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The biphasic immunoregulation of pyrimidylpiperazine (Y-40138) is IL-10 sensitive and requires NF-κB targeting in the alveolar epithelium

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1 Pyrimidylpiperazine (Y-40138), a synthetic derivative of *N*-[1-(4-{[4-(pyrimidin-2-yl)piperazin-1-yl]methyl}phenyl)cyclopropyl] acetamide, is a novel dual regulator of pro- and anti-inflammatory cytokines *in vivo*. The aim of the present study was to determine the signal transduction mechanisms implicated *in vitro*.

2 In alveolar epithelial cells, pre-treatment (30 min) with Y-40138 reduced LPS-induced biosynthesis of IL-1β, IL-6 and TNF-α, an effect paralleled by up-regulating an anti-inflammatory counter-loop mediated through IL-10.

3 This differential regulation of pro- and anti-inflammatory signals was accompanied by an inhibition of the nuclear localization of selective NF-κB subunits, particularly NF-κB$_1$ (p50), RelA (p65), the major transactivating member of the *Rel* family, RelB (p68) and c-Rel (p75). In addition, Y-40138 blockaded, in a dose-dependent manner, the LPS-induced nuclear activation of NF-κB.

4 Analysis of the upstream pathway involved in Y-40138-dependent retardation of LPS-induced NF-κB translocation/activation revealed the involvement of an IκB-α sensitive pathway. Pre-treatment with Y-40138 ameliorated LPS-induced degradation of IκB-α in the cytosolic compartment and retarded its phosphorylation, suggesting the involvement of an upstream kinase.

5 Recombinant IL-10 (0–10 ng ml^{-1}) blockaded, in a dose-dependent manner, LPS-induced biosynthesis of IL-1β, IL-6 and TNF-α. Furthermore, rhIL-10 reduced the DNA binding activity of NF-κB. Immunoneutralization of endogenous IL-10 by a polyclonal αIL-10 (5 μg ml^{-1}) reversed the inhibitory effect of Y-40138 on pro-inflammatory cytokines and partially restored the DNA binding activity of NF-κB.

6 These results indicate that Y-40138 mediated dual immunoregulation of pro- and anti-inflammatory cytokines is IL-10 sensitive and mediated through the IκB-α/NF-κB signal transduction pathway.

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Keywords: Cytokines; endotoxin; IκB-α; IL-10; immunopharmacology; NF-κB; pathophysiology

Abbreviations: AEBSF, 4-(2-Aminoethyl)]-benzene sulphonyl fluoride-HCl; BSA, Bovine serum albumin; DTT, Dithiothreitol; DMEM, Dulbecco's modified Eagle medium; EMSA, electrophoretic mobility shift assay; ELISA, enzyme-linked immuno-sorbent assay; fATII, foetal alveolar type II epithelial cells; FCS, foetal calf serum; HBSS, Hanks' balanced salt solution; HRP, horseradish peroxidase; IκB-α, inhibitory-κB; IKK, IκB kinase; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NIK, NF-κB inducing kinase; PBS, phosphate buffered saline; pyrimidylpiperazine (Y-40138), *N*-[1-(4-{[4-(pyrimidin-2-yl)piperazin-1-yl]methyl}phenyl)cyclopropyl] acetamide; rhIL-10, recombinant human interleukin 10; SDS–PAGE, sodiumdodecyl polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-tetramethyl-benzidine dihydrochloride; TNF-α, tumour necrosis factor-α

Introduction

Endotoxins of gram-negative microbes, components of the outer membrane of the cell wall, perform a vital function for bacterial viability but, if set free, induce in mammalians pathophysiological effects. Chemically, they are lipopolysaccharides (LPS) recognized as potent toxins that act as major factors mediating toxic manifestations of severe infections, endotoxemia and generalized sepsis (Rietschel *et al*., 1994).

The complex effects of LPS include non-specific activation of immunity, activation of the complement cascades and induction of the characteristic shock syndrome (Morrison & Ryan, 1987). Many of the effects of LPS are secondary to the overproduction of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor (TNF)-α. Specifically, TNF-α has been considered a central

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mediator of septic shock (Tracey *et al*., 1986), since it has been observed that an anti-TNF-α neutralizing serum was effective in preventing death from LPS in mice (Beutler *et al*., 1985). Moreover, intravenous injection of IL-1 has been shown to induce septic shock and acute lung injury with the sequestration of neutrophils and subsequent permeability oedema (Goldblum *et al*., 1987). In addition, both IL-6 and IL-8 have been recognized to exacerbate the pathophysiology of bacterial sepsis in primates (Redl *et al*., 1991). In its capacity to affect immune and non-immune cells, endotoxin, recognized as a pleiotropic macroamphiphile that interacts with several types of host cells, constitutes perhaps the most potent and multivalent molecule of bacterial origin.

The down-regulation of pro-inflammatory cytokines and augmentation of an anti-inflammatory response is a major focus of the rational approach to the treatment of inflammatory diseases, such as chronic inflammation, sepsis and rheumatoid arthritis. For instance, a novel recent study by Haskó *et al*. (2000) reported a potential role for extracellular purines, including adenosine and ATP, and inosine, a degradation product of these purines, as potent endogenous immunomodulatory molecules that inhibit inflammatory cytokine biosynthesis and protect against endotoxin-induced shock. It has also been reported, in addition, that selective inhibition of phosphodiesterases, a family of enzymes involved in the degradation of cyclic AMP (Haskó *et al*., 1998; Doherty, 1999), and steroids, such as glucocorticoids (Visser *et al*., 1998), differentially regulate the release of pro- and anti-inflammatory cytokines, thereby contributing to a crucial protection strategy in endotoxemia. Furthermore, experimental studies *in vivo* based on this approach also led to identification of a synthetic immunoregulator of cytokines, pyrimidylpiperazine *N*-[1-(4-{[4-(pyrimidin-2-yl)piperazin-1-yl]methyl}phenyl)cyclopropyl] acetamide (Y-39041 and its soluble hydrochloride salt, Y-40138) (Hanano *et al*., 2000; Hisadome *et al*., 2000). The immunopharmacological potential of pyrimidylpiperazine and its effect on LPS-induced release of pro-inflammatory mediators, however, have not been considerably recognized. It was previously observed that pyrimidylpiperazine exhibited a pharmacological property in muscle and cutaneous circulation *in vivo* (Laubie *et al*., 1971). However, it was Fukuda *et al*. (2000) who firstly reported pyrimidylpiperazine as a novel dual immunoregulator by exercising a selective anti-inflammatory role *in vivo* (suppressing LPS-induced TNF-α release and augmenting IL-10 biosynthesis), and that this compound has the potential to retard the onset of death due to septic shock. Furthermore, pyrimidylpiperazine was recently recognized as an anti-rheumatic drug with potent anti-inflammatory potential *in vivo* (Hisadome *et al*., 2000). Whether the immunomodulatory property assigned to pyrimidylpiperazine is manifested *in vitro* is not known and the underlying molecular mechanism involved has yet to be defined.

These observations, therefore, prompted us to investigate the mechanism of action of this novel pyrimidylpiperazine in the alveolar epithelium. We particularly show that pyrimidylpiperazine derivative (Y-40138) down-regulated LPS-induced biosynthesis of pro-inflammatory cytokines and that this effect is accompanied by up-regulating an anti-inflammatory signal through IL-10. Moreover, Y-40138 inhibited the degradation of IκB-α, the major cytosolic inhibitor of NF-κB, allowing its accumulation, blocked its phosphorylation, and subsequently retarded the nuclear translocation of selective NF-κB subunits. This compound also reduced the DNA-binding activity of NF-κB within the nuclear compartment. Our results indicate that the dual immunoregulatory potential of pyrimidylpiperazine is IL-10 sensitive and requires the selective targeting of the IκB-α/NF-κB signalling transduction pathway.

Methods

Chemicals and reagents

Unless otherwise indicated, chemicals of the highest analytical grade were purchased from Sigma-Aldrich (Dorset, U.K.). The pyrimidylpiperazine derivative, Y-40138, the water-soluble hydrochloride salt of *N*-[1-(4-{[4-(pyrimidin-2-yl)piperazin-1-yl]methyl}phenyl)cyclopropyl] acetamide (Y-39041), was synthesized at Welfide Corporation and is a generous gift of Dr Tetsuko Fukuda (Japan). IL-10 (human rDNA; rhIL-10) was purchased from the National Institute for Biological Standards and Control (NIBSC, U.K.). rhIL-10 was reconstituted in deionized H_2O, temporarily stored in aliquots at $-20^{\circ}C$, and its biological activity was authenticated as supplied by the manufacturer (1000 ng $ml^{-1} \approx 5000$ IU ml^{-1}). Rabbit polyclonal anti-rat IL-10 neutralizing antibody (αIL-10) was obtained from BioSource International Corporation (U.K.), purified from rabbit serum by protein A/G affinity column chromatography. The immunoglobulin solution was constituted in phosphate buffered saline (PBS; pH 7.3), and the endotoxin level was verified to be <0.01 ng μg^{-1} of protein. For the purpose of immunoneutralization of endogenously produced IL-10, αIL-10 was used at a concentration of 5 $\mu g\ ml^{-1}$, which is required to neutralize 5 ng ml^{-1} rat IL-10, as recommended (BioSource International). Antibodies for Western analysis with IκB and NF-κB subunits were all obtained from Santa Cruz Biotechnology (Wiltshire, U.K.). All experimental procedures involving the use of live animals were reviewed, approved and strictly adhered to under the Animals legislation (Scientific Procedures) Act, 1986 (U.K.).

Primary cultures of alveolar epithelia

Foetal alveolar type II (fATII) epithelial cells were isolated from lungs of foetuses, essentially as reported elsewhere (Haddad & Land, 2000a). Briefly, foetal rats were removed from pregnant Sprague-Dawley rats by caesarean section at day 19 of gestation (term = 22 days), the lungs excised, teased free from heart and upper airway tissue, and were finely minced then washed free of erythrocytes using sterile, chilled Mg^{2+}- and Ca^{2+}-free Hank's balanced salt solution (0.5 ml foetus^{-1}) (HBSS). The cleaned lung tissue was re-suspended in 1 ml foetus^{-1} HBSS containing trypsin (0.1 mg ml^{-1}), collagenase (0.06 mg ml^{-1}) and DNase I (0.012% w v^{-1}1), and was agitated at 37°C for 20 min. The solution was then centrifuged at $100 \times g$ for 2 min to remove undispersed tissue, the supernatant was saved to a fresh sterile tube and an equal volume of Dulbecco's Modified Eagle Medium (DMEM) with 10% (v v^{-1}) foetal calf serum (FCS) was added to the supernatant. After passing the supernatant through a 120 μm

pore sterile mesh, the filtrate was centrifuged at $420 \times g$ for 5 min, the pellet re-suspended in 20 mls DMEM/FCS and the cells were placed into a T-150 culture flask for 1 h at 37°C to enable fibroblasts and non-epithelial cells to adhere. Unattached cells were washed three times by centrifugation at $420 \times g$ for 5 min each and then seeded onto 24 mm diameter Transwell-clear permeable supports (Costar; 0.4 μm pore size) at a density of 5×10^6 cells per filter and were allowed to adhere overnight at 152 Torr ($\approx$21% O_2/5% CO_2). DMEM/FCS was exchanged for 4 ml of serum free PC-1 media (Biowhittaker, MD, U.S.A.) pre-equilibrated to $pO_2 = 152$ Torr and 37°C 24 h later and cells were maintained at this pO_2 until the experiment. In each case, and under conditions of independent pre-treatments, the adenylate energy charge, an index of cell viability and competence, remained $\geqslant 0.7$ and transepithelial monolayer resistance was monitored constantly at 250–350 $\Omega.cm^2$ (Haddad & Land, 2000a; Haddad *et al*., 2000).

Drug treatment and measurement of pro- and anti-inflammatory cytokines by ELISA

Epithelial cells were pre-treated for 30 min with Y-40138 (0, 0.01, 0.1, 1, 2.5, 5, 10 & 20 μg ml^{-1}, corresponding to $\approx$0, 0.025, 0.25, 2.5, 6.5, 13, 25 and 50 mM) and subsequently challenged with LPS (1 μg ml^{-1}) for 24 h. Cell-free supernatants were assayed for pro-inflammatory (IL-1β, IL-6, and TNF-α) (R&D Systems, U.K.) and anti-inflammatory (IL-10) (BioSource International Incorporation, U.K.) cytokine biosynthesis by two-site, solid phase, sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, rabbit immunoaffinity purified polyclonal anti-rat IL-1β (1 μg ml^{-1}), IL-6 (2 μg ml^{-1}), TNF-α (2 μg ml^{-1}) and IL-10 (2 μg ml^{-1}) antibodies were used to coat high-binding microtitre plates (MaxiSorp, Nunc) in bicarbonate buffer (0.1 M $NaHCO_3$ and 0.1 M NaCl, pH 8.2), as described previously (Safieh-Garabedian *et al*., 1997). After blocking in 3% bovine serum albumin (BSA), recombinant (standard) and biotinylated (recognition) immunoaffinity purified sheep anti-rat cytokine antibodies were employed for secondary detection. The colour was developed using streptavidin-poly-HRP (Amersham, U.K.) coupled with 3,3′,5,5′-tetramethyl-benzidine dihydrochloride (TMB) and 1 mM H_2O_2. The optical density was read at 450 nm against a background filter measuring at 595 nm, where the inter- and intra-assay coefficients of variations were reported at $\leqslant$10%. Results were extracted from the positive linear regression of the positive slope and cytokine concentrations expressed in pg ml^{-1}.

Preparation of subcellular extracts followed by Western immunoelectroblotting analysis (SDS–PAGE) and electrophoretic mobility shift assay (EMSA)

The signalling mechanism mediating Y-40138 dependent regulation of NF-κB translocation and activation in the alveolar epithelium is not well characterized. Accordingly, we designed a series of experiments to span NF-κB translocation/activation in response to LPS and pyrimidylpiperazine pre-treatment. Epithelial cells were pre-treated for 30 min with Y-40138, prior to exposure to LPS (1 μg ml^{-1}) for 24 h. Subcellular cytosolic/nuclear extracts were subsequently prepared, followed by Western analysis and electrophoretic mobility gel shift assay (EMSA), essentially as described previously (Haddad & Land, 2000a,b; Haddad *et al*., 2000). Briefly, cytosolic/nuclear extracts were prepared from monolayer filters washed twice in 5 ml ice-cold, pre-equilibrated PBS and cells ($1-2 \times 10^7$) were collected and centrifuged at $420 \times g$ for 5 min at 4°C. Nuclei were released by re-suspending the pellet in 250 μls buffer A containing (in mM): Tris-HCl (pH 7.8) 10, KCl 10, NaH_2PO_4 2.5, $MgCl_2$ 1.5, Na_3VO_4 1, dithiothreitol (DTT) 0.5, [4-(2-aminoethyl)]-benzene sulphonyl fluoride-HCl (AEBSF)] 0.4 and 2 μg ml^{-1} each of leupeptin, pepstatin A and aprotinin. The suspension was left in ice for 10 min followed by a 45-s homogenization at a moderate speed. Nuclei were collected by centrifuging the slurry at $4500 \times g$ for 5 min at 4°C and re-suspending in 100 μl buffer B (Buffer A adjusted to (in mM): Tris-HCl (pH 7.8) 20, KCl, 20% (v v^{-1}) Glycerol) 420. The supernatants thus obtained were termed cytosolic extracts. The nuclei were then lysed at 4°C for 30 min with gentle agitation, the debris cleared by centrifugation at $10{,}000 \times g$ for an additional 30 min at 4°C and the supernatants, termed nuclear extracts, were frozen in liquid nitrogen and stored at −70°C until used. In all cases, protein contents were determined by the Bradford method using BSA as a standard (Haddad & Land, 2000a,b).

Cytosolic and nuclear proteins (20–25 μg) were resolved over sodiumdodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE; 7.5%) gels at RT, blotted onto nitrocellulose membrane, and non-specific binding sites were subsequently blocked. Mouse monoclonal IgG_1 anti-IκB-α (H-4), IgG_{2b} anti-*p*IκB-α (B-9), rabbit polyclonal IgG anti-p50 (NLS), anti-p52 (K-27), anti-p65 (RelA; A), anti-p68 (RelB; C-19), and anti-p75 (c-Rel; N) (Santa Cruz Biotechnology, U.K.) antibodies were used for primary detection. Anti-rabbit Ig-biotinylated antibody (Amersham Life Science, U.K.) was employed for secondary detection followed by the addition of streptavidin-HRP conjugate and visualized on film by chemiluminescence. β-Actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable. Western blots were scanned by NIH MagiScanII and subsequently quantitated by UN-Scan-IT automated digitizing system (version 5.1; 32-bit), and the ratio of the density of the band to that of β-actin was subsequently performed.

Custom deoxy-oligonucleotide probe sequences were purchased from Genosys, U.K.: NF-κB, 5′-AGTTGAGGG-GACTTTCCCAGGC-3′ (binding sequence underlined). Gel-purified double-stranded DNA was end-labelled with [γ^{32}P]-ATP (NEN Life Sciences Products). Identical amounts of radioactive probe ($1-2 \times 10^4$ counts min^{-1}) were added to binding reactions containing 1–5 μg fATII nuclear extracts in a final volume of 40 μl in DNA binding buffer (20 mM HEPES (pH 7.9); 1 mM $MgCl_2$; 4% Ficoll). Reaction mixtures were incubated for 30 min at 25°C before separating on non-denaturing 4% polyacrylamide gels at RT and subjected to electrophoresis with 1 : 10 5 × Tris-Borate-EDTA buffer. A non-specific competitive polydeoxyinosinic-deoxycytidylic acid [poly(dI-dC)] (Amersham Pharmacia Biotech, U.K.) was added to reaction mixtures after addition of labelled probe. Gels were transferred to ion-exchange chromatography paper, vacuum dried and then electronically visualized on a Packard Instant phosphorimager. Specific quantitation of the corresponding DNA gel shift bands was

performed with phosphorimaging (Haddad & Land, 2000a; Haddad *et al*., 2001c).

Pre-treatment with rhIL-10 and αIL-10 and measurement of cytokines and NF-κB activation

Epithelial cells were pre-treated for 30 min with rhIL-10 (0–10 ng ml^{-1}), washed twice with sterile, pre-equilibrated PBS, then exposed to LPS (1 μg ml^{-1}) for 24 h. Cell-free supernatants were assayed for pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) by ELISA. Nuclear extracts were prepared as above and the DNA binding activity was determined by EMSA. In separate experiments, epithelial cells were exposed to Y-40138 in the absence or presence of αIL-10 (5 μg ml^{-1}) antibody, prior to exposure to LPS (1 μg ml^{-1}) for 24 h. Supernatants were withdrawn and analysed for cytokines and nuclear extracts were used to determine NF-κB activation.

Statistical analysis and data presentation

Data are the means and the error bars the s.e.mean. Statistical evaluation of the difference in mean separation was performed by one-way analysis of variance (ANOVA), followed by *post hoc* Tukey's test, and the *a priori* level of significance at 95% confidence level was considered at $P<0.05$.

Results

The effect of Y-40138 on LPS-induced release of pro- and anti-inflammatory cytokines

The inhibitory action of Y-40138 on LPS-induced release of IL-1β is markedly effective at doses $\geqslant$2.5 μg ml^{-1}, as shown in Figure 1A. On the other hand, this compound suppressed IL-6 biosynthesis within the lower range of the dose-response curve ($\geqslant$0.1 μg ml^{-1}) (Figure 1B). In a manner more prominent than its effect on either IL-1β or IL-6, Y-40138 abrogated the induced release of TNF-α at lower concentrations falling within the dose-response curve ($\geqslant$0.01 μg ml^{-1}) (Figure 1C). The maximum inhibition of pro-inflammatory cytokine biosynthesis by Y-40138 was observed at a dose of 20 μg ml^{-1}, a concentration corresponding to $\approx$50 mM. As shown in Figure 2, there was no detectable effect of Y-40138 on LPS-induced IL-10 release at concentrations $\leqslant$0.1 μg ml^{-1}. The minimum effective concentration exhibiting significant induction in IL-10 release appeared at a dose $\geqslant$1 μg ml^{-1}, an effect concomitant with depressing LPS-induced release of pro-inflammatory cytokines.

The involvement of Y-40138 in IκB-α signalling and phosphorylation

Cell treatment with LPS (1 μg ml^{-1}; 24 h) induced IκB-α degradation in the cytosolic compartment, as compared with the control (Figure 3). Pre-treatment with Y-40138, prior to exposure to LPS, blocked IκB-α degradation and allowed its cytosolic accumulation, in a dose-dependent manner. Of note, Y-40138 effect on IκB-α was accompanied by dose-dependent inhibition of its phosphorylation (*p*IκB-α), suggesting the interference of an upstream kinase (Figure 3).

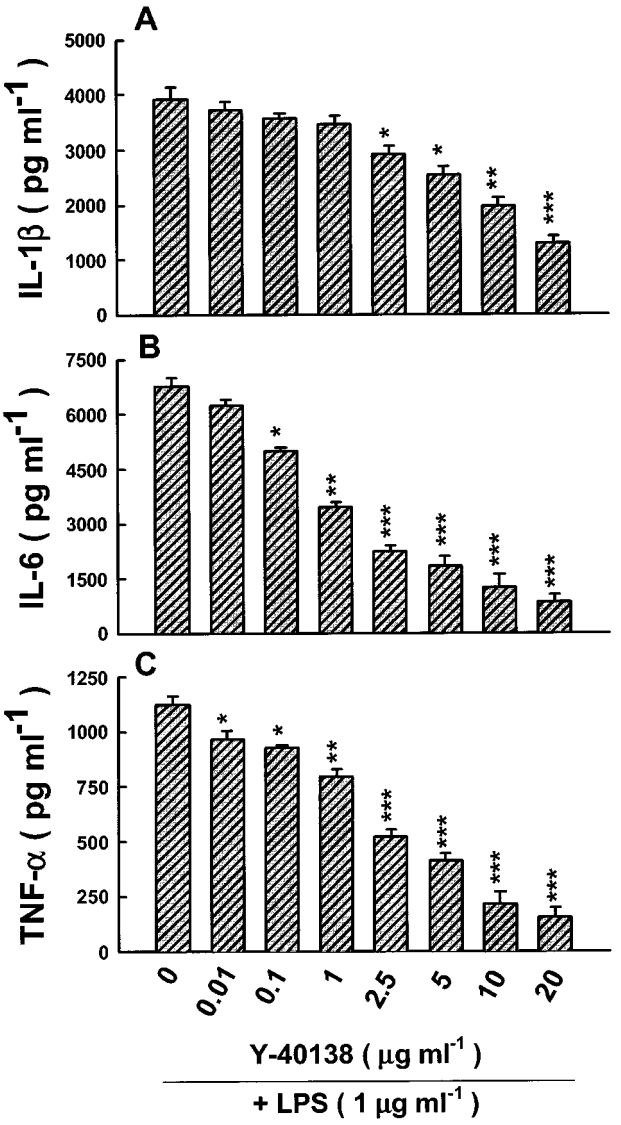

Figure 1 Selective amelioration of LPS-induced release of pro-inflammatory cytokines by Y-40138. (A) The effect of Y-40138 on IL-1β is prominent at doses $\geqslant$2.5 μg ml^{-1}. (B) The inhibitory effect of Y-40138 on IL-6 is evident at doses $\geqslant$1 μg ml^{-1}. (C) Y-40138 mitigated the effect of LPS on TNF-α within the range $\geqslant$0.01 μg ml^{-1}. *$P<0.05$, **$P<0.01$, ***$P<0.001$, as compared to control (LPS alone). Data are means and histograms the s.e.mean of four independent experiments run in duplicate.

The inhibitory role of Y-40138 in LPS-induced translocation/activation of NF-κB

In parallel to its ability to ameliorate, in a dose-dependent manner, the induced release of pro-inflammatory mediators by LPS, potentiate LPS-induced release of anti-inflammatory cytokines, and interfere in the transduction pathway governing IκB-α phosphorylation and degradation, Y-40138 provoked a dose-dependent inhibition of LPS-induced NF-κB translocation/activation (Figure 4). Western immunolelectroblotting analysis of NF-κB subunit composition in response to LPS challenge has been previously determined (Haddad *et al*., 2001c). The abundance of NF-κB$_2$ (p52) was not affected

and that of RelB (p68) was marginally induced in the nuclear compartment, whereas those of NF-κB_1 (p50), RelA (p65) and c-Rel (p75) were increased with ascending LPS concentration in a dose- and time-dependent manner (Haddad *et al*., 2001c). Pre-treatment with Y-40138 blocked, in a dose-dependent manner, the LPS-mediated translocation of p50, p65 and p75, but marginally affected the abundance of p68, as shown in Figure 4. In parallel to down-regulating NF-κB translocation, Y-40138 suppressed the nuclear DNA binding activity of this transcription factor, in a dose-dependent manner (Figure 5A). Histogram analysis of the corresponding gel-shifted bands shows quantitative depression of NF-κB activity with Y-40138 (Figure 5B). Negative linear inverse correlation simulating the per cent inhibition of pro-inflammatory cytokine biosynthesis and the per cent

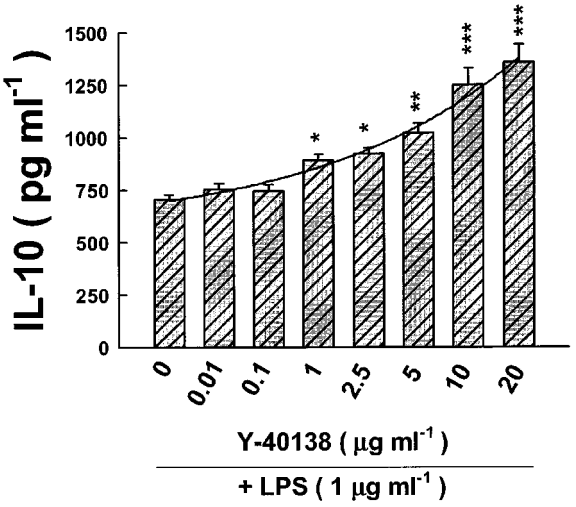

Figure 2 The role of Y-40138 in amplifying an anti-inflammatory loop through enhancement of IL-10. Spanning the dose-response curve revealed a prominent amplification effect of Y-40138 on LPS-induced IL-10 release at doses $\geqslant 1$ μg ml^{-1}. Regression analysis by *pseudo-voigt* curve fitting shows the prominent amplification loop through LPS-induced release of the anti-inflammatory cytokine IL-10. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, as compared to control (LPS alone). Data are means and histograms the s.e.mean of four independent experiments run in duplicate.

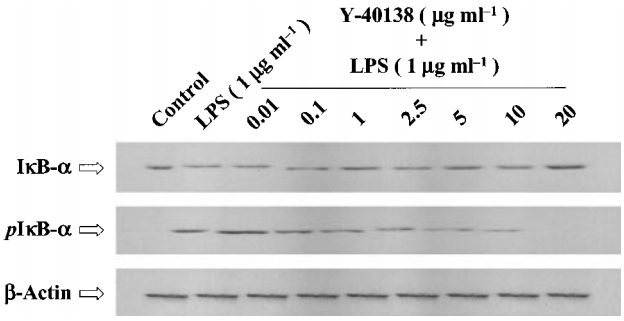

Figure 3 The regulatory potential of Y-40138 in governing IκB-α phosphorylation and degradation in the cytosolic compartment. Incubation of epithelial cells with LPS (1 μg ml^{-1}) for 24 h induced IκB-α degradation, relative to control. Pre-treatment with Y-40138, before exposure to LPS, blocked IκB-α degradation and allowed its accumulation within the cytosolic compartment. Y-40138 also blocked IκB-α phosphorylation, in a dose-dependent manner. The housekeeping β-actin protein was used as an internal reference for semi-quantitative loading in parallel lanes. $n=4$, which represents the number of independent experiments.

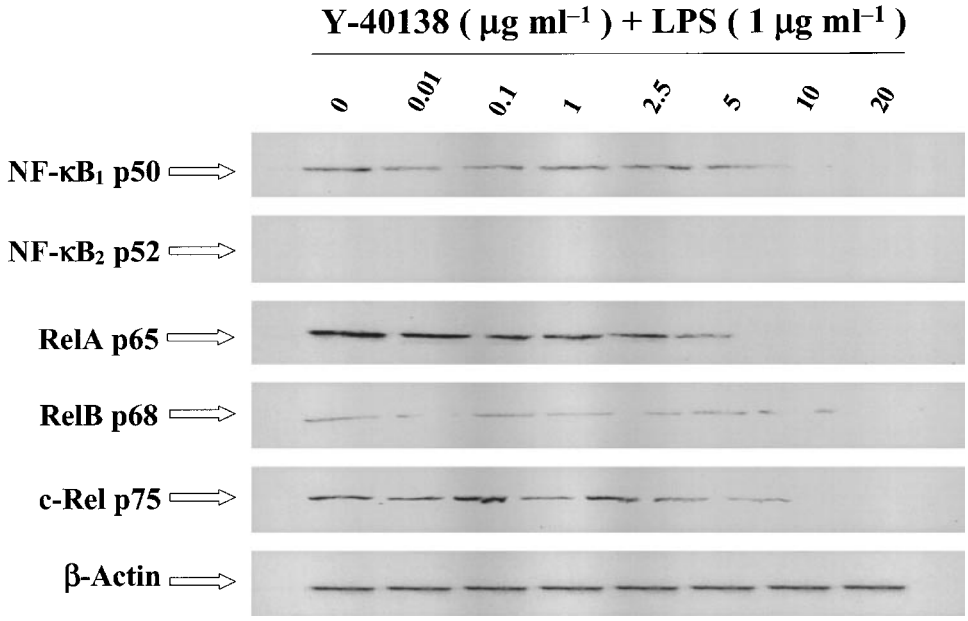

Figure 4 The effect of Y-40138 on LPS-induced NF-κB nuclear localization. Western immunoblotting shows the dose-dependent inhibition of LPS-induced translocation of NF-κB subunits. LPS (1 μg ml^{-1}; 24 h) induced the nuclear accumulation of NF-κB_1 (p50), RelA (p65), and c-Rel (p75), but it has no effect on NF-κB_2 (p52) and a mild effect on RelB (p68). Pre-treatment with Y-40138, prior to exposure to LPS, reduced LPS-induced nuclear accumulation of p50, p65 and p75, with mild effect on p68. The housekeeping β-actin protein was used as an internal reference for semi-quantitative loading in parallel lanes. $n=4$, which represents the number of independent experiments.

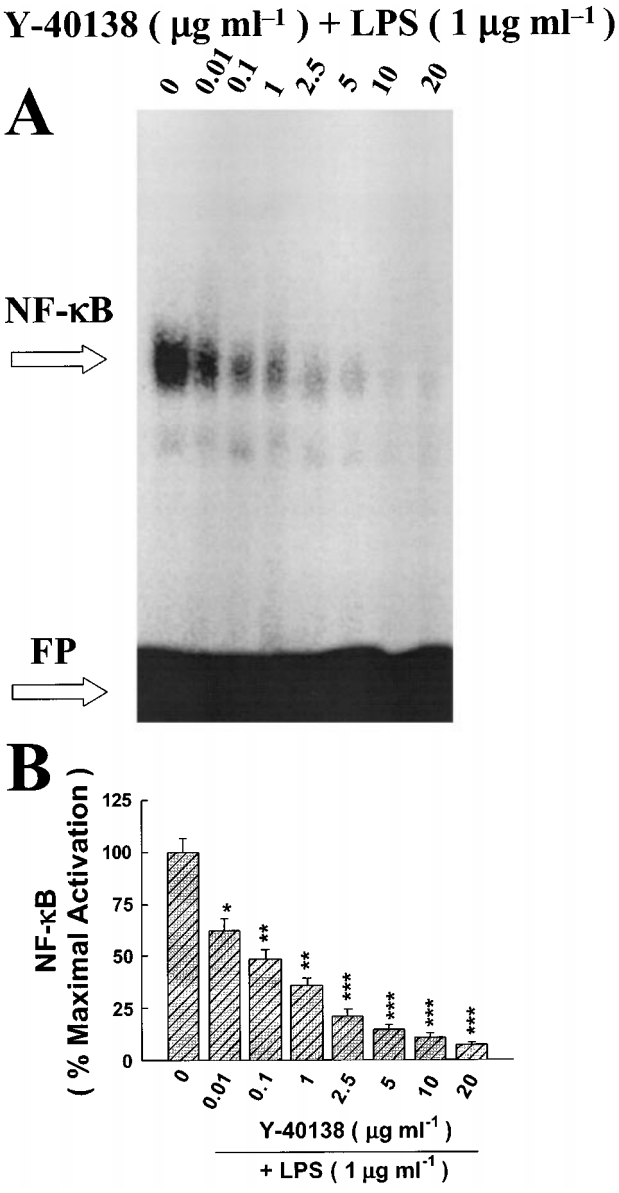

Figure 5 Electrophoretic mobility gel shift assay revealing the effect of Y-40138 on NF-κB DNA binding activity. (A) A representative EMSA showing the effect of Y-40138 on LPS-induced NF-κB activation. Y-40138 mitigated the induced activation of NF-κB across the dose-response curve. The upper open arrow indicates the NF-κB/DNA complex, and the lower open arrow indicates the faster migrating unbound free probe. (B) Histogram analysis of the intensity of the corresponding bands performed by phosphorimaging. $*P<0.05$, $**P<0.01$, $***P<0.001$, as compared to control (LPS alone). Data are means and histograms the s.e.mean of three independent experiments.

amplification of IL-10 is given in Figure 6 (A–C). Positive linear correlation simulating the per cent inhibition of pro-inflammatory cytokines and the per cent inhibition of NF-κB is shown in Figure 6 (D–E) (dotted lines represent the correlation interval at 95% confidence limit). Analysis of the 50% minimum effective inhibitory (IC_{50}) and stimulatory (EC_{50}) concentrations of Y-40138 and rhIL-10 on LPS-induced cytokine release, IκB-α abundance and NF-κB translocation/activation is shown in Table 1.

The potential role of rhIL-10 in regulating pro-inflammatory cytokines and NF-κB activation

As shown in Figure 7, rhIL-10 reduced, in a dose-dependent manner, LPS-induced activation of NF-κB (Figure 7A), concomitant with abrogating the LPS induction of pro-inflammatory cytokines IL-1β ($\geqslant$0.1 ng ml^{-1}), IL-6 ($\geqslant$1 ng ml^{-1}), and TNF-α ($\geqslant$0.1 ng ml^{-1}) (Figure 7B).

The effect of anti-IL-10 antibody (αIL-10) on Y-40138 dependent inhibition of pro-inflammatory cytokines and NF-κB activation

Simultaneous pre-treatment with αIL-10 and Y-40138 reversed the inhibitory effect of Y-40138 on LPS-induced NF-κB activation (Figure 8A) and pro-inflammatory cytokine biosynthesis (Figure 8B).

Discussion

There is increasing evidence that the alveolar epithelium plays an important role in regulating the inflammatory and metabolic response to oxidative stress and the accompanying inflammatory signal (Freeman *et al*., 1993; Haddad *et al*., 2000, 2001a,b,c), sepsis, endotoxemia, and other critical illnesses in the lung (Pittet *et al*., 1995; Matuschak & Lechner, 1996; Laffon *et al*., 1999; Matthay *et al*., 2000). Many of the side effects of lipopolysaccharide endotoxin (LPS) are secondary to the overproduction of pro-inflammatory mediators, such as the pleiotropic cytokines, which exacerbate the pathophysiological condition by activating and recruiting inflammatory cells. Therefore, the suppression of a pro-inflammatory signal, and the downstream conjugated inflammatory pathways, and augmentation of a counter-inflammatory response has been a major focus of the approach to the treatment of inflammatory diseases. For instance, glucocorticoids (Visser *et al*., 1998), extracellular purines (Haskó *et al*., 2000), phosphodiesterase selective inhibitors (Haskó *et al*., 1998; Doherty, 1999), pyrimidylpiperazine (Fukuda *et al*., 2000; Hisadome *et al*., 2000), and adrenoreceptor agonism/antagonism (Zhang *et al*., 1999) have been widely used to counteract the effects of inflammatory cytokines and subsequently suppress the protracted pathophysiological conditions *in vitro* and *in vivo*.

Although the immunopharmacological, anti-inflammatory potential of pyrimidylpiperazine (*N*-[1-(4-{[4-(pyrimidin-2-yl)piperazin-1-yl]methyl}phenyl)cyclopropyl] acetamide; Y-40138) has been previously recognized *in vivo* (Fukuda *et al*., 2000; Hanano *et al*., 2000; Hisadome *et al*., 2000), the underlying mechanism of action is not known. It has been reported that pyrimidylpiperazine acts as a dual regulator of pro- and anti-inflammatory cytokines and that it has the capacity to retard the onset of death due to septic shock (Fukuda *et al*., 2000; Hisadome *et al*., 2000). Furthermore, it is recognized as a potent anti-inflammatory drug in adjuvant arthritis (Hisadome *et al*., 2000). However, the signal transduction pathways involved in pyrimidylpiperazine-mediated immunoregulation are not well characterized, neither in the alveolar epithelium nor in other tissues. This study provides evidence that the biphasic immunopharmaco-

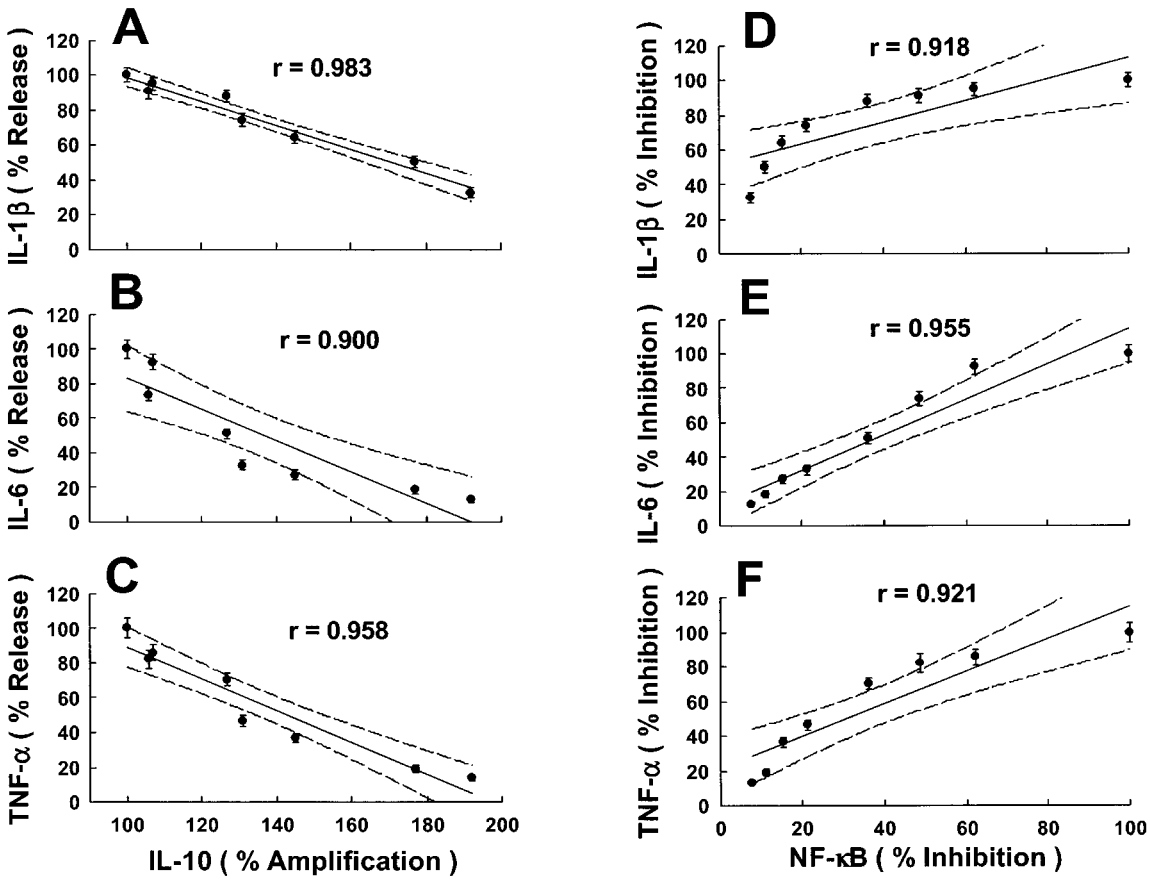

Figure 6 Negative sigmoidal inverse correlation with best subsets regression of cytokine per cent inhibition on the per cent amplification of a counter-inflammatory response through IL-10. (A) IL-1β, (B) IL-6 and (C) TNF-α correlation subsets with IL-10 amplification. Positive sigmoidal correlation with best subsets regression of cytokine per cent inhibition on the per cent inhibition of NF-κB. (D) IL-1β, (E) IL-6 and (F) TNF-α correlation subsets with NF-κB inhibition. Dotted lines correspond to limits of confidence interval at 95%. Data represent the mean$\pm$s.e.mean of four independent measurements run in duplicate.

Table 1 Analysis of the 50% minimum effective inhibitory (IC_{50}) and stimulatory (EC_{50}) concentrations of Y-40138 and rhIL-10 on LPS-induced cytokine release, IκB-α and NF-κB in the alveolar epithelium

	Y-40138 (μg ml^{-1}) *IC_{50}*	*Y-40138* (μg ml^{-1}) *EC_{50}*	*rhIL-10* (ng ml^{-1}) *IC_{50}*
IL-1β	12.45$\pm$1.28	–	3.35$\pm$0.27
IL-6	5.11$\pm$0.36	–	7.57$\pm$0.32
IL-10	–	8.02$\pm$0.75	–
TNF-α	6.55$\pm$0.47	–	4.12$\pm$0.18
IκB-α	–	5.25$\pm$0.42	–
*p*IκB-α	2.14$\pm$0.08	–	–
NF-κB Subunit Translocation			
NF-κB$_1$ p50	1.36$\pm$0.15	–	–
NF-κB$_2$ p52	–	–	–
RelA p65	3.21$\pm$0.23	–	–
RelB p68	0.95$\pm$0.07	–	–
c-Rel p75	2.58$\pm$0.18	–	–
NF-κB Activation			
NF-κB/DNA	0.72$\pm$0.08	–	1.25$\pm$0.16

Data are presented as mean$\pm$s.e.mean. $n=4$, each. IC_{50} and EC_{50} were determined from the negative and positive slopes of the linear regression curves, respectively.

logical potential of pyrimidylpiperazine is mediated through the IκB-α/NF-κB sensitive pathway *in vitro*.

Expectedly, pyrimidylpiperazine (Y-40138) suppressed, in a dose-dependent manner, the LPS-induced biosynthesis of pro-inflammatory cytokines, an effect accompanied by augmenting a counter-inflammatory signal mediated by IL-10. This compound blocked LPS-induced degradation of IκB-α, the major cytosolic inhibitor of NF-κB (Haddad *et al.*,

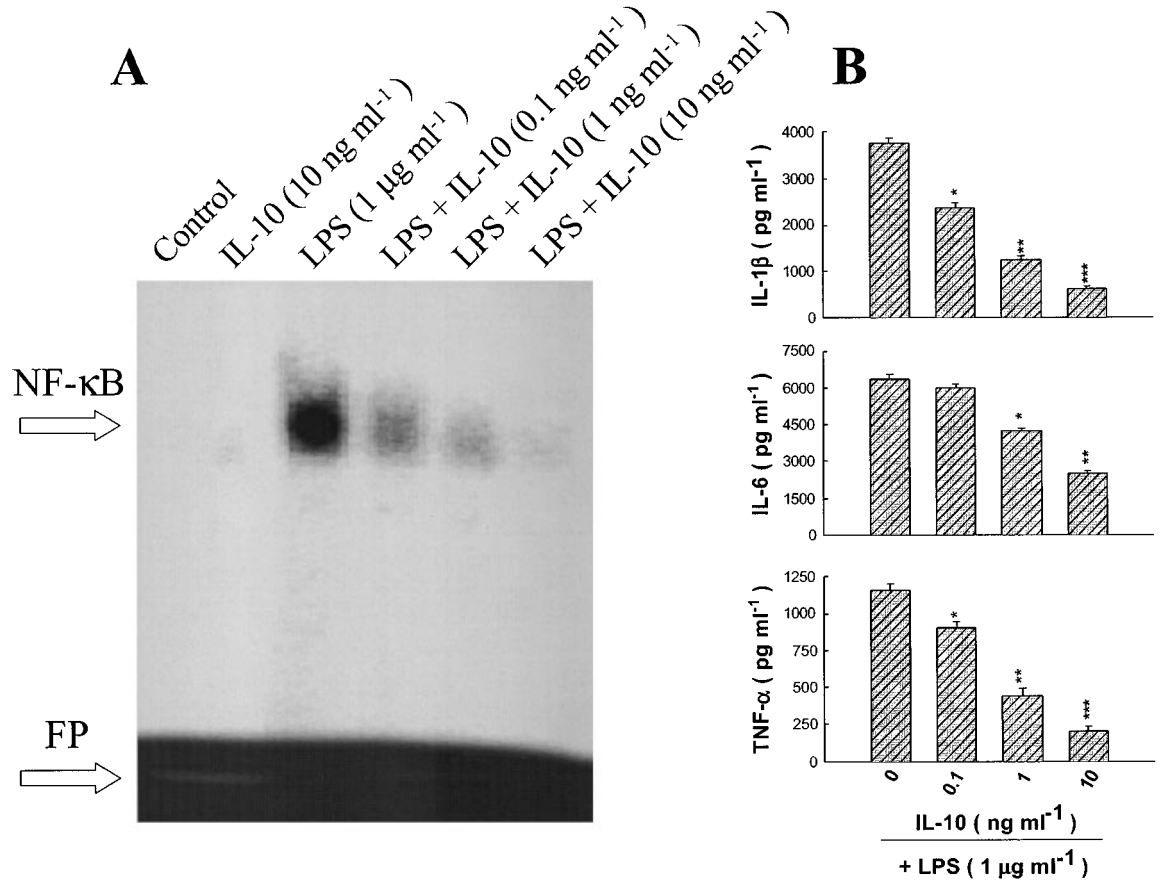

Figure 7 The effect of rhIL-10 on LPS-induced activation of NF-κB and pro-inflammatory cytokine biosynthesis. (A) rhIL-10 reduced, in a dose-dependent manner, LPS-induced up-regulation of NF-κB DNA binding activity, as shown by the representative EMSA. The upper open arrow indicates the NF-κB/DNA complex, and the lower open arrow indicates the faster migrating unbound free probe. (B) rhIL-10 abrogated the stimulatory effect of LPS on pro-inflammatory cytokine release. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, as compared to control (LPS alone). Data are means and histograms the s.e.mean of three independent experiments.

2001c), and prevented its phosphorylation, suggesting the involvement of an upstream kinase. In addition, pyrimidylpiperazine differentially, but selectively, intervened at the level of NF-κB subunit composition translocating onto the nucleus, thereby preventing the DNA binding activity of this transcription factor that is essentially involved in regulating genes encoding cytokines (Mercurio & Manning, 1999). The observation that immunoneutralization of endogenously-produced IL-10 by the action of pyrimidylpiperazine blocked the inhibitory effect of this compound on LPS-induced NF-κB activation and restored the biosynthesis of cytokines suggested the involvement of an IL-10 sensitive pathway. Interestingly, exogenous rhIL-10 suppressed NF-κB activation and reduced cytokine release, which supports the notion that pyrimidylpiperazine mediated inhibition of the IκB-α/NF-κB signal transduction pathway is dependent, at least in part, on a counter-inflammatory loop transduced through an IL-10 sensitive mechanism.

Although the transcription factor NF-κB has been originally recognized in regulating gene expression in B-cell lymphocytes (Sen & Baltimore, 1986), subsequent studies demonstrated that it is one member of a ubiquitously expressed family of *Rel*-related transcription factors that serve as critical regulators of many genes, including those of pro-inflammatory cytokines (Baldwin, 1996). The translocation and activation of NF-κB in response to various stimuli are sequentially organized at the molecular level. In its inactive state, the heterodimeric NF-κB, which is mainly composed of two subunits, p50 (NF-κB$_1$) and p65 (RelA), is present in the cytoplasm associated with its inhibitory protein, IκB (Schreck *et al*., 1992; Siebenlist *et al*., 1994). Upon stimulation, such as with cytokines and LPS, IκB-α undergoes phosphorylation on serine/threonine residues, ubiquitination and subsequent proteolytic degradation, thereby unmasking the nuclear localization signal on p65 and allowing nuclear translocation of the complex. This sequential propagation of signalling ultimately results in the release of NF-κB subunits from IκB-α inhibitor, allowing translocation and promotion of gene transcription. In this report, we have provided evidence supporting the involvement of an IκB-α/NF-κB sensitive signalling pathway mediating the immunomodulatory potential of pyrimidylpiperazine. However, from this data alone we are unable to identify which components of the upstream signalling transduction pathways converging on IκB-α, thereby regulating its phosphorylation and degradation, are likely to be targets for pyrimidylpiperazine. Signals emanating from membrane receptors, such as those for IL-1 and TNF-α, activate members of the MEKK-related family, including NF-κB inducing kinase (NIK) and MEKK$_1$, both of which are

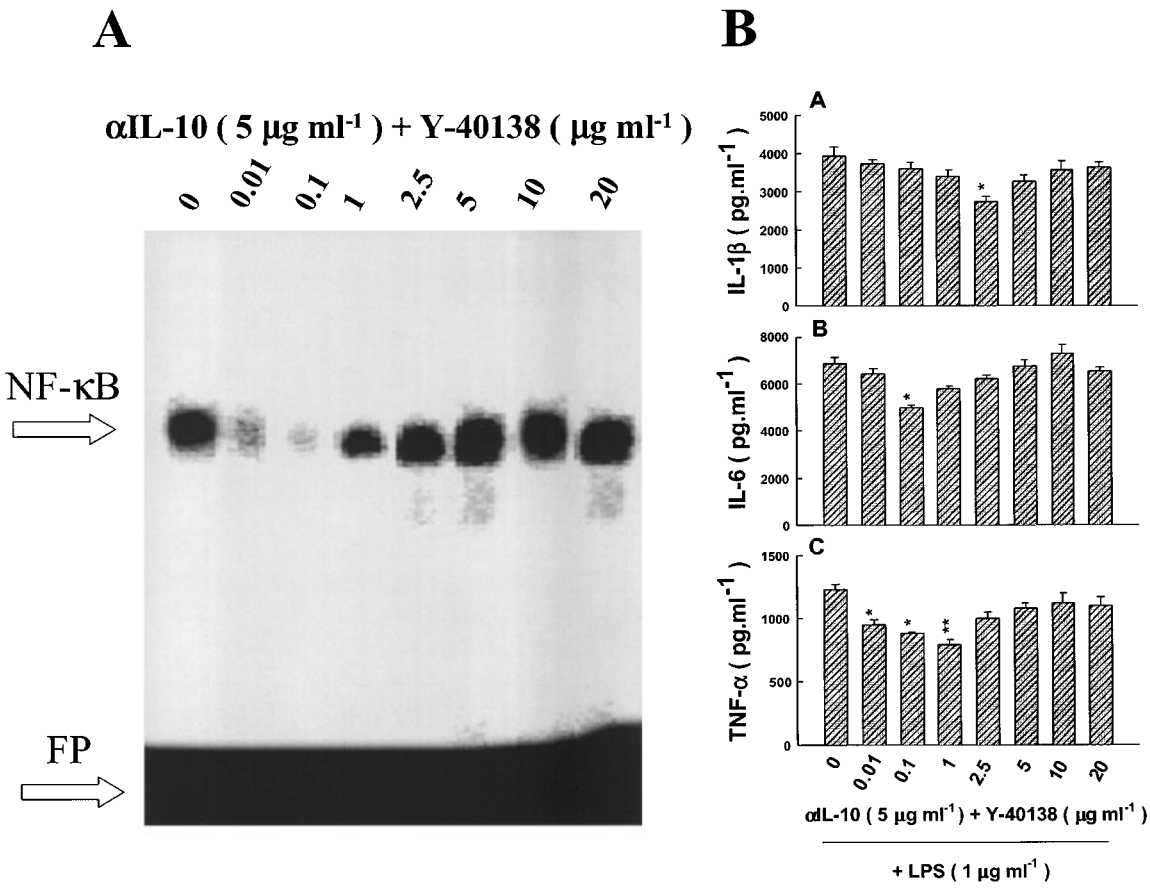

Figure 8 The effect of rabbit (polyclonal) anti-rat IL-10 neutralizing antibody (αIL-10; 5 μg ml^{-1}) on Y-40138 dependent inhibition of LPS-induced activation of NF-κB and pro-inflammatory cytokine biosynthesis. (A) αIL-10 mitigated the effect of Y-40138 and restored the NF-κB DNA binding activity, as shown by the representative EMSA. The upper open arrow indicates the NF-κB/DNA complex, and the lower open arrow indicates the faster migrating unbound free probe. (B) αIL-10 abrogated the inhibitory effect of Y-40138 on LPS-induced pro-inflammatory cytokine release. $^{*}P<0.05$, $^{**}P<0.01$, as compared to control (LPS alone). Data are means and histograms the s.e.mean of three independent experiments.

involved in the activation of IκB kinases, IKK_1 and IKK_2, components of the IKK signalsome. IKK_1 and IKK_2 were identified as components of the high-molecular weight complex containing a number of proteins involved in NF-κB regulation (Zandi *et al*., 1997; Mercurio *et al*., 1997). These kinases phosphorylate members of the IκB family, including IκB-α, at specific serines within their amino termini, thereby leading to site-specific ubiquitination and degradation by the 26S proteasome. The ability of pyrimidylpiperazine to down-regulate the phosphorylation of IκB-α and its subsequent degradation suggested the involvement of an upstream kinase, probably the IKK complex, as a potential target for the anti-inflammatory action of this novel compound.

The promoters of genes encoding cytokines contain multiple *cis*-acting motifs including those that bind such transcription factors as NF-κB. Furthermore, the release of free NF-κB upon extracellular stimulation due to IκB phosphorylation and degradation, leads to DNA binding to initiate transcription of related genes, including immuno-receptors, cytokines and, interestingly, its own inhibitor, IκB (Mercurio *et al*., 1997; Kim *et al*., 1999; Haddad *et al*., 2001c). Two unique features of the NF-κB/IκB complex system are deduced from its feedback regulation. The transcriptional activation of NF-κB triggers the synthesis of IκB, and NF-κB activated transcription is maintained by continuous degradation of IκB, which is sustained by an extracellular stimulus (Ghosh & Baltimore, 1990; Manna & Aggarwall, 1998). Thus, the expression of IκB parallels both NF-κB activity and the duration of the activating extracellular stimulation, suggesting that this temporal parallelism between IκB accumulation/degradation and an effective external stimulation is a mechanism allowing dual, biphasic, regulation of NF-κB within the alveolar space. The selective interference of pyrimidylpiperazine in regulating the activation of NF-κB and the expression of its inhibitor IκB-α is of particular interest since it suggests that this compound's anti-inflammatory role within the alveolar space resides within and/or above the upstream pathway regulating the phosphorylation of IκB-α, thereby regulating the downstream pathway governing NF-κB translocation and activation. However, the possibility of pyrimidylpiperazine directly interacting with the NF-κB complex, thus preventing its binding to the κB DNA moiety, cannot be excluded.

Interleukin-10 (IL-10) was originally identified as a cytokine inhibitory factor, which suppresses the biosynthesis of an array of pro-inflammatory mediators, including IL-1β, IL-4, IL-6, IL-8, TNF-α, TNF-β, interferon-γ (IFN-γ), and granulocyte/macrophage colony stimulating factor (GMCSF)

(Wang *et al*., 1995). The level of regulation by IL-10 on cytokines is both transcriptional and translational, raising the possibility of IL-10 acting on transcription factors involved in regulating inflammatory genes (Wang *et al*., 1995). It has been shown that IL-10 selectively inhibited NF-κB activation, a phenomenon well correlated with a dose-dependent inhibition of the release of pro-inflammatory cytokines (Wang *et al*., 1995). Our results are in agreement with this study as we have shown that exogenous rhIL-10 suppressed NF-κB activation, accompanied by a dose-dependent inhibition of cytokine biosynthesis. On the mechanism of action of IL-10, it seems that it involves the blockade of a reaction required for the release of IκB from the complex in intact cells. For example, certain experiments using cell-free preparations have suggested that certain protein kinases phosphorylate IκB causing its release and allowing activation of the NF-κB complex (Shirakawa & Mizel, 1989). Our data, however, do not rule out the possibility that IL-10 is acting on transcription factors other than NF-κB, since NF-IL-6, AP-1 and 2, CREB, Sp-1 and Oct have been reported to regulate the transcriptional activity of inflammatory genes (Libermann & Baltimore, 1990; Yasumoto *et al*., 1992; Rhoades *et al*., 1992; Shirakawa *et al*., 1993). Since IL-10 interfered with the activation of NF-κB and subsequently suppressed the release of cytokines suggest that this transcription factor is a target for the anti-inflammatory action of this cytokine. Of note, immunoneutralization of endogenous IL-10 blocked pyrimidylpiperazine-dependent inhibition of LPS-induced cytokine biosynthesis and the binding activity of NF-κB. Although it is evident that the immunopharmacological potential of pyrimidylpiperazine is IL-10 sensitive and requires NF-κB targeting, it is also possible that the effect of pyrimidylpiperazine on NF-κB is closely dependent on IL-10. This is rather supported by the unequivocal evidence that neutralizing the effect of IL-10 induced by pyrimidylpiperazine partially restored the DNA binding activity of NF-κB. Therefore, the ability of pyrimidylpiperazine to down-regulate NF-κB requires, at least in part, IL-10. In short, the involvement of an IL-10 sensitive pathway implicated in the immunoregulatory role of pyrimidylpiperazine is supported by: (i) the ability of exogenous rhIL-10 to suppress NF-κB activation, concomitant with down-regulating the biosynthesis of pro-inflammatory mediators, and (ii) that immunoneutralization of endogenous IL-10 induced by Y-40138 restored LPS-dependent NF-κB activation and the release of pro-inflammatory cytokines. We are extending these observations to investigate a particular role of pyrimidylpiperazine in regulating selective upstream protein kinases, such as PKC, mitogen-activated protein kinase (MAPK p38, p44/p42) and NF-κB inducing kinase (NIK), and whether this mechanism is likely to intervene in the pathways controlling the activation of NF-κB. The proposed model of molecular pathways involved in pyrimidylpiperazine mediated immunomodulation in the alveolar epithelium is schematized in Figure 9.

This study provides a novel evidence for a dual immunopharmacological potential of pyrimidylpiperazine in the alveolar epithelium. Our findings are highlighted as follows: (i) pyrimidylpiperazine ameliorated, in a dose-dependent manner, LPS-induced release of pro-inflammatory cytokines; (ii) this mechanism is accompanied by up-regulating an anti-inflammatory loop dependent on IL-10; (iii) the immunoregulatory effect of pyrimidylpiperazine on cytokine release is paralleled by selective targeting of the nuclear localization/activation of NF-κB; analysis of the upstream pathway implicated revealed that pyrimidylpiperazine is targeting the phosphorylation and degradation of IκB-α, the major cytosolic inhibitor of NF-κB; (iv) exogenous rhIL-10 suppressed, in a dose-dependent manner, NF-κB activation, concomitant with down-regulating LPS-induced pro-inflammatory cytokines; and (v) immunoneutralization of endogenous IL-10 augmented by pyrimidylpiperazine reversed the inhibitory effect of this compound on LPS-induced NF-κB activation and pro-inflammatory cytokine biosynthesis. We conclude that the immunomodulatory potential of pyrimidylpiperazine in the alveolar epithelium follows a biphasic pattern by down-regulating a pro-inflammatory signal and amplifying an anti-inflammatory loop through IL-10, an effect accompanied by selective interference in the signal transduction pathways mediating NF-κB translocation and activation.

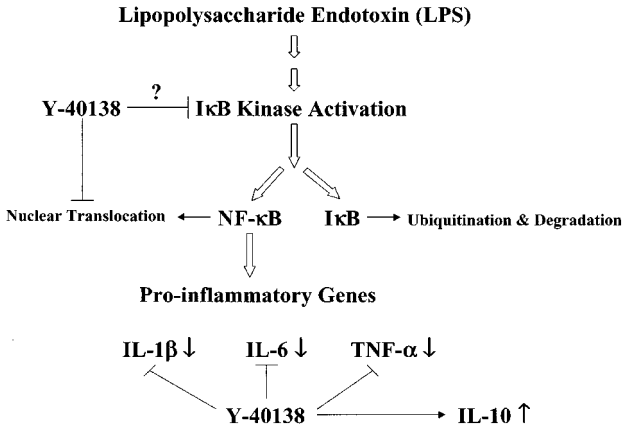

Figure 9 Schematic representation of the signalling pathways mediating the immunopharmacological property of Y-40138 in the alveolar epithelium. Exposure to endotoxin (LPS) triggers a sequential cascade that ultimately lead to activation of a specific IκB kinase, which in turn phosphorylates the inhibitor of NF-κB, IκB, marking its ubiquitination and proteasome degradation. This mechanism unmasks the nuclear localization sequence of NF-κB allowing its dissociation from IκB and subsequent nuclear translocation. NF-κB binds to promoters of genes containing the κB moiety and thereby mediates the expression of pro-inflammatory genes encoding cytokines. The pyrimidylpiperazine derivative, Y-40138, inhibits NF-κB translocation, probably by inhibiting IκB kinase activity. This subsequently ameliorates the induced production of cytokines, an effect partially amplified through activation of an anti-inflammatory loop mediated by IL-10.

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