Title Page

Inhibitory influence of protease activated receptor-2 and E-Prostanoid receptor stimulants in lipopolysaccharide models of acute airway inflammation

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**Running title page**

a) PAR$_2$ activators blunt LPS-induced neutrophilia in mouse lung

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d) BAL bronchoalveolar lavage

COX cyclooxygenase

KC keratinocyte-derived chemokine

LPS lipopolysaccharide
MIP-2  macrophage-inflammatory protein-2
NK    neurokinin
PAR   protease-activated receptor
PGE$_2$  prostaglandin E$_2$
TNF-$\alpha$  tumour necrosis factor-alpha

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Abstract

Protease-activated receptors (PARs) are widely expressed throughout the respiratory tract, and PAR2 has been investigated as a potential drug target for inflammatory airway diseases. The primary focus of this study was to determine the extent to which PAR2-activating peptides modulate lipopolysaccharide (LPS)-induced airway neutrophilia in mice and to establish the underlying mechanisms. Intranasal administration of LPS induced dose- and time-dependent increases in the number of neutrophils recovered from bronchoalveolar lavage (BAL) fluid of mice. Co-administration of the PAR2-activating peptide f-LIGRL inhibited LPS-induced neutrophilia at 3 and 6 hours post-inoculation. PAR2-mediated inhibition of LPS-induced neutrophilia was mimicked by PGE2 and butaprost (selective EP2 receptor agonist), and blocked by parecoxib (COX-2 inhibitor) and AH6809 (EP1/EP2 receptor antagonist). PAR2-activating peptides also blunted early increases in the levels of the key neutrophil chemoattractants KC and MIP-2 in the BAL of LPS-exposed mice. However, neither PAR2-activating peptides nor PGE2 inhibited LPS-induced generation of MIP-2 in cultures of primary murine alveolar macrophages. In summary, PAR2-activating peptides and PGE2 suppressed LPS-induced neutrophilia in murine airways, independently of an inhibitory action on MIP-2 generation by alveolar macrophages.
Introduction

Protease-activated receptors (PAR₁, PAR₂, PAR₃ and PAR₄) are a unique family of G protein-coupled receptor (Steinhoff et al., 2005). PARs are targets of specific proteases, which cleave the extracellular N-terminus of the PAR exposing a newly-formed tethered ligand that activates the receptor (Schmidlin and Bunnett, 2001). PAR₁, PAR₂ and PAR₄ are also targeted and activated by small peptide sequences that mimic the first 5 – 8 amino acids of the tethered ligand (Devlin et al., 2007). For example, peptide sequences such as SLIGRL and f-LIGRL activate murine PAR₂ (Kawabata et al., 2004). f-LIGRL appears more suited to in vivo investigations as it is a more potent activator of PAR₂ than SLIGRL, and is resistant to degradation by aminopeptidases (Kawabata et al., 2004). Partially scrambled analogues of SLIGRL and f-LIGRL, such as LSIGRL and f-LRGIL, do not activate PAR₂ receptors, and are regularly used as control peptides in experiments involving PAR₂-activating peptides (PAR₂-APs) (Abey et al., 2006).

PARs are widely expressed, with high levels of expression reported in the cardiovascular, gastrointestinal and respiratory systems. Of specific relevance to this study, PAR₂ is abundantly expressed by many resident structures within the airways, including airway epithelial cells, fibroblasts, myocytes, sensory neurons, endothelial cells and vascular smooth muscle (Cocks et al., 1999; Peters and Henry, 2009). PAR₂ is also expressed on a variety of immune cells including macrophages, lymphocytes, neutrophils, eosinophils and mast cells, suggesting a role for PAR₂ in inflammatory processes (Steinhoff et al., 2005). PAR₂ expression is increased in asthmatic bronchi as well as airways exposed to respiratory tract viruses, allergens, inflammatory cytokines and smoke (Knight et al., 2001; Lan et al., 2004; Freund-Michel and Frossard, 2006). However, the functional significance of increased
PAR₂ expression in inflammatory lung disorders is controversial because there is evidence of PAR₂ mediating both pro- and anti-inflammatory activities (Peters & Henry, 2009).

Activators of PAR₂ have been shown to variably influence the level of bronchomotor tone, the development of airways hyperresponsiveness and the extent of inflammatory cell influx in animal models of respiratory tract disease induced by allergens, viruses and bacterial products (Peters and Henry, 2009; Khoufache et al., 2009). For example, PAR₂-APs reduced the airway inflammatory responses induced by the bacterially-derived product lipopolysaccharide (LPS) (Moffatt et al., 2002). However, the underlying mechanism for SLIGRL-induced inhibition of LPS-induced airway neutrophilia has not been determined, and elucidating this mechanism is a primary focus of the current study. One possibility to be explored is that PAR₂-APs generate prostanoids, such as prostaglandin E₂ (PGE₂), which exert downstream anti-inflammatory effects within the lungs. Evidence supporting this mechanism includes the finding that PAR₂-APs elevate PGE₂ levels in respiratory epithelial cells, intact airway segments and in vivo (Kawao et al., 2005; Lan et al., 2001; De Campo and Henry, 2005). Furthermore, PGE₂ has been reported to produce a wide range of anti-inflammatory effects within the airways (Pavord and Tattersfield, 1995; Vancheri et al., 2004), including inhibition of LPS-induced airway neutrophilia (Goncalves de Moraes et al., 1996). This may be a novel therapeutic approach in COPD and severe asthma, where neutrophils are thought to play an important role in exacerbations of disease (Beeh and Beier, 2006).

An initial aim of this study was to examine the inhibitory influence of a range of doses of the PAR₂-APs SLIGRL and f-LIGRL (and the control peptide LSIGRL)
on the time-course of LPS-induced neutrophilia in mice. The putative mediator role of endogenous PGE$_2$ in these inhibitory processes was subsequently investigated pharmacologically by pretreatment of mice with cyclooxygenase inhibitors and prostanoid receptor antagonists. These were complimented by experiments which determined the influence of exogenous PGE$_2$ and an EP$_2$ receptor-selective agonist butaprost on LPS-induced neutrophilia. Additional mechanistic studies were conducted using isolated alveolar macrophages to determine the influence of PAR$_2$-APs and PGE$_2$ on the levels of cytokines and chemokines thought to play an important role in the recruitment of neutrophils to LPS-exposed mouse lungs, including tumour necrosis factor-alpha (TNF-α) and macrophage-inflammatory protein-2 (MIP-2).
Methods

Materials. The PAR2-APs SLIGRL and f-LIGRL were synthesized by Proteomics International (Perth, Australia), as were the control peptides LSIGRL and f-LRGIL. Lipopolysaccharide (E.coli strain 0127:B8), butaprost, indomethacin, and L-703,606 were purchased from Sigma Chemical Company (St. Louis, MO) and parecoxib from Pfizer (Paris, France). PGE₂ was purchased from Cayman Chemical Company (Ann Arbor, MI). Anti-mouse TNF-α antibody and goat IgG isotype control were purchased from R&D Systems (Minneapolis, MN), as were the ELISA kits to measure levels of KC and MIP-2.

Animals. Male BALB/c mice (specified pathogen free) at 8–10 weeks old, weighing approximately 20 grams were purchased from the Animal Resource Centre (Murdoch, Australia). Animals were housed on a 12 hour : 12 hour light dark cycle and given sterile food and water ad libitum. Experiments were performed in accordance with the National Health and Medical Research Council (NHMRC) guidelines as outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition) (NHMRC, 2004).

Intranasal inoculation. Mice were lightly anesthetized with methoxyfluorane vapour, held upright, and a 20 μl aliquot of inoculum applied to each of the external nares. As obligate nose breathers, mice inhaled the inoculum into their airways. The inoculum contained either LPS or vehicle (sterile saline). LPS derived from the cell wall of Escherichia coli strain 0127:B8 was selected for use as this strain has previously been demonstrated to induce airway inflammation in BALB/c mice.
Appropriate LPS dose (0.3 – 30 μg/mouse) and periods of exposure (3 – 72 hours) were determined from dose-response and time-course studies. In selected experiments, the intranasal inoculum also contained a PAR2-AP (SLIGRL; 25 – 400 μg/mouse, f-LIGRL; 3 – 30 μg/mouse) or an EP receptor agonist (PGE2; 10 μg/mouse, butaprost; 10 or 50 μg/mouse). The partially scrambled peptide LSIGRL (400 μg/mouse), which does not activate PAR2, was administered in control experiments.

In mechanistic studies, mice were administered a cyclooxygenase inhibitor (indomethacin, 1 mg/kg i.p. or parecoxib, 1 mg/kg i.p.), an EP receptor antagonist (AH8609, 5 mg/kg i.p.) or a NK1 receptor antagonist (L-703,606, 3 mg/kg i.p.) one hour prior to intranasal peptide or LPS inoculation.

**Bronchoalveolar lavage.** Mice were euthanased with pentobarbitone sodium (980 mg/kg i.p) and the trachea intubated with a teflon coated cannula. Lungs were lavaged with 6 X 0.5 ml aliquots of ice cold pyrogen free PBS pH 7.4 and the resultant BALF centrifuged (1500 rpm, 7 min, 4˚C). The cell pellet was resuspended in sterile red blood cell lysis buffer (Tris Ammonium Chloride buffer; 0.83% NH4Cl in Tris pH 7.65) for 8 min at RT. After washing, BAL cells were resuspended in PBS pH 7.4 with 1% BSA or complete RPMI 1640 supplemented with 10% FCS, 100ug/ml gentamycin and 2mM Glutamax (Gibco). Total leucocyte number was determined using a haemocytometer and cell viability (always >95%) established by trypan blue (0.4%) exclusion. Cytospin preparations of BAL cells were stained with Rapid I, II (Amber Scientific, Perth, Australia) and differential cell counts of 400 cells performed at X40 magnification (Olympus BX-50 light microscope, Olympus Optical Co, Japan). In some instances, cell numbers in PAR2-AP-treated mice are
expressed as a percent reduction of the mean recruitment of cells in the absence of drug.

**Alveolar macrophage culture**  Primary alveolar macrophages were isolated from BALF by a simple adhesion method. BALF from 20 mice was pooled, centrifuged and the cell pellet purged of red blood cells by Tris ammonium chloride treatment as described above. The washed cell pellet was resuspended in 1.0 ml of complete RPMI, a cell count performed and the cells diluted such that 4 X 10⁴ viable cells were plated / well in 100 μL in a 96 well plate (Nunc, Denmark). Macrophages were allowed to adhere for 1 hour at 37°C in a 5% CO₂ humidified incubator. Non adherent cells and debris were removed by washing. The adherent macrophages were cultured in 100 μL fresh complete medium and treated with either the PAR₂-APs SLIGRL (1, 10 or 100 μM / well), f-LIGRL (20 μM) or their scrambled control counterparts LSIGRL (100 μM) and f-LRGIL (20 μM), or PGE₂ (10 μM) 10 mins prior to stimulation with LPS (10 ng/ml). Combinations were tested at least in triplicate / plate and each individual experiment repeated on at least 4 separate occasions. In order to reduce the metabolic rate of the macrophages and hence slow down the very rapid synthesis of cytokines, at various time points (separate experiments) the culture plates were put on ice and 100 μL of ice cold medium added / well. Culture supernatants were quickly harvested and stored at -80°C until assayed for MIP-2, KC, TNF-α and PGE₂. In some experiments, cultures were treated with an anti-TNF-α antibody 10 mins prior to exposure to LPS.

**Chemokine and cytokine ELISAs.** Levels of the chemokines MIP-2 and KC, and the cytokine TNF-α and the prostaglandin PGE₂ were determined in the culture
supernatants of peptide- or PGE$_2$-treated, LPS-stimulated primary alveolar macrophages. MIP-2, KC and TNF-α EIA kits were purchased from R&D Systems (Minneapolis, MN) and the PGE$_2$ kit from Cayman Chemical Co (Ann Arbor, MI). All supernatants were assayed according to individual kit instructions.

**Statistical analyses.** Data are presented as the mean ± SEM of the group. All data was checked for normality and equal variance prior to statistical analysis. Statistical analysis was performed using a commercial software package (SigmaStat version 3.5). Statistical analyses for multiple comparisons were completed using analysis of variance with an appropriate post hoc test. All pairwise comparisons were completed using the Holm-Sidak method. A p-value of less than 0.05 was considered statistically significant.
Results

LPS model of acute airway inflammation. As expected, intranasal inoculation of mice with LPS caused significant dose and time-dependent increases in total BAL cell number (Figure 1A). The influx of cells into the airways occurred rapidly, with the total BAL cell number recovered at 3 hours post-inoculation being 6.6 ± 0.8 fold higher in LPS-treated mice than in control mice (p < 0.001, Figure 1A), with the peak increase occurring between 3 and 24 hours post-inoculation. Up until at least 48 hours post-inoculation, the increase in total BAL cell number in LPS-treated mice was almost entirely due to an increase in the number of neutrophils (Figure 1B). Thereafter, the number of neutrophils steadily declined to levels that were only marginally elevated at 72 hours (Figure 1B). The number of BAL macrophages remained relatively constant during the first 48 hours after administration of LPS, but were significantly elevated at 72 hours (Figure 1C).

Over the dose range of 0.3 – 30 μg LPS / mouse, a strong positive relationship was observed between dose and total BAL cell number (Figure 1D), and dose and BAL neutrophil number (Figure 1E) at 6 hours post-inoculation. In contrast, the number of macrophages recovered from BAL fluid (at 6 hours post-inoculation) was independent of the dose of LPS administered (Figure 1F). In summary, intranasal administration of LPS to mice caused a profound inflammatory response in the airways, dominated by a transient and reversible neutrophilia.

PAR2-APs suppressed LPS-induced increases in BAL neutrophil numbers. To determine the influence of PAR2-APs on the time-course of LPS-induced neutrophilia, groups of mice were intranasally inoculated with a sterile solution
containing 10 μg LPS, with or without SLIGRL. In these experiments, a dose of 400 μg SLIGRL/mouse was used because it had previously been reported to be effective in a murine model of airway inflammation (Moffatt et al., 2002). At 3 hours post-inoculation, BAL fluid recovered from SLIGRL-treated LPS mice had 78 ± 4% fewer neutrophils than LPS-only mice (Figure 2A, p < 0.01). However, SLIGRL did not significantly reduce the number of neutrophils in BAL fluid recovered from LPS-mice at either 6 or 24 hours post-inoculation (Figure 2A).

Additional studies were conducted using f-LIGRL, an aminopeptidase resistant, longer-acting PAR2-AP (Kawabata et al., 2004). A dose of 30 μg f-LIGRL reduced LPS-induced BAL neutrophil numbers by 77 ± 7% at 3 hours post-inoculation and by 35 ± 2% at 6 hours post-inoculation (Figure 2B, p < 0.001). However, f-LIGRL did not significantly inhibit the number of BAL neutrophils recovered from LPS-exposed mice at 24 hours post-inoculation (Figure 2B). In summary, f-LIGRL was able to reduce LPS-induced BAL neutrophil numbers for a longer period than SLIGRL, although not over the entire time-course of LPS-induced inflammation.

In subsequent studies, the dose-response relationship for SLIGRL- and f-LIGRL-induced inhibition of BAL neutrophil numbers was determined at 3 hours post-inoculation (Figure 3). Administration of 25, 100 and 400 μg SLIGRL/mouse reduced the numbers of BAL neutrophils recovered from LPS-exposed mice by 36 ± 3%, 47 ± 2% and 74 ± 2%, respectively (Figure 3A, p<0.001). f-LIGRL also produced a dose-dependent inhibition of LPS-induced BAL neutrophil numbers, with 10 and 30 μg f-LIGRL/mouse reducing numbers of BAL neutrophils by 37 ± 6% and 65 ± 4%, respectively (Figure 3B, p<0.05). Neither SLIGRL nor f-LIGRL produced any alteration in the BAL cell profile of control mice (Figure 3A,B). The partially
scrambled peptide sequence LSIGRL (400 μg/mouse) did not significantly alter BAL neutrophil numbers in either LPS-treated or control mice (Figure 3C).

**Cyclooxygenase inhibitors blocked responses to PAR$_2$-APs.** To determine whether COX products mediated the inhibitory effects of PAR$_2$-APs on BAL neutrophilia, a non-selective COX inhibitor, indomethacin, was administered i.p. to mice one hour before inoculation with LPS and/or PAR$_2$-APs. As expected, 30 μg/mouse f-LIGRL reduced LPS-induced BAL neutrophil numbers (by 77 ± 7%, Figure 5A). However, f-LIGRL did not significantly reduce BAL neutrophil numbers in indomethacin-treated, LPS-exposed mice (Figure 4A).

Parecoxib, a selective COX-2 inhibitor, was used to determine if the COX-2 isoform played a primary role in f-LIGRL-induced inhibition of LPS-induced neutrophilia. In these studies, 30 μg f-LIGRL/mouse reduced the number of BAL neutrophils recovered from LPS-exposed mice by 68 ± 9% (p < 0.001, Figure 4B). However, in LPS-exposed mice treated with parecoxib, f-LIGRL failed to significantly reduce BAL neutrophil numbers (Figure 4B). Parecoxib did not significantly alter the numbers of BAL neutrophils in saline- or LPS-treated mice in the absence of f-LIGRL. Thus, inhibition of COX-2 attenuated the suppressive effect of PAR$_2$-APs on LPS-induced neutrophilia.

**EP receptor agonists mimicked, and EP receptor antagonists inhibited, responses to PAR$_2$-APs.** PGE$_2$ is a product of COX-2 that mediates a range of effects induced by PAR$_2$-APs. Consistent with this, PGE$_2$ reduced the number of LPS-induced BAL neutrophils recovered at 3 hours post-inoculation by 54 ± 5% (Figure 5A, p < 0.001). Similarly, a selective EP$_2$ receptor agonist, butaprost,
produced an 83 ± 5% reduction in neutrophils recovered from LPS-treated mice (Figure 5B, p < 0.001). In additional studies, a mixed EP<sub>1</sub>/EP<sub>2</sub>/DP receptor antagonist AH6809 significantly inhibited the effects of f-LIGRL on LPS-induced airway neutrophilia (Figure 6A). In summary, these findings are consistent with the postulate that f-LIGRL-induced suppression of LPS-induced airway neutrophilia was mediated via PGE<sub>2</sub> and its activation of EP<sub>2</sub> receptors.

**NK<sub>1</sub> receptor antagonists did not inhibit responses to PAR<sub>2</sub>-APs.** As PAR<sub>2</sub>-APs have been reported to activate NK<sub>1</sub> receptors (Abey et al., 2006), experiments were performed to determine if a NK<sub>1</sub> receptor antagonist, L-703,606, altered f-LIGRL-induced decreases in LPS-induced BAL neutrophil numbers. Similar to previous observations, LPS caused a marked increase in the number of BAL neutrophils and f-LIGRL reduced this effect by 65 ± 3% (Figure 6B). The NK<sub>1</sub> receptor antagonist, L-703,606 (3 mg/kg i.p. one hour prior to LPS and/or peptides) did not significantly alter the number of BAL neutrophils recovered in response to LPS, in the presence or absence of f-LIGRL (Figure 6B).

**f-LIGRL suppressed the early LPS-induced increases in BAL fluid levels of KC and MIP-2.** Neutrophil recruitment into LPS-exposed airways is preceded and promoted by increases in the levels of chemokines such as KC and MIP-2. Consistent with this, the levels of KC and MIP-2 in BAL fluid recovered one hour after exposure to LPS were 26-fold and 4.2-fold higher, respectively than in saline-exposed mice (Figure 7A). Of particular interest, BAL fluid recovered from LPS-exposed mice which received f-LIGRL (30 μg/mouse) had levels of KC and MIP-2 40 – 45% lower than that recovered from mice receiving LPS alone (P<0.01, Figure
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7A). However, f-LIGRL did not suppress the elevated levels of KC and MIP-2 detected in BAL fluid recovered three hours post-LPS (Figure 7B).

PAR2-APs and PGE2 did not inhibit LPS-induced MIP-2 production in murine alveolar macrophages. To further investigate the underlying mechanisms for PAR2- and PGE2-mediated suppression of chemokine levels in vivo, additional studies were conducted using primary cultures of murine alveolar macrophages. LPS induced profound time- and concentration-dependent increases in MIP-2 levels in isolated alveolar macrophages (Figure 8A and 8B). However, LPS-induced increases in MIP-2 levels were not significantly inhibited by f-LIGRL (or control peptide f-LRGIL, Figure 8C), SLIGRL (or control peptide LSIGRL, Figure 8D) or PGE2 (Figure 8E).

TNF-α played a minor role in LPS-induced increases in MIP-2 production in murine alveolar macrophages LPS-exposed alveolar macrophages generated high levels of TNF-α (Figure 9A). LPS-induced increases in TNF-α were unaffected by f-LIGRL (Figure 9A), but were significantly inhibited by either 1 or 10 μM PGE2 (Figure 9B). Despite the high levels of TNF-α produced in response to LPS, mTNF-α was a very weak activator of MIP-2 generation in murine alveolar macrophages (Figure 9C). Consistent with this, a TNF-α absorbing monoclonal antibody did not significantly reduce LPS-induced MIP-2 generation (Figure 9C).

Effect of SLIGRL on PGE2 levels in cultures of alveolar macrophages Exposure of alveolar macrophage cultures to SLIGRL or to control peptide (LSIGRL) for up to 2 hours did not induce any measurable increase in PGE2 levels (Figure
The elevated levels of PGE$_2$ observed in SLIGRL-treated alveolar macrophage cultures at 24 hours (Figure 10) were not significantly different from levels observed in response to either LSIGRL (Figure 10) or vehicle (data not shown).
Discussion

In the current study, intranasal administration of PAR2-APs produced dose- and time-dependent inhibition of LPS-induced pulmonary neutrophilia in mice. Additional in vivo and in vitro mechanistic studies indicate that this process involves PAR2-mediated, COX-2-dependent production of PGE2, which suppresses airway neutrophilia independently of a direct inhibitory action on MIP-2 generation by alveolar macrophages.

Inhalation of bacterial-derived LPS produces a profound immune response characterised by a rapid recruitment of neutrophils into the lung. In the current study, LPS-induced increases in BAL neutrophils were evident within 3 hours of intranasal inoculation, peaked between 6 and 24 hours post-inoculation and had resolved by 72 hours post-inoculation. Furthermore, the number of neutrophils recovered from the BAL fluid of LPS-exposed mice was directly related to the dose of LPS administered. Dose- and time-dependent increases in LPS-induced neutrophilia have been previously reported (Szarka et al., 1997; Bozinovski et al., 2004), as has the rebound increase in macrophages observed at 72 hours (Moffatt et al., 2002; Bozinovski et al., 2004), which is required to phagocytose the large numbers of neutrophils undergoing apoptosis (Cox et al., 1995).

In mice, the magnitude of LPS-induced neutrophilia was variably inhibited by the intranasal administration of PAR2-APs. At 3 hours post-inoculation, both SLIGRL and f-LIGRL inhibited BAL neutrophilia in a dose-dependent manner, and as expected for a PAR2-mediated response, f-LIGRL was more potent than SLIGRL (Kawabata et al., 2004). The duration of PAR2-mediated inhibition of LPS-induced neutrophilia was also peptide-dependent. Whereas both SLIGRL and f-LIGRL inhibited LPS-induced airway neutrophilia at 3 hours post-inoculation, only f-LIGRL
was effective 6 hours post-inoculation. Neither PAR₂-AP inhibited LPS-induced neutrophilia at 24 hours post-inoculation. A likely explanation for these differences in duration of effect is the greater resistance of f-LIGRL to peptide degrading aminopeptidases (Kawabata et al., 2004). The finding that SLIGRL inhibited LPS-induced neutrophilia is consistent with a previous report (Moffatt et al., 2002), although differences in the onset and duration of action were evident. In contrast to the current study, Moffatt and coworkers found that SLIGRL-induced inhibition of LPS-induced pulmonary neutrophilia was considerably delayed, with no significant reduction in neutrophils observed at either 3 or 6 hours post-inoculation. The reasons for these differences are unclear, but cannot be explained on the basis of the strain of mouse used, the method used for peptide and LPS administration, or the dose of SLIGRL used, as these were similar in both studies.

In addition to PAR₂ receptors, PAR₂-APs are known to activate NK₁ receptors (Abey et al., 2006). However, f-LIGRL-induced reductions in LPS-induced neutrophilia were unaffected by administration of a NK₁ receptor antagonist, indicating that these receptors were not involved in PAR₂-AP-induced reductions in LPS-induced neutrophilia. In summary, data obtained from studies using control peptides (LSIGRL, f-LRGIL) and NK₁ receptor antagonists indicate that f-LIGRL- and SLIGRL-induced reductions in LPS-induced neutrophilia are mediated via activation of PAR₂.

Several PAR₂-mediated effects within the airways have been attributed to the generation of prostanoids such as PGE₂. For example, PAR₂-mediated relaxation of airway smooth muscle and inhibition of allergic inflammation were both blocked by inhibitors of cyclooxygenase such as indomethacin (Cocks et al., 1999; Lan et al., 2001; Decampo & Henry, 2005). Likewise, in the current study, indomethacin
blocked f-LIGRL-induced inhibition of pulmonary neutrophilia in LPS-exposed mice.

A similar effect was produced by parecoxib, indicating the particular involvement of COX-2 in this process. Consistent with the likely mediator role played by endogenous PGE₂ in f-LIGRL-induced effects, we observed that the direct intranasal application of exogenous PGE₂ also inhibited neutrophilia in LPS-exposed mice. These data concur with earlier findings that intranasal application of PGE₂ inhibited neutrophil recruitment in response to LPS (Goncalves de Moraes et al., 1996). Furthermore, intranasal administration of the selective EP₂ receptor agonist butaprost inhibited the increase in BAL neutrophils observed in LPS-exposed mice. A role for the EP₂ receptor in PAR₂-mediated inhibition of LPS-induced neutrophilia is also supported by the findings that an EP₁/EP₂ receptor antagonist AH6809 inhibited f-LIGRL-induced effects in the LPS model. Together, these data indicate a role for EP₂ receptors in PAR₂-mediated reductions in LPS-induced neutrophilia, although it is possible that other EP receptors and COX products are involved in PAR₂-mediated anti-inflammatory effects.

The chemokines MIP-2 and KC are functionally related to human IL-8 and play a key role in the recruitment of neutrophils in LPS-exposed rodents (Lee et al., 1995; Schmal et al., 1996; De Filippo et al., 2008). In line with this chemoattractant role, markedly elevated levels of MIP-2 and KC were detected in the BAL fluid of LPS-exposed mice prior to significant increases in the number of neutrophils. Moreover, the levels of both chemokines were significantly lower in LPS-exposed mice treated with f-LIGRL. To investigate whether direct inhibition of MIP-2 release by alveolar acrophages underpinned the inhibitory effect of PAR₂-APs on LPS-induced neutrophilia, additional in vitro studies were performed using primary cultures of alveolar macrophages, a significant cellular source of neutrophilic
chemokines (Merrill et al., 1980; Goodman et al., 1998; Amano et al., 2000; Thorley et al., 2007). In these studies, LPS produced profound concentration and time-dependent increases in MIP-2 levels in murine alveolar macrophage cultures. Of particular relevance, neither PGE\(_2\) nor PAR\(_2\)-APs (SLIGRL, f-LIGRL) significantly affected LPS-induced increases in MIP-2 levels in murine alveolar macrophage cultures. Although the effect of PAR\(_2\)-APs on MIP-2 release from alveolar macrophages has not previously been reported, the findings with PGE\(_2\) are consistent with several previous reports that this prostanoid failed to inhibit IL-8 release from human alveolar macrophages (Strieter et al., 1989; Standiford et al., 1992), despite an inhibitory action on human blood monocytes (Standiford et al., 1992). Thus, the inhibitory effect of PAR\(_2\)-APs and PGE\(_2\) on LPS-induced increases in BAL fluid MIP-2 levels and neutrophil numbers observed \textit{in vivo} cannot be readily explained by \textit{direct} inhibition of MIP-2 generation by LPS-exposed alveolar macrophages.

The cytokine TNF-\(\alpha\) has also been implicated in LPS-induced pulmonary neutrophilia. For example, the lung neutrophil influx observed following LPS exposure in mice could be suppressed by neutralising anti-TNF-\(\alpha\) antibodies (Goncalves de Moraes et al., 1996) or by using mice that were deficient in receptors for TNF-\(\alpha\) (and IL-1\(\beta\)) (Jones et al., 2005; Mizgerd et al., 2001). Thus, processes that inhibit LPS-induced TNF-\(\alpha\) generation may reduce neutrophil influx. In this context, we observed that LPS-induced generation of TNF-\(\alpha\) by murine alveolar macrophages was suppressed by PGE\(_2\) but not by f-LIGRL. This raises the possibility that PAR\(_2\)-APs promote the synthesis and release of PGE\(_2\) which then inhibits LPS-induced generation of TNF-\(\alpha\) by alveolar macrophages (Smith et al., 2004; Ratcliffe et al., 2007). The cellular source of PGE\(_2\) is unlikely to be the
alveolar macrophage as these cells did not produce PGE₂ in response to PAR₂-APs, but may be airway epithelial cells which are known to generate PGE₂ rapidly in response to PAR₂-APs (Lan et al., 2001; Kawao et al. 2005; Saleh et al., 2008). Although it is currently unclear how TNF-α might mediate LPS-induced neutrophil influx, one possibility is that TNF-α produced by LPS-exposed alveolar macrophages stimulates the generation of neutrophilic chemokines such as MIP-2 and IL-8 (Thorley et al., 2007). However, LPS-induced generation of TNF-α was unlikely to play a major role in MIP-2 production by alveolar macrophages because inhibiting the actions of TNF-α with a blocking monoclonal antibody did not suppress LPS-induced generation of MIP-2 and, exogenous murine TNF-α was a weak activator of MIP-2 generation by alveolar macrophages.

At present, the precise mechanisms underlying the inhibitory actions of PAR₂-APs and PGE₂ on LPS-induced neutrophilia are unclear, although they are unlikely to involve direct inhibition of MIP-2 generation by alveolar macrophages. Preliminary studies in murine alveolar macrophages provide supportive evidence that suppression of TNF-α generation may play a contributory role, although other effects mediated by PAR₂ in the airways may potentially inhibit LPS-induced neutrophilia. For example, given the strong dose-dependency demonstrated for LPS-induced generation of neutrophilic chemokines by alveolar macrophages, and for LPS-induced increases in pulmonary neutrophilia, consideration should be given to the possibility that PAR₂-APs act by promoting the early removal of the LPS stimulus from the airways. Indeed, several studies have proposed a role for PAR₂ in stimulating mucociliary clearance (Kunzelmann et al., 2005; Cho et al., 2010), which facilitates the removal of noxious stimuli such as LPS from the airways. The rapid, but incomplete, removal of LPS in PAR₂-treated mice may explain why these mice
had lower MIP-2 levels (at 1 hour post LPS) and neutrophil numbers (at 3 and/or 6 hour post-LPS) in BAL fluid. In addition, actions of PAR2-APs at sites located downstream of MIP-2 production, some of which maybe TNF-α-dependent (e.g. PAR2-mediated inhibition of adhesion molecule expression; PAR2-mediated inhibition of neutrophil motility) may also contribute to their inhibitory activity against LPS-induced neutrophilic inflammation, and require further investigation. In summary, stimulation of airway PAR2 may offer a mechanism for protecting the host against excessive lung injury caused by extreme neutrophil accumulation, without jeopardising the critical role of the neutrophil in antibacterial host defence.
References


Footnotes

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d)
Legends for figures

Figure 1
Number of (A) total cells, (B) neutrophils and (C) macrophages recovered at selected time-points after the intranasal inoculation of mice with 10μg LPS (filled circles) or sterile vehicle (open circles). Number of (D) total cells, (E) neutrophils and (F) macrophages recovered 6 hours after intranasal inoculation of mice with increasing doses of LPS. Data is presented as the mean ± s.e.m of 4 – 7 mice / group.

Figure 2
(A) Number of BAL neutrophils recovered at 3, 6 and 24 hours after intranasal inoculation with LPS (10 μg/mouse, white bars) or LPS plus SLIGRL (400 μg/mouse, black bars). (B) Number of BAL neutrophils recovered at 3, 6 and 24 hours after intranasal inoculation with LPS (10 μg/mouse, white bars) or LPS plus f-LIGRL (30 μg/mouse, black bars). Data is presented as the mean ± s.e.m of 5 – 6 mice / group (* indicates p < 0.05, compared to corresponding LPS-alone group, white bar).

Figure 3
Effect of selected doses of (A) SLIGRL (μg/mouse), (B) f-LIGRL (μg/mouse) and (C) LSIGRL (μg/mouse) on the number of BAL neutrophils recovered 3 hours after intranasal inoculation with 10μg LPS or vehicle. Data is presented as the mean ±
s.e.m of 5 – 6 mice / group (* indicates p < 0.05, compared to LPS-alone group, white bar).

**Figure 4**
Effect of (A) indomethacin (INDO, 1 mg/kg i.p. one hour before inoculation) and (B) parecoxib (COXIB, 1 mg/kg i.p. one hour before inoculation) on f-LIGRL-induced (30 μg/mouse, i.n.) reductions in BAL neutrophil numbers recovered 3 hours after intranasal inoculation with LPS (10 μg/mouse). Data is presented as the mean ± s.e.m of 5 – 6 mice / group (* indicates p < 0.05, compared to LPS-alone group, white bar).

**Figure 5**
Effect of (A) PGE₂ (10 μg/mouse, i.n.) and (B) butaprost (10 and 50 μg/mouse, i.n.) on the number of BAL neutrophils recovered 3 hours after intranasal inoculation with LPS (10 μg/mouse) or sterile vehicle. Data is presented as the mean ± s.e.m of 5 – 12 mice / group (* indicates p < 0.05, compared to LPS-alone group, white bar).

**Figure 6**
Effect of (A) an EP₁/EP₂ receptor antagonist (AH6809, 100 μg, one hour before inoculation) and (B) a neurokinin-1 receptor antagonist (NK₁-R-A, L-703,606, 3 mg/kg i.p., one hour before inoculation) on f-LIGRL-induced (30 μg/mouse, i.n.) reductions in BAL neutrophil numbers recovered 3 hours after intranasal inoculation with LPS (10 μg/mouse) or vehicle. Data is presented as the mean ± s.e.m of 5 mice / group (* indicates p < 0.01, compared to LPS-alone group, white bar).
Figure 7
Effect of a PAR2-AP (f-LIGRL, 30 μg/mouse, i.n.) and control peptide (f-LRGIL, 30 μg/mouse, i.n.) on LPS-induced increases in MIP-2 (black bars) and KC (white bars) levels in BAL fluid recovered from LPS-exposed mice at (A) 1 hour and (B) 3 hours post LPS. Data is presented as the mean ± s.e.m of 4-9 mice / group (* indicates p < 0.01, compared to LPS-alone group).

Figure 8
Influence of PAR2-APs and PGE2 on MIP-2 levels in mouse alveolar macrophage cultures. MIP-2 levels were determined (A) following exposure to 10 ng/ml LPS (black bars) or vehicle (white bars) for varying periods, and (B) following a 3 hour exposure to varying concentrations of LPS. The effects of f-LIGRL (20 μM), SLIGRL (1, 10 and 100 μM) and PGE2 (1 and 10 μM) on MIP-2 levels in the presence and absence of LPS (10 ng/ml, 3 hours exposure) were also determined (panels C, D and E, respectively). Data is presented as the mean ± s.e.m (* indicates p < 0.05, compared to control values (white bars)).

Figure 9
Influence of (A) f-LIGRL or control peptide f-LRGIL (20 μM) and (B) 1 and 10 μM PGE2 on TNF-α levels in mouse alveolar macrophage cultures exposed to either 10 ng/ml LPS (black bars) or vehicle (white bars) for 3 hours. Panel (C) shows the effect of a 3 hour exposure to TNF-α (10 or 100 ng/ml) and LPS (10 ng/ml) on MIP-2 levels in cultures. The final bar shows the effect of an anti-TNF-α antibody (20 μg/ml) on MIP-2 levels in cultures exposed to 10 ng/ml LPS for 3 hours. Data is
presented as the mean ± s.e.m († indicates p < 0.05 compared to no PGE₂ in control cultures, and * indicates p < 0.05 compared to no PGE₂ in LPS-treated cultures).

Figure 10

PGE₂ levels in cultures of alveolar macrophages following selected periods of exposure to a PAR₂-AP (SLIGRL, black bars) or to a control peptide (LSIGRL, white bars). Data is presented as the mean ± s.e.m.
Figure 1

A

B

C

D

E

F

Total BAL cells (millions)

Neutrophils (millions)

Macrophages (millions)

Time (hours)

Dose of LPS (µg/mouse)
Figure 2

A

Neutrophils (millions)

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>2.5</td>
</tr>
<tr>
<td>24</td>
<td>3.0</td>
</tr>
</tbody>
</table>

B

Neutrophils (millions)

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>4.0</td>
</tr>
<tr>
<td>24</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Figure 4

A

![Bar graph for Neutrophils (millions)]

B

![Bar graph for Neutrophils (millions)]
Figure 9

A

TNF-α (pg/ml)

LPS
f-LIGRL
Control

B

TNF-α (pg/ml)

LPS
PGE₂

C

MIP-2 (pg/ml)

LPS
TNF-α
Anti-TNF

* Indicates significant difference from control.
† Indicates trend towards significance.
Figure 10

![Graph showing PGE<sub>2</sub> levels over time](image-url)