, we found that TNF- α could rapidly induce the phosphorylation of p38 MAPK in both EoL-1 cells and blood eosinophils (Figs 4a and b). This finding concurs with our recent data on human peripheral blood eosinophils [38], thereby confirming the activation of both NF- κ B and p38 MAPK signalling pathways in TNF- α -stimulated EoL-1 cells. In an attempt to further investigate the role of p38 MAPK in TNF- α -induced ICAM-1 expression on EoL-1 cells, we used a specific p38 MAPK inhibitor, SB 203580, a pyridinyl imidazole inhibitor of p38 MAPK that competitively binds to the ATP-binding pocket of p38 MAPK, thereby inhibiting its downstream kinase activity [10]. As shown in Fig. 2(b), inhibition of p38 MAPK with SB 203580 did not inhibit TNF- α -induced ICAM-1 expression on EoL-1 cells, suggesting that the p38 MAPK signalling pathway did not regulate the post-transcription level of ICAM-1 in EoL-1 cells. TNF- α could induce I κ B- α degradation, which could be significantly inhibited by MG-132 but not SB 203580 (Fig. 5a). It was also found to significantly induce the phosphorylation of I κ B- α , which could not be suppressed by both MG-132 and SB 203580 (Fig. 5b). This is because MG-132 affected the proteolytic activity of proteasome, and treatment with MG-132 might not significantly suppress the level of TNF- α -induced I κ B- α phosphorylation. Previous studies have found that the p38 MAPK pathway might not interfere with NF- κ B DNA binding activity [37, 39]. In our EMSA study, treatment with MG-132 but not SB 203580 inhibited the NF- κ B band shift in TNF- α -stimulated EoL-1 cells (Fig. 6). These results indicate that TNF- α can activate NF- κ B translocation and DNA binding activity in EoL-1 cells, whereas the TNF- α -activated p38 MAPK do not affect the NF- κ B translocation and hence its activity. Our results were also supported by other findings showing that p38 MAPK regulates the expression of VCAM-1 but not the surface expression of ICAM-1 on human umbilical vein endothelial cells stimulated by TNF- α [27], as well as in the A549 human

epithelial cell line, p38 MAPK is not required for the IL-1 β -induced surface expression of ICAM-1 [40].

In conclusion, the above mechanistic findings suggest that TNF- α -induced surface ICAM-1 expression through the NF- κ B activation pathway is independent of the TNF- α -activated p38 MAPK activation in EoL-1 cells. This implies that NF- κ B plays a critical role in the adhesion and migration of eosinophils in allergic inflammation. Our results should provide some biochemical basis for the future development of more effective drugs for allergy and inflammatory diseases.

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References

- Westerhof F, Timens W, van Oosten A et al. Inflammatory cell distribution in guinea pig airways and its relationship to airway reactivity. *Med Inflamm* 2001; 10:143–54.
- Walsh GM. Human eosinophils: their accumulation, activation and fate. *Br J Haematol* 1997; 97:701–9.
- Erger RA, Casale TB. Tumor necrosis factor alpha is necessary for granulocyte-macrophage-colony-stimulating-factor-induced eosinophil transendothelial migration. *Int Arch Allergy Immunol* 1998; 115:24–32.
- Ebisawa M, Liu MC, Yamada T et al. Eosinophil transendothelial migration induced by cytokines. II. Potentiation of eosinophil transendothelial migration by eosinophil-active cytokines. *J Immunol* 1994; 152:4590–6.
- Jagels MA, Daffern PJ, Zuraw BL, Hugli TE. Mechanisms and regulation of polymorphonuclear leukocyte and eosinophil adherence to human airway epithelial cells. *Am J Respir Cell Mol Biol* 1999; 21:418–27.
- Giembycz MA, Lindsay MA. Pharmacology of the eosinophil. *Pharmacol Rev* 1999; 51:213–340.
- Barnes PJ, Chung KF, Page CP. Inflammatory mediators of asthma: an update. *Pharmacol Rev* 1998; 50:515–96.
- Yang L, Cohn L, Zhang DH et al. Essential role of nuclear factor B in the induction of eosinophilia in allergic airway inflammation. *J Exp Med* 1998; 188:1739–50.
- Baldwin AS Jr. The NF- κ B and I κ B proteins: new discoveries and insights. *Annu Rev Immunol* 1996; 14:649–83.
- Herlaar E, Brown Z. p38 MAPK signalling cascades in inflammatory disease. *Mol Med Today* 1999; 5:439–47.
- Calderon E, Lockey RF. A possible role for adhesion molecules in asthma. *J Allergy Clin Immunol* 1992; 90:852–65.
- Rojas AI, Ahmed AR. Adhesion receptors in health and disease. *Crit Rev Oral Biol Medical* 1999; 10:337–58.
- Hynes RO. Cell adhesion: old and new questions. *Trends Cell Biol* 1999; 9:M33–7.
- Joseph-Silverstein J, Silverstein RL. Cell adhesion molecules: an overview. *Cancer Invest* 1998; 16:176–82.
- Staunton DE, Marlin SD, Stratowa C et al. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. *Cell* 1988; 52:925–33.
- van de Stolpe A, van der Saag PT. Intercellular adhesion molecule-1. *J Mol Med* 1996; 74:13–33.
- Hellewell PG. Adhesion molecule strategies. *Pulm Pharmacol Ther* 1999; 12:137–41.
- Hartnell A, Moqbel R, Walsh GM et al. Fc gamma and CD11/CD18 receptor expression on normal density and low density human eosinophils. *Immunology* 1990; 69:264–70.
- Czech W, Krutmann J, Budnik A et al. Induction of intercellular adhesion molecule 1 (ICAM-1) expression in normal human eosinophils by inflammatory cytokines. *J Invest Dermatol* 1993; 100:417–23.
- Smith CH, Barker JN, Lee TH. Adhesion molecules in allergic inflammation. *Am Rev Respir Dis* 1993; 148:S75–8.
- Fiedler MA, Wernke-Dollries K, Stark JM. Inhibition of TNF- α -induced NF- κ B activation and IL-8 release in A549 cells with the proteasome inhibitor MG-132. *Am J Respir Cell Mol Biol* 1998; 19:259–68.
- Musso M, Ghiorzo P, Fiorentini P et al. An upstream positive regulatory element in human GM-CSF promoter is recognized by NF- κ B/Rel family members. *Biochem Biophys Res Commun* 1996; 223:64–72.
- Gerondakis S, Grumont R, Rourke I et al. The regulation and roles of Rel/NF- κ B transcription factors during lymphocyte activation. *Curr Opin Immunol* 1998; 10:353–9.
- Pahl HL. Activators and target genes of Rel/NF- κ B transcription factors. *Oncogene* 1999; 18:6853–66.
- Hashimoto S, Matsumoto K, Gon Y et al. p38 MAP kinase regulates TNF- α -, IL-1 α - and PAF-induced RANTES and GM-CSF production by human bronchial epithelial cells. *Clin Exp Allergy* 2000; 30:48–55.
- Underwood DC, Osborn RR, Kotzer CJ et al. SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. *J Pharmacol Exp Ther* 2000; 293:281–8.
- Pietersma A, Tilly BC, Gaestel M et al. p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem Biophys Res Commun* 1997; 230:44–8.
- Kampen GT, Stafford S, Adachi T et al. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood* 2000; 95:1911–7.
- Wong CK, Ho CY, Lam CW et al. Differentiation of a human eosinophilic leukemic cell line, EoL-1: characterization by the expression of cytokine receptors, adhesion molecules, CD95 and eosinophilic cationic protein (ECP). *Immunol Lett* 1999; 68:317–23.
- Mayumi M. EoL-1, a human eosinophilic cell line. *Leuk Lymphoma* 1992; 7:243–50.
- Seguchi M, Nakajima S. Induction of ICAM-1 expression on the eosinophilic leukemia cell line-3 (EoL-3) by platelet-activating factor and platelet factor 4. *Ann Allergy Asthma Immunol* 1995; 74:255–7.
- Matsuyama G, Ochiai K, Ishihara C et al. Heterogeneous expression of tumor necrosis factor- α receptors I and II on human peripheral eosinophils. *Int Arch Allergy Immunol* 1998; 117 (Suppl. 1):28–33.
- Tachimoto H, Bochner BS. The surface phenotype of human eosinophils. *Chem Immunol* 2000; 76:45–62.
- Hansel TT, Braunstein JB, Walker C et al. Sputum eosinophils from asthmatics express ICAM-1 and HLA-DR. *Clin Exp Immunol* 1991; 86:271–7.
- Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 1998; 8:397–403.
- Carter AB, Knudtson KL, Monick MM et al. The p38 mitogen-activated protein kinase is required for NF- κ B-dependent gene expression. The role of TATA-binding protein (TBP). *J Biol Chem* 1999; 274:30858–63.
- Bergmann M, Hart L, Lindsay M et al. I κ B α degradation and nuclear factor- κ B DNA binding are insufficient for interleukin-1 β and tumor necrosis factor- α -induced κ B-dependent

- transcription. Requirement for an additional activation pathway. *J Biol Chem* 1998; 273:6607–10.
- 38 Wong CK, Zhang JP, Ip WK et al. Activation of p38 mitogen-activated protein kinase and nuclear factor- κ B in tumour necrosis factor-induced eotaxin release of human eosinophils. *Clin Exp Immunol* 2002; 128:483–9.
- 39 Wesselborg S, Bauer MK, Vogt M et al. Activation of transcription factor NF- κ B and p38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. *J Biol Chem* 1997; 272:12422–9.
- 40 Chen CC, Chen JJ, Chou CY. Protein kinase calpha but not p44/42 mitogen-activated protein kinase, p38, or c-Jun NH (2)-terminal kinase is required for intercellular adhesion molecule-1 expression mediated by interleukin-1 β . Involvement of sequential activation of tyrosine kinase, nuclear factor- κ B-inducing kinase, and I κ B kinase 2. *Mol Pharmacol* 2000; 58:1479–89.