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Factors modulating TLR2, TLR4 and HBD2 expression

in Respiratory Epithelium in COPD.

Thesis – Degree of M.D. 2006
University of Dublin, Trinity College
Dr Ruth MacRedmond
MB, BCh, BAO, MRCPI

Department of Respiratory Research
Beaumont Hospital and Royal College of Surgeons in Ireland
Dublin 9
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Summary

The innate immune response of the respiratory epithelium, employing mechanisms of germ-line encoded, genetically conserved pattern recognition to “non-self” organisms, is an important component of host defence against inhaled pathogens. Key components of innate immunity include the toll-like receptors, including TLR4 that signals the response to Gram-negative elements and TLR2 signalling Gram-positive patterns, and antimicrobial peptides such as human beta-defensin 2. Airway infection is important in COPD, and this project explores elements of innate immunity pertinent to COPD.

The respiratory epithelial cell line A549 and the renal epithelial cell line, HEK 293 (which are unresponsive to LPS) were used as a model system. Gene expression was determined by semi-quantitative rtPCR, Real Time PCR and by promoter-linked luciferase activity. Western blotting, immunohistochemistry, laser scanning cytometry and ELISA determined protein expression. The role of specific components of signalling pathways was further explored by transfection of functionally active or inactive transgene constructs. Human respiratory epithelial cells in vivo were harvested by brushing of nasal and tracheo-bronchial epithelium.

In the first part of this study, I provide evidence for a critical role for TLR4 in LPS-induced HBD2 expression in airway epithelial cells. Transcriptional regulation of HBD2 is further elucidated with demonstration of the role of the adaptor proteins MyD88 and Mal in this reaction.

I next looked at the effect of potential modulators of TLR4 expression in respiratory epithelium, which are pertinent to COPD. Using the same cell culture model, I found a dose-dependent reduction in TLR4 mRNA and protein following stimulation with the corticosteroids fluticasone and dexamethasone. The
functional significance of this effect was demonstrated by impaired IL-8 and HBD2 induction in response to LPS. Stimulation with salmeterol (10^6 M) caused upregulation of TLR4 membrane protein expression with no upregulation of mRNA, suggesting a post-translational effect. The effect of dexamethasone and salmeterol in combination was additive, with downregulation of TLR4 gene expression, and no change in membrane receptor expression. Stimulation of airway epithelial cells with cigarette smoke extracts resulted in a dose dependent downregulation in TLR4 mRNA and protein expression. A similar effect was found in vivo, with significantly reduced expression of TLR4 in the nasal epithelium of smokers, harvested by brushing, compared to healthy controls.

The final part of my study examines expression of TLR2/4 and HBD2 in COPD patients in-vivo, to see if our observations in the laboratory could be translated to the “real world”. I demonstrate that TLR4 and HBD2 expression is significantly downregulated nasal epithelium of patients with severe stable COPD compared to those with less severe disease, while there is no significant difference in TLR2 expression. I further demonstrate excellent correlation between nasal and tracheobronchial expression of TLR4. This correlates with the increased incidence of Gram-negative pneumonia in severe COPD.

This study provides new data regarding innate immune function of the respiratory epithelium and modulation of this important part of host defense by inhaled compounds and airways disease. It is hoped that an understanding of these processes may ultimately guide future therapies aimed at augmenting or preserving innate immunity, thus protecting against pulmonary infection.
Chapter 1

Introduction
1.1 General Introduction

The lung represents the largest epithelial surface in the body and as such is a major portal of entry for pathogenic microorganisms. Despite being regularly exposed to particulate matter during breathing, which contains bacteria from the commensal flora in the nasopharynx and from the environment, the healthy lung is kept sterile by efficient defence mechanisms. These defence mechanisms consist firstly of non-specific physical barriers to entry, and failing this, the specific innate and adaptive immune responses, representing a dynamic interaction of host and pathogen. Mucins of the mucociliary blanket lining the surface of airways act by trapping micro-organisms that are then cleared by ciliary movement. Particles that pass this barrier are met by a range of soluble mediators, some constitutive, some induced by specific activation, produced by cells of the respiratory tract. Previous studies have focused on factors relating to bacterial adherence (impaired mucociliary clearance, epithelial damage and unmasking of extracellular matrix) as possible contributors to bacterial colonisation of the lower respiratory tract (Plotkowski, Chevillard et al. 1991). The innate immune system, which is integral to early pathogen recognition and elimination, may also have an important role in this process. This project will focus on elements of the innate immunity of respiratory epithelium, namely the anti-microbial peptide human beta-defensin 2 (HBD2) and the pathogen recognition receptors, the Toll-like receptors (TLRs), particularly in the context of Chronic Obstructive Pulmonary Disease.
1.2 The Toll-like receptors

The immune system responds to microbial pathogens using both innate and adaptive components. The key features of adaptive immunity are the clonal expansion of lymphocytes in response to a particular pathogen and the ability to evoke an immunologic memory. Specific T and B-cell receptors for each clone of cells respond to a specific antigen. Each clonal receptor is structurally unique and is not encoded in the germ line, but rather is established in each generation following exposure to antigen. There is a lag period of more than one week for a primary host and of several days for the primed host. Host defence in this period relies on the innate immune response. The immediate innate response is mediated largely by white cells such as neutrophils and macrophages, cells that phagocytose and kill pathogens, and that synchronise the additional host response by synthesising a wide range of inflammatory mediators and cytokines. In macrophages, the infectious agent is killed and degraded in the phagosome and components of the pathogen are presented to the T cells resulting in activation of the adaptive immune response. (Aderem and Underhill 1999)

Unlike adaptive immunity, innate immune responses are germ line encoded and highly conserved. The challenge for this system is the recognition and discrimination of the enormous numbers of potential pathogens in order to mount the appropriate response. The problem is compounded by the tendency of pathogens to mutate. Innate immune recognition is based on a pattern recognition system. Because microbes are extremely heterogenous and have high mutation rates, the system focuses on highly conserved structures collectively present in many types of microorganisms. These motifs have essential roles in the biology of
the invading agents and are therefore not subject to high rates of mutation. They have been termed pathogen associated molecular patterns (PAMPs) and their cognate binding patterns on the host cell pattern recognition receptors (PRRs) (Janeway and Medzhitov 1998). Examples of PAMPs include mannans in the yeast cell wall, formylated peptides and various bacterial cell wall components such as lipopolysaccharide (LPS) in Gram-negative bacteria, peptidoglycans and lipoteichoic acid in Gram-positive bacteria. It is estimated that the total number of receptors involved in innate recognition of antigens is less than 100, but using this pattern recognition system the innate immune system can recognise, discriminate and initiate an appropriate response to thousands of different pathogens (Hallman, Ramet et al. 2001). The Toll-like receptors are such a family of PRRs, and the rapid expansion of knowledge about these molecules in recent years has illustrated their pivotal importance in orchestrating the innate immune response.

Toll receptor was originally identified in Drosophila as an essential receptor for the establishment of dorso-ventral pattern in developing embryos (Hashimoto, Hudson et al. 1988). Despite the lack of an adaptive immune system, Drosophila is highly resistant to microbial infections (Imler and Hoffmann 2000). Hoffman and colleagues found that Toll-mutant flies were highly susceptible to fungal infection, suggesting a role for this receptor in host defence (Lemaitre, Nicolas et al. 1996), and subsequently demonstrated that activation of members of the Toll-like receptor (TLR) family in Drosophila, dToll and 18-wheeler, resulted in synthesis of the anti-fungal peptide drosomycin and the antibacterial peptide attacin respectively (Lemaitre, Nicolas et al. 1996; Williams, Rodriguez et al. 1997). Furthermore, activation of these receptors initiates an intracellular cascade
culminating in translocation of the transcription factors Dif and Relish to the nucleus. Dif and Relish are homologues of the mammalian transcription factor nuclear factor-kappa B (NF-κB), known to activate a variety of inflammatory mediators and cytokines including TNF-α and IL-12 (Anderson 2000).

In their search for receptors within the innate immune system, Janeway and colleagues recognised the highly conserved nature of PRRs and began searching for dToll related proteins. Their efforts were rewarded with the discovery of the first human homologue of Drosophila dToll, initially termed human Toll, and subsequently TLR4 (Medzhitov, Preston-Hurlburt et al. 1997). Activation of this protein resulted in upregulation of NF-κB controlled pro-inflammatory genes IL-1, IL-6 and IL-8 as well as members of the B7 family, molecules that are required for the activation of naïve T-cells by antigen presenting cells (Medzhitov, Preston-Hurlburt et al. 1997). A mutation of the TLR4 gene was identified in C3H/HeJ mice that are hyporesponsive to LPS (Poltorak, He et al. 1998). This and other studies established TLR4 as the principal pathogen recognition receptor for the PAMP LPS. Binding of ligand to receptor activates a signalling cascade resulting in upregulation of effector genes of the immune response. A further nine TLRs have since been described in humans, bringing the total to 10, and at least one ligand has been described for each TLR except TLR10: triacylated lipoprotein for TLR1, peptidoglycan for TLR2, double stranded DNA for TLR3, LPS for TLR4, flagellin, a component of bacterial flagella for TLR5, diacylated lipoprotein for TLR6, imidazoquinoline, an anti-viral drug, and it’s derivative R-848 for TLR7, GU rich ssRNA for TLR8, and bacterial unmethylated CpG DNA for TLR9 (Heil, Hemmi et al. 2004; Yamamoto, Takeda et al. 2004). (See figure 1). The best described and most widely studied of these TLRs are TLR4 and TLR2, which principally signal the response to Gram-negative and Gram-positive bacteria.
respectively. TLR11 has recently been described in mice, displaying a distinct pattern of expression in macrophages and liver, kidney, and bladder epithelial cells. Cells expressing TLR11 fail to respond to known TLR ligands but instead respond specifically to uropathogenic bacteria. TLR11 sequences are present in the human genome but it is suggested that humans may not express full length TLR11 protein, due to the presence of stop codons in the open reading frame of the TLR11 gene (Zhang, Zhang et al. 2004).
### Complete list of TLR ligands

#### TLR2
- **Gram + Bacteria**
  - PGN, LTA
- **Bacteria**
  - Lipoprotein
- **Spirochetes**
  - Glycolipids
- **Mycobacteria**
  - Lipoarabinomannan
- **Porphyromonas gingivalis**
  - LPS, fimbriae
- **Spirochetes (Leptospira)**
  - LPS
- **Yeast**
  - Zymosan
- **Trypanosoma Cruzi**
  - GPI anchor
- **Klebsiella**
  - Outer membrane protein A
- **Neisseria**
  - Porin, OMV-LPS
- **Host**
  - HSP60, HSP70, HSPgp96

#### TLR2/TLR1
- **Neisseria meningitides**
  - Soluble factor
- **Bacteria**
  - Triacylated lipoproteins
- **Chemicals**
  - JBT3002

#### TLR3
- **Virus**
  - dsRNA
- **Chemicals**
  - poly(I:C)

#### TLR4
- **Gram-negative bacteria**
  - LPS, LTA
- **Chlamydia**
  - HSP60
- **Flavobacteria**
  - Flavolipin
- **RS virus**
  - F protein
- **Murine retrovirus**
  - Envelope proteins
- **Plant**
  - Taxol
- **Host**
  - HSP60, HSP70, HSPgp96, Fibronectin EDA
  - Oligosaccharides of Hyaluronan

#### TLR5
- **Bacteria with flagellin**
  - Flagellin

#### TLR2/TLR6
- **Mycoplasma**
  - Di-acylated lipoprotein
- **Group B Streptococcus**
  - Heat labile soluble factor (GBS-F)
- **Staphlococcus**
  - Phenol soluble modulin

#### TLR7
- **Chemicals**
  - Imidazoquinolones, Loxoribine, Broprimine

#### TLR8
- **Virus**
  - GU rich ssRNA

#### TLR9
- **Bacteria, virus, insects**
  - Unmethylated CpG DNA
- **Host**
  - Chromatin IgG complexes
The TLRs consist of a cytoplasmic portion that is homologous to that of the interleukin-1 (IL-1) receptor and has been termed the Toll/IL-1 receptor (TIR) domain and an extra-cellular leucine rich repeat (LRR) domain. The signalling cascade downstream of TLR is generated from this TIR domain (Bowie and O'Neill 2000). MyD88 is an adaptor protein possessing a TIR domain in the C-terminal portion and a death domain in the N-terminal portion. MyD88 associates with the TIR domain of TLRs. Upon stimulation, MyD88 recruits IL-1 receptor – associated kinase (IRAK), a serine threonine kinase related to the Pelle kinase of *Drosophila*, through interaction of the death domains of both molecules (Muzio, Ni et al. 1997; Medzhitov, Preston-Hurlburt et al. 1998). Four members of the IRAK family have been identified so far: IRAK-1, IRAK-2, IRAK-4 and IRAK-M. IRAK-4 acts upstream of, and phosphorylates, IRAK-1, while IRAK-M plays a negative inhibitory role (Kobayashi, Hernandez et al. 2002; Li, Strelow et al. 2002). IRAK then dissociates from the receptor complex and associates with tumour necrosis factor (TNF) receptor-activated factor 6 (TRAF-6). The IRAK/TRAF-6 complex then associates with the TGF-β-activated kinase 1(TAK-1) and TAK-1 binding proteins TAB1 and TAB2. IRAK is degraded, while the TRAF-6, TAK1, TAB1 and TAB2 complex forms a larger complex in the cytoplasm with other proteins such the E2 ligases Ubc13 and Uev1A (Deng, Wang et al. 2000). TAK-1 is activated and phosphorylates the IKK-complex consisting of IKKα, IKKβ and NEMO/IKKɛ, and the cJun NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) family, resulting in activation of the transcription factors NF-κB and AP-1 respectively (Takeda and Akira 2004). (See Figure A)
MyD88 binds to the cytoplasmic portion of TLRs through interaction between individual TIR domains. Upon stimulation, IRAK-4, IRAK-1, and TRAF6 are recruited to the receptor, which induces association of IRAK-1 and MyD88 via the death domains. IRAK-4 then phosphorylates IRAK-1. Phosphorylated IRAK-1, together with TRAF6, dissociates from the receptor and then TRAF6 interacts with TAK1, TAB1, and TAB2. The complex of TRAF6, TAK1, TAB1, and TAB2 further forms a larger complex with Ubc13 and Uev1A, which induces the activation of TAK1. Activated TAK1 phosphorylates the IKK complex, consisting of IKKα, IKKβ, and NEMO/IKKγ, and MAP kinases, such as JNK, and thereby induces the activation of the transcription factors NF-κB and AP-1, respectively. (Takeda and Akira 2004)
It was previously thought that MyD88 was essential for TLR signalling. MyD88 knockout mice showed no cellular response, in terms of cytokine production, to ligands of TLRs such as LPS (TLR4), peptidoglycan (TLR2), CpG DNA (TLR9), imidazoquinolone (TLR7) and flagellin (TLR5) (Kawai, Adachi et al. 1999; Hacker, Vabulas et al. 2000; Takeuchi, Kaufmann et al. 2000; Hemmi, Kaisho et al. 2002). Stimulation of MyD88-deficient cells with the TLR4 ligand LPS was however shown to result in activation of NF-κB and JNK, albeit with delayed kinetics (Kawai, Adachi et al. 1999). It has since emerged that MyD88-deficient cells are intact in their ability to induce interferon (IFN)-inducible genes, such as IP-10, GARG-16 or IRG-1, and that this is achieved through activation of an MyD88-independent pathway involving activation of the transcription factor IRF-3, resulting in upregulation of IFN-β. IFN-β in turn activates Stat-1 leading to activation of several IFN-inducible genes (Kawai, Takeuchi et al. 2001; Doyle, Vaidya et al. 2002; Toshchakov, Jones et al. 2002). It has subsequently been shown that the TLR3 ligand dsRNA can also activate this MyD88-independent signalling pathway (Yoneyama, Suhara et al. 1998).

Analysis of the MyD88-independent signalling pathways has led to the identification of two further TIR domain containing adaptors: TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) and TIR domain-containing adaptor inducing IFN-β (TRIF)/TOR domain-containing adaptor molecule (TICAM-1). Studies with knockout mice demonstrated that TIRAP/Mal is essential for MyD88-dependent signalling via TLR4 and TLR2, but not for the MyD88-independent signalling, and is not involved in signalling responses to TLR3, TLR5, TLR7 and TLR9 ligands (Horng, Barton et al. 2002; Yamamoto, Sato et al. 2002). TRIF is essential for the TLR3- and TLR4- mediated MyD88-independent signalling pathway (Yamamoto, Sato et al. 2003). Thus, the TIR
domain containing adaptor proteins confer specificity on the TLR signal transduction pathways.
Activation of the innate immune response results in upregulation of a variety of cytokines, chemokines and adhesion molecules essential for pathogen destruction and induction of the adaptive immune response. Similar to *Drosoplila*, which produces fungicides and bactericides in response to infection, the mammalian innate immune system also produces a variety of anti-microbial peptides (AMPs) as part of its host defence repertoire. Indeed, these proteins constitute the first line of defence against a broad spectrum of microbes, including Gram-positive and Gram-negative bacteria, fungi and certain viruses. In addition, these peptides can interact with the host itself, triggering events that complement their role as antibiotics. This diverse group of small proteins include the cathelicidins (including LL-37), the defensins, mucins, lysozyme, lactoferrin, fibronectin and many others (Bals and Hiemstra 2004).

The defensins are a broadly dispersed group of gene-encoded AMPs. These small cationic peptides are typically 28-44 amino acids long and contain 6 to 8 cysteine residues that form characteristic intramolecular disulfide bridges. The alignment of disulfide bridges and molecular structure is used to classify defensins into three distinct families: the α-defensins, the β-defensins and the θ-defensins. The β-defensins contain 6 cysteine motifs connected by three disulfide bonds. To date, four types of β-defensin have been identified in humans, (human β-defensin (HBD) 1 through 4), although genomic studies suggest many more have yet to be discovered (Raj and Dentino 2002; Schutte, Mitros et al. 2002).

The molecular structure of the defensins determines their biological activity. Like most antimicrobial peptides, defensins are cationic (polar) molecules with spatially separated hydrophobic and charged regions. This arrangement allows
them to insert themselves into phospholipid membranes so that their hydrophobic regions are buried within the oily membrane interior and their cationic regions interact with anionic phospholipid head groups and water. In the membrane, some defensins assemble into multimeric pores. Defensins and other AMPs preferentially disrupt bacterial membranes that are rich in negatively charged phospholipids. Conversely, the lower anionic phospholipid content of the cell membranes of higher animals may provide relative protection from collateral damage (Ganz 1999).

HBD1 was originally isolated from haemofiltrates, but was subsequently found in human urine and has been determined to be constitutively expressed in the kidney and female urogenital tract (Valore, Park et al. 1998). A second β-defensin, HBD2, was first isolated from the skin of patients with psoriasis, and was found to be expressed in epithelial cells of the trachea, skin and lung tissues (Harder, Bartels et al. 1997). Expression has since been demonstrated in gingival and intestinal epithelial cells (Mathews, Jia et al. 1999; O'Neil, Porter et al. 1999), and the peptide has been isolated from epithelial lining fluid (ELF) and bronchoalveolar lavage (BAL) fluid, as well as from the supernatants of cultured respiratory epithelial cells in vitro (Singh, Jia et al. 1998; Hiratsuka, Mukae et al. 2003).

Like other defensins, HBD2 has a broad spectrum of anti-microbial activity. HBD2 displays potent microbicidal activity against many Gram-negative bacteria, including *Escherischa coli* and *Pseudomonas aeruginosa* (LD$_{90}$:10 μg/ml), as well as the yeast *Candida albicans* (LD$_{90}$:25 μg/ml) (Singh, Jia et al. 1998). It is less effective against Gram-positive organisms however, demonstrating bacteriostatic as opposed to bactericidal activity against *Staphlococcus aureus*
(LD₅₀ >100 μg/ml). HBD3 has much more potent anti-Staphlococcal activity compared to either HBD1 or HBD2 and it is postulated that this is due to its ability to form amphipathic dimers in solution with increased positive surface charge density compared to the other two structures (Schibli, Hunter et al. 2002).

As well as having direct anti-microbial action, HBD2 has additional immunomodulatory roles which augment its function in host defence. Both HBD2 and HBD1 have been shown to be chemotactic in vitro. At submicromolar concentrations, these defensins attracted both immature dendritic cells and memory T cells, which initiate primary and recall immune responses, respectively. The effect was evidently mediated by the CCR6 chemokine receptor because β-defensins effectively competed with the receptor's ligand, MIP-3α. If the same mechanism functions in vivo, the release of these two defensins from injured epithelial cells would recruit dendritic cells and memory T cells to infected tissues, thereby promoting the development of adaptive (antibody and T cell-mediated) immunity (Yang, Chertov et al. 1999). HBD2 can also promote histamine release and prostaglandin D2 production in mast cells, suggesting a role in allergic reactions (Befus, Mowat et al. 1999; Niyonsaba, Ogawa et al. 2004).

In contrast to HBD1, which is constitutively and stably expressed, HBD2 expression is induced in response to infective stimuli, including Gram-negative and, less potently, Gram-positive bacteria or their components (LPS and peptidoglycan) or to pro-inflammatory stimuli including tumour necrosis factor (TNF)-α and interleukin (IL)-1β in vitro (Singh, Jia et al. 1998; Harder, Meyer-Hoffert et al. 2000). A similar effect has been demonstrated in vivo with the demonstration of increased HBD2 in tracheal aspirates from neonates with pulmonary or systemic infection (Schaller-Bals, Schulze et al. 2002). HBD2 can
thus act as a dynamic component of the host defence, whereby the epithelium can elicit an immediate anti-microbial response to a pathogen, which is independent of leukocyte mediated immune defence mechanisms.

HBD2 was first described in skin, and many investigators have looked at the role of HBD2 in chronic inflammatory skin conditions. Atopic dermatitis is a chronic inflammatory disease of skin, frequently found in association with asthma and allergic rhinitis, which is complicated by bacterial, viral and fungal infections. Skin lesions from these patients have been found to have reduced levels of HBD2 mRNA and protein compared to normal controls and to patients with psoriasis (Ong, Ohtake et al. 2002). Patients with psoriasis, another chronic inflammatory skin condition, have a reduced incidence of several types of infection. It has been proposed that this relative resistance to infection is due to increased local production of anti-microbial peptides in psoriatic skin (Christophers and Henseler 1987; Frohm, Agerberth et al. 1997; Harder, Bartels et al. 1997). Taken together, this data demonstrates clinically significant disease arising from local innate immunodeficiency, while relative protection is afforded by overproduction of anti-microbial peptides under similar conditions. Increased HBD2 expression has also been demonstrated in inflamed intestinal epithelium in the context of ulcerative colitis, peptic ulcer disease and *Helicobacter pylori* infection (O'Neil, Porter et al. 1999).

Much of the work regarding the role of the defensins in the lung has focused on Cystic Fibrosis, a disease in which pulmonary mucosal defences are impaired, leading to bacterial colonisation, first with *Haemophilis influenza* and *Staphylococcus aureus* and later with mucoid strains of the Gram-negative
organism *Pseudomonas aeruginosa* (Konstan M. 1993). This bacterial colonisation and chronic inflammation is associated with increased morbidity and mortality in CF. The production of the mucoid extracellular polysaccharide by *P. aeruginosa* has been linked to virulence, and it is these mucoid strains rather than non-mucoid strains which induce HBD2 in respiratory epithelium *in vivo* (Harder, Meyer-Hoffert et al. 2000). Although defensins are found in normal concentration in the lungs of patients with CF, it is postulated that they exhibit reduced activity in the local environment of the CF lung (Goldman, Anderson et al. 1997). The defective chloride channel (CFTR) in the CF lung results in elevated salt concentration on epithelial cells. Investigators have demonstrated a dose dependent reduction in anti-microbial activity of HBD2 according to salt concentration *in vitro* (Bals, Wang et al. 1998; Harder, Meyer-Hoffert et al. 2000). CF airway epithelia fail to kill apically applied *P. aeruginosa*, contrasting with the anti-bacterial properties of normal airway epithelium (Smith, Travis et al. 1996). A recent study also demonstrates degradation and inactivation of HBD2 by the serine proteases cathepsin B, L, and S, all of which are present in increased concentrations in CF BAL, and indeed by CF BAL itself (Taggart, Greene et al. 2003). It is postulated that impaired β-defensin activity in the CF lung may be an important factor in the impaired host defence of these patients, resulting in failure to clear pathogens and increased susceptibility to bacterial colonisation.

Increased HBD2 expression has been demonstrated in a number of acute and chronic respiratory conditions. HBD2 is found in the BAL of patients with bacterial pneumonia and is upregulated in these patients (Hiratsuka, Nakazato et
al. 1998). Increased levels have also been demonstrated in other infective conditions, such as diffuse panbronchiolitis, a progressive condition characterised by frequent episodes of superimposed infection, typically caused by *P. aeruginosa* (Hiratsuka, Mukae et al. 2003). Singh et al also demonstrated increased levels of HBD2 in BAL from patients with non-infective chronic inflammatory conditions such as sarcoidosis and idiopathic pulmonary fibrosis, with increased levels also found in the context of the non-specific inflammatory insult of inhalation injury (Singh, Jia et al. 1998; Milner, Cole et al. 2003). This correlates with the observed upregulation of HBD2 expression in response to pro-inflammatory cytokines observed in vitro. Analysis of the nasal mucosa of patients with common cold also showed a reduced up-regulation of HBD2 in response to inflammatory stimuli (Dauletbaev, Gropp et al. 2002). HBD2 expression and regulation has not been investigated in the context of COPD to date, however a polymorphism of the HBD1 gene has been found to be significantly associated with the disease (Matsushita, Hasegawa et al. 2002).

**1.4 Linking Toll to HBD2**

Like other elements of the innate immune system, the defensins are highly conserved structures, and the bovine β-defensin tracheal antimicrobial peptide (TAP) serves as a paradigm for induction of innate immunity in the airway in man. The TAP gene is expressed in ciliated airway epithelium in response to bacterial infection. *In vitro* incubation of bovine tracheal epithelium with LPS increases TAP mRNA levels via a CD14-mediated response, culminating in activation of NF-κB and transcriptional up-regulation of the TAP gene (Diamond, Russell et al. 1996; Diamond, Kaiser et al. 2000). Applying this paradigm to human tracheobronchial epithelial (hTBE) cells in culture, Becker et al
demonstrated that LPS-induced HBD-2 expression was similarly dependent on CD-14. (Becker, Diamond et al. 2000)
LPS is the primary inducer of Gram-negative septic shock, and as such, enormous effort has been devoted to the identification of the LPS receptor. Several molecules bind LPS and subsequently activate resting monocytes/macrophages including β₂-integrins (CD11/CD18), the macrophage scavenger receptor for acetylated Low-density lipoprotein (LDL), L-selectin and CD14. These molecules also play an important role in the internalization and detoxification of endotoxin. However, CD14 is the main 'LPS-receptor' that can activate monocytes in conjunction with serum LPS-binding protein (LBP) at low (<10 ng mL⁻¹) clinically significant concentrations of LPS, as observed in the sera of septic patients (Wright, Ramos et al. 1990). Furthermore, CD14 interacts with different components of other Gram-negative and-positive bacteria and fungi, defining CD14 as a central pattern recognition molecule in innate immunity (Pugin, Heumann et al. 1994). Based on its cDNA sequence, CD14 is a membrane glycoprotein of 356 amino acids and a 19 amino acid long N-terminal leader peptide. The CD14 gene is on chromosome 5 (region q23-21), consisting of two exons and encoding a single 1.4-kb mRNA transcript. After translation, the C-terminal leader sequence of 28-30 amino acids is replaced by a glycosyl-phosphatidyl inositol (GPI) anchor. So, CD14 is not a transmembrane protein but is attached to the plasma membrane via the GPI tail (Haziot, Chen et al. 1988). The GPI-anchor has no role in the signal transduction.

Because CD14 lacks a transmembrane or cytoplasmic domain, it is must act in concert with other signal transduction molecules in order to influence gene transcription. Several intracellular kinases have been reported to be activated by LPS bound in a CD14-dependent way: protein tyrosine kinases, mitogen activated
tyrosine kinases (ERK1/2, JNK, p38) and protein kinase A and C, as has activation and translocation of NF-κB to the nucleus (Sweet and Hume 1996).

The TLRs are transmembrane pathogen recognition receptors, possessing a cytoplasmic domain, binding to which activates an intracellular signalling cascade including the kinases and transcription factors described above. This suggests that the TLRs would be attractive candidate co-factors to work in concert with CD14 and LPS binding protein. Of the 11 TLRs identified to date, TLR4 has emerged as the most important in LPS signalling. I postulated therefore that LPS-induced HBD-2 upregulation in epithelial cells is TLR4-dependent.
1.6 COPD

COPD is a major public health problem. It is the fourth most common cause of chronic morbidity and mortality in the USA, and it is projected by the World Health Organisation/World Bank that by the year 2020, it will rank fifth in the worldwide burden of disease (1995; Murray and Lopez 1996). The American Thoracic Society previously defined COPD physiologically by the presence of irreversible or partially reversible airflow obstruction in patients with chronic bronchitis or emphysema (Murray and Lopez 1996). Chronic bronchitis is defined clinically by the presence of productive cough for most days of at least three months on two consecutive years, where other causes of cough are excluded (1965). Emphysema is defined clinically as permanent dilatation of the airspaces distal to the terminal bronchioles, accompanied by destruction of the alveolar septa in the absence of fibrosis (Snider 1989). This rather cumbersome definition has been revised by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) committee, which defines COPD as follows: “COPD is a disease state characterised by airflow obstruction that is not fully reversible. The airflow limitation is usually both progressive and associated with abnormal inflammatory response in the lungs to noxious gases or particles.” (www.goldcopd.com/revised).

This new definition highlights the increasingly recognised importance of airway inflammation in the pathogenesis of this disease. The natural history of COPD is one of a progressive decline in ventilatory function, exercise tolerance and health status that is punctuated by exacerbations of symptoms (Fletcher and Peto 1977). Patients who suffer the most exacerbations have significantly lower health status and there is also evidence that exacerbation frequency predicts accelerated decline in lung function (Seemungal, Donaldson et al. 1998; Kanner, Anthonisen et al. 2001; Donaldson, Seemungal et al. 2002). Acute respiratory tract infection has
been shown to have a significant effect on decline in lung function in smokers with COPD (Kanner, Anthonisen et al. 2001), while chronic mucus hypersecretion was significantly and consistently associated with both an excess FEV1 decline and an increased risk of subsequent hospitalization because of COPD in the Copenhagen City Health study (Vestbo, Prescott et al. 1996).

The pathogenesis of COPD remains unclear, but three factors appear to be important: cigarette smoking, inhalation of dust and infections. The role of bacteria in the pathogenesis and acceleration of COPD remains the subject of some debate, but increasing evidence in recent years supports the importance of bacteria in this disease, as a stimulus to chronic inflammation and a cause of exacerbations. In their state of the art review of bacterial infection in COPD, Sethi and Murphy outline five potential ways in which bacteria could contribute to the course and pathogenesis of COPD:

(i) **Childhood lower respiratory tract infection impairs lung growth, reflected in smaller lung volumes in adulthood.** Several studies have demonstrated a lower forced expiratory volume in one second (FEV1) in subjects with a reliable history of childhood lower respiratory tract infections compared to normal controls. Controversy remains as to whether this is a causative relationship or represents a genetic vulnerability to both infection and airflow obstruction.

(ii) **Bacteria cause a substantial proportion of acute exacerbations of chronic bronchitis which cause considerable morbidity and mortality.** Bacteria are isolated from sputum in 40-60% of acute exacerbations of COPD (Sethi 2000). There has been much controversy over the years as to whether these bacteria were pathogenic or were merely innocent
bystanders in the exacerbation process, isolated from common colonisers of the respiratory tract. Advances in diagnostic and research techniques in the last decade have allowed this issue to be examined more rigorously. Several studies using bronchoscopic sampling of the lower respiratory tract with protected specimen brush for quantitative culture have demonstrated significant bacterial infection of the distal airway in approximately half of patients experiencing an acute exacerbation (Fagon, Chastre et al. 1990; Monso, Ruiz et al. 1995; Pela, Marchesani et al. 1998; Soler, Ewig et al. 1999). Molecular typing of bacteria has shown that colonisation is not stable but that there is a frequent turnover of discrete strains of *H. influenzae* (Murphy, Sethi et al. 1999), and that there is an association between an exacerbation of COPD and isolation of a new strain of a bacterial pathogen (Sethi, Evans et al. 2002). Furthermore, strain-specific anti-bodies to surface outer membrane proteins of *H. influenzae* have been shown to develop in relation to exacerbations (Yi, Sethi et al. 1997). Strain-specific antibody responses to *B. catarrhalis* and *H. parainfluenzae* have also been demonstrated (Chapman, Musher et al. 1985.; Hill, Mitchell et al. 2000) This data suggests that a change in strain but not necessarily organism may be responsible for an exacerbation of COPD, and earlier studies which failed to discriminate between different strains may have missed evidence of a new infection.

(iii) **Chronic colonization of the lower respiratory tract by bacterial pathogens amplifies the chronic inflammatory response present in COPD and leads to progressive airway obstruction (vicious circle hypothesis).** *In vitro* studies have shown that cell-free supernatants of cultures of *H. influenzae, S. pneumoniae* and *P. aeruginosa* can induce
mucus hypersecretion and impair ciliary kinetics (Murphy, Sethi et al. 1999), and that non-typeable *H. influenzae* causes epithelial injury (Read, Wilson et al. 1991). *H. influenza* LPS has been shown to increase epithelial expression of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α *in vitro* (Williams, Rodriguez et al. 1997), providing a potential mechanism for upregulation of inflammation. There has been further *in vivo* evidence to support this concept of bacterial colonisation resulting in airway inflammation. Patients with a higher positive culture of *H. influenzae* have increased concentration of TNF-α in their sputum (Bresser, Out et al. 2000), while a study of patients with stable chronic bronchitis demonstrated a direct correlation between bacterial load and markers of neutrophilic inflammation, regardless of the pathogen isolated (Hill, Campbell et al. 2000). Similarly, the isolation of pathogenic microbes from broncho-alveolar lavage fluid is strongly associated with increased neutrophils and TNF-α. Eradication or reduction of bacteria is associated with reduced inflammation (Soler, Ewig et al. 1999). In this study, bacterial colonisation of the lower airways was also seen in smokers who did not have significant airflow obstruction, suggesting that bacterial colonisation may be an early phenomenon in the course of the disease. Bacterial colonisation of the lower airways in COPD may impair host defence, cause direct airway epithelial damage and promote chronic inflammation, which in turn may contribute to symptoms and disease progression.

(iv) **Bacterial pathogens invade and persist in respiratory tissues, alter the host response to cigarette smoke, or induce a chronic inflammatory response and thus contribute to the pathogenesis of COPD.** Having
previously been regarded as an extracellular pathogen, recent studies have demonstrated that nontypeable \textit{H. influenza} invades beyond the surface of the respiratory epithelium both \textit{in vitro} and \textit{in vivo}. Bacteria in tissues are protected from anti-biotics and may act as reservoirs of infection. Chronic \textit{Chlamydia pneumoniae} infection has also been demonstrated in COPD, with incidence increasing with severity of disease (Von Hertzen, Alakarppa et al. 1997).

\textbf{(v)} \textbf{Bacterial antigens in the lower airway induce hypersensitivity that enhances airway hyperreactivity and induces eosinophilic inflammation.} In COPD, there is a delayed clearance of bacteria from the lower respiratory tract, resulting in prolonged contact between airway lymphoid tissue and bacterial antigens. This could lead to production of IgE and eosinophil recruitment. An increased number of eosinophils is characteristic of airway inflammation in most patients with COPD and this eosinophilia increases with exacerbations. Endotoxin from \textit{H. influenza} has been shown to stimulate release of histamine, a potent bronchoconstrictor, from mast cells obtained from BAL \textit{in vitro} (Clementsen, Larsen et al. 1995), while patients with exacerbations of COPD have been found to have basophil-bound and serum IgE to homologous strains of \textit{H. influenza} and \textit{S. pneumoniae} (Kjaergard, Larsen et al. 1996). Bacterial pathogens may thus contribute to the eosinophilia, airway hyper-reactivity and bronchoconstriction in patients with COPD.
Several studies in recent years have shown a relationship between lung function impairment and the type of bacterial flora colonising the lower respiratory tract and causing infective exacerbations of COPD. Examining sputa from patients during exacerbations, Eller et al found that patients with worse lung function had a higher rate of Gram-negative *Enterobacteriaceae* and *Pseudomonas* species (Eller, Ede et al. 1998). Similarly, in a study looking at 91 ambulatory patients, Miravitlles found that individuals with severe pulmonary function impairment, as defined by FEV1<50% predicted, were at a six fold higher risk of suffering acute exacerbations caused by *H influenza* and *P. aeruginosa* than were patients with FEV1 >50% (Miravitlles, Espinosa et al. 1999). *Pseudomonas* was found in only one of 14 patients with FEV1 >50%, and active smoking was independently and significantly associated with *H influenza* in the sputum. Looking at patients with severe exacerbations requiring mechanical ventilation, 88% of whom had an FEV1 <50%, Soler reported a high rate of Gram-negative bacteria including *Pseudomonas/Stenotrophomonas* in respiratory samples (Soler, Torres et al. 1998). Patel found *H. influenza* to be the most common colonising organisms in stable patients with moderately severe COPD (mean FEV1 38.7% predicted), and this was not related to smoking status. Colonisation with any species was associated with increased exacerbation frequency (Patel, Seemungal et al. 2002). Increased rates of oropharyngeal carriage of aerobic Gram-negative bacilli in severe COPD versus less severe disease, representing a potential source of pneumonia/infective exacerbation, has also been reported (Mobbs, van Saene et al. 1999).

It would appear that as lung function deteriorates in COPD, the respiratory tract is more frequently colonised with, and exacerbations are more frequently caused by, Gram-negative organisms. The reason for this is unclear. One reason may be
the ecological pressure exerted by the use of previous antibiotics. Patients with more severe disease are likely to have disease of longer duration and therefore have been exposed to more antibiotics. Increased age and frequent hospitalisation were independent risk factors for Gram-negative bacteria and *Pseudomonas* infection in Soler’s study (Soler, Torres et al. 1998). Other hypotheses include increased adherence of bacteria to damaged epithelium, such as that which is assumed to exist in patients with more severe disease (Plotkowski, Chevillard et al. 1991). Other potential factors relating to host defence are also likely to prove important. Failure to mount an immediate and effective host response resulting in eradication of potentially pathogenic organisms may leave the host susceptible to colonisation, chronic inflammation and acute exacerbations.

Patients with cystic fibrosis are also frequently colonised by Gram-negative organisms, particularly *Pseudomonas*. Proposed reasons for this susceptibility include abnormalities of airway surface liquid leading to impaired mucociliary clearance, increased availability of bacterial receptors, reduced ingestion of pathogens by CF cells and impaired defence related to low levels of molecules such as nitric oxide or glutathione. As outlined above, impaired production, survival and activity of AMPs may also be important. The inducible expression of AMPs in response to bacteria is likely to be critically linked to the TLRs, and through this and its other immunomodulatory functions, the TLRs may also prove important. TLR4 is the predominant signal transducer of the Gram-negative pathogen recognition receptor LPS. Changes in TLR4 expression in the respiratory tract could potentially result in an altered immediate immune response to Gram-negative organisms, contributing to the failure to eradicate these bacteria.
1.7 Corticosteroids:

Airway inflammation is a prominent feature of both COPD and asthma. The benefits of maintenance inhaled corticosteroids in asthma are well established (1987). The use of systemic steroids have long been a mainstay of treatment in acute exacerbation of COPD, but the use of maintenance low dose inhaled corticosteroids in stable disease remains the subject of much debate. Specific features distinguish asthma from COPD with regard to the characteristics of the key inflammatory changes. In particular, asthma is characterized by bronchial eosinophilic infiltration and airway remodeling, which are supposed to be orchestrated by a predominant CD4+/T helper 2 (Th2) response (Maddox and Schwartz 2002), whereas in COPD there is an infiltration of CD8+ T lymphocytes, macrophages, and neutrophils (Jeffery 2000).

There have been a number of large scale multi-centre placebo controlled trials of inhaled corticosteroids in COPD, looking at a number of different end points (Renkema, Schouten et al. 1996; Paggiaro, Dahle et al. 1998; Pauwels, Lofdahl et al. 1999; 2000; Burge, Calverley et al. 2000). None has established a significant reduction in all cause mortality. A small early improvement in FEV1 was reported in some studies but this was not maintained and there was no effect on the rate of decline in lung function compared to the placebo treated group. There were important improvements in other end points however. Regular use of inhaled corticosteroids reduced the chance of an exacerbation, and the total exacerbation rate, by up to one third. A slower deceleration in disease-specific health status, better symptom control, improved exercise tolerance and fewer pulmonary symptoms have been variably reported. As a result, inhaled coricosteroids are
increasingly used in the treatment of stable COPD, often in combination with long-acting beta-2 agonists.

Airway inflammation is effectively suppressed by low doses of inhaled corticosteroids in most patients with asthma, but a similar effect in COPD has not been firmly established. Studies with high doses of inhaled steroids over short periods (2-4 weeks) failed to show a reduction in inflammatory cells, cytokines or proteases (Keatings, Jatakanon et al. 1997; Culpitt, Maziak et al. 1999). Other studies, with longer treatment periods of 8 weeks and more, have demonstrated a reduction in neutrophilic and lymphocytic infiltration, and in protease activity (Llewellyn-Jones, Harris et al. 1996; Confalonieri, Mainardi et al. 1998; Gizycki, Hattotuwa et al. 2002).

Although corticosteroids have been used as anti-inflammatory agents for a long time, it is only during the past few years that the molecular mechanisms underlying their biological and pharmacological effects have begun to be elucidated. Recent significant advances made within this research field are beginning to identify a broad spectrum of molecular targets of glucocorticoids including genomic DNA, histone-modifying enzymes, transcription factors, and signalling cascades activated by a wide range of stimuli.

Glucocorticoids exert their action by binding to a specific, intracellular glucocorticoid receptor (GR). Unbound GR is associated in the cytoplasm in a nonactive oligomeric complex with some regulatory proteins including the 90kDa heat shock protein (hsp90). The interaction between hsp90 and GR is required to maintain the C-terminal domain of the GR in a favourable conformation for ligand binding. Binding of hormone agonists releases GR from its interactions with hsp90, thus inducing a conformational change, which results in unmasking of the
receptor nuclear localization signal. Upon activation, GR thereby translocates to the nucleus and binds as a dimer to DNA through its central domain, which is structurally characterized by a DNA binding motif consisting of two "zinc fingers", each containing a zinc ion tetrahedrally coordinated to four cysteine residues. GR interacts with DNA by targeting specific nucleotide palindromic sequences termed "glucocorticoid response elements" (GRE). In particular, the dimeric GR places its two DNA-binding fragments into adjacent major grooves of the DNA double helix in correspondence to appropriately spaced GRE half palindromes (Luisi, Xu et al. 1991). The overall process results in either stimulation or inhibition of gene transcription.

Transcriptional stimulation of anti-inflammatory genes contributes little to the anti-inflammatory effects of corticosteroids in asthma. They increase the expression of anti-inflammatory proteins such as lipocortin-1, which inactivates the enzyme phospholipase A₂ thus inhibiting the production of lipid mediators (i.e., platelet-activating factor, leukotrienes and prostaglandins). Glucocorticoids also enhance the production of other anti-inflammatory molecules such as interleukin-1 receptor antagonist (IL-1ra), interleukin-10 (IL-10), secretory leukocyte inhibitory protein and neutral endopetidase (Adcock and Ito 2000). The physiological effect of this is small, however. It is also relevant the GRs induce the synthesis of IxB-α, the endogenous inhibitor of NF-κB (McKay and Cidlowski 1998).

Effective control of airway inflammation is largely mediated by inhibition of the transcriptional activity of several different genes encoding pro-inflammatory proteins such as (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13, TNF-α, GM-CSF), chemokines (IL-8, RANTES, MIP-1, MCP-1, MCP-3, MCP-4, eotaxin),
adhesion molecules (ICAM-1, VCAM-1, E-selectin), and mediator-synthesizing enzymes (i-NOS, COX-2, cytoplasmic PLA₂) (Adcock and Ito 2000). This inhibition occurs at a genomic level in the case of a limited number of genes, but more important appears to be the non-genomic mechanism of transpression, due to the effect of activated GR on the function of several transcription factors. Direct physical interactions between monomeric GR and subunits of the transcription factors AP-1 and NF-κB result in inhibition of their transcriptional properties (Pfahl 1993).

Glucocorticoids are thought to inhibit histone acetylation by counteracting the actions of several transcription factors such as CREB, Sp1, Ets, NF-AT and STATs. All the latter can indeed interact with the CBP/p300 coactivator complex thus stimulating its intrinsic histone acetyltransferase activity, whereas GR binding to CBP results in inhibition of histone acetylation (Ito, Barnes et al. 2000). GR may also stimulate histone deacetylation by binding corepressors such as N-Cor and SMRT, resulting in recruitment and activation of macromolecular complexes that have deacetylating function (Ayer 1999; Ito, Barnes et al. 2000). It has been postulated that the relative inability of corticosteroids to effectively suppress inflammation in COPD may be due, at least in part, on the effects of oxidative stress and cigarette smoke on the balance between histone acetylation and deacetylation (Rahman 2002; Barnes, Ito et al. 2004).

Corticosteroids can further indirectly inhibit gene transcription by interfering with the signal transduction pathways involved in regulation of transcription factors, including the mitogen activated protein kinases (MAPK). In human pulmonary endothelial cells, it has been demonstrated that dexamethasone inhibits phosphorylation-dependent activation of the MAPK subgroups JNK (c-Jun N-
terminal kinases), **ERK1/2** (extracellular signal-regulated kinases 1 and 2) and p38, induced by oxidative stress and by the pro-inflammatory cytokines IL-1β and TNF-α (Pelaia, Cuda et al. 2001). Budesonide, a topical corticosteroid widely used as inhaled anti-inflammatory therapy in asthma and increasingly in COPD, exerts a potent inhibitory effect on p38 phosphorylation stimulated by transforming growth factor-β (TGF-β), a molecule which has potent pro-apoptotic effect. At the molecular level, glucocorticoid-induced MAPK inhibition seems to be mediated by an increased expression, as well as a decreased proteolytic degradation, of the MAP kinase phosphatase-1 (MKP-1) (Kassel, Sancono et al. 2001). Corticosteroids may also modulate gene expression at a post-translational level by reducing the half-life of some mRNAs. (See Figure B)

Corticosteroids are widely used for their potent anti-inflammatory effects, however one of the many side effects of steroid treatment is increased susceptibility to infection. There is an enormous body of literature concerning specific effects of corticosteroids on host defences, including affects on leukocyte kinetics, phagocytic immunity, cell-mediated immunity, and humoral immunity. There is relatively little information to date regarding the specific effect on innate immune function. Gene profiling studies have shown that glucocorticoids induce expression of TLRs in peripheral blood mononuclear cells (Galon, Franchimont et al. 2002), while another study showed synergistically enhanced Non-typeable *H. influenzae* (NTHi)-induced TLR2 expression in human airway epithelial cells treated with dexamethasone. The mechanism was specific up-regulation of the MAPK phosphatase-1 (MKP-1), which, in turn, leads to dephosphorylation and inactivation of p38 MAPK, the negative regulator for TLR2 expression (Imasato, Desbois-Mouthon et al. 2002).
Figure B:

Glucocorticoids could modulate TLR signalling in a number of ways:

1. Direct genomic interaction resulting in stimulation or inhibition of effector genes
2. Transpression – inhibition of the transcription factors NF-κB and AP-1
3. Inhibition of MAP kinases, which may be due in part to
4. Induction of MAP kinase phosphatase 1

(Adapted from Takeda and Akira 2004)
The effect of corticosteroids on HBD2 expression has not been clarified. Using an airway epithelial cell line, Tomita et al demonstrated reduced LPS-induced HBD2 expression following pre-treatment with dexamethasone (Tomita, Nagase et al. 2002). Duits et al, however, using primary bronchial epithelial cells, found that expression of HBD3 but not HBD2 or HBD2 following stimulation with heat killed *P. aeruginosa* was inhibited by dexamethasone (Duits, Rademaker et al. 2001). There has been little work regarding the effect of corticosteroids on TLR expression.
1.8 Long acting beta-agonists

Although long-term domiciliary oxygen has been shown to improve survival in a selected subgroup of patients with hypoxic chronic obstructive pulmonary disease (1981), smoking cessation remains the only therapeutic intervention known to alter the natural progression of COPD. Pharmacological therapies are therefore aimed at relieving symptoms and reducing exacerbations of the disease. Inhaled bronchodilators are the mainstay of treatment and, at present, short-acting beta agonists such as salbutamol and anti-cholinergics e.g. ipratropium bromide, given four times daily, are the most commonly used. The long-acting beta-agonists have a 12-hour duration of action and are now widely used in both asthma and COPD. As well as being more convenient for the patient, there are increased benefits in terms of lung function, exercise performance and health status. A recent Cochrane review estimated that combining a long-acting beta2-agonist with an inhaled corticosteroid resulted in an approximate 30% (RR, 0.70; 95% CI, 0.62-0.78) reduction in exacerbations (Man, McAlister et al. 2003).

Long acting beta-2 agonists such as salmeterol and formoterol are effective bronchodilators, and they are used for this reason in obstructive airways disease. COPD and asthma are characterised by both airflow obstruction and airways inflammation, although of different types. Beta-2 adrenergic receptors are widely distributed, occurring not only in airway smooth muscle cells but also on inflammatory cells such as mast cells, monocytes, eosinophils, T-lymphocytes, and neutrophils which are implicated in the pathophysiology of respiratory disease (Johnson 2002). In recent years, several studies have looked at the potential anti-inflammatory effects of long-acting beta agonists, with evidence emerging of both...
direct and indirect effects on cell activation, inflammatory mediator release and cell recruitment, which involves adhesion, chemotaxis, proliferation and survival mechanisms. Both short acting and long acting beta-agonists have been found to stabilise mast cells and inhibit inflammatory mediator release from these cells in vitro (Butchers, Vardey et al. 1991), although this effect has not been reproduced in vivo. Salmeterol has been demonstrated to inhibit both spontaneous and LPS-induced IL-8 production in peripheral blood monocytes (Pantelidis P 1995). Neutrophils have a relatively low beta 2 adrenergic receptor density, however salmeterol has been shown to inhibit a range of neutrophil activities in vitro and in vivo including adhesion to bronchial epithelial cells, chemotaxis, superoxide and IL-8 release (Jeffery, Venge et al. 2002; Johnson 2002). In vivo, treatment of mild asthma patients with salmeterol for six weeks resulted in significant reduction in neutrophil content and activation in the bronchial mucosa (Li D 1997). One study showed a reduced number of eosinophils in the lamina propria of asthmatics treated with salmeterol (Li, Ward et al. 1999), but other studies have not replicated this finding (Jeffery, Venge et al. 2002). There does appear to be an inhibition of eosinophil activation, with reduction in eosinophil cationic protein and eosinophil protein X release after antigen challenge (Pedersen, Dahl et al. 1993). Similarly, the long acting beta agonists appear to have little effect on lymphocyte numbers in BAL or bronchial biopsy specimens, but there may be inhibition of lymphocyte profile and activity, with a reduction in CD4+ cells and expression of HLA-DR and IL-2 receptor reported in some studies (Johnson 2002).

Long acting beta-agonists (LABA) are usually used in combination with corticosteroids, and have an additive and/or synergistic effect in clinical practice. The addition of LABA to inhaled corticosteroid has been demonstrated to be more...
effective in improving asthma control than increasing the dose of inhaled corticosteroid, while combination therapy has resulted in reduced exacerbation rates and improved quality of life scores in patients with COPD. The molecular basis for this may be twofold. Corticosteroids can modulate the beta\textsubscript{2} adrenergic receptor (\(\beta_2\)AR) by activating the \(\beta_2\)AR gene. Topical intra-nasal beclometasone, a steroid frequently used in COPD, has been shown to increase the density of \(\beta_2\)AR on the nasal mucosa (Baraniuk, Ali et al. 1997), although Aksoy and colleagues were able to demonstrate only a brief and unsustained increase in \(\beta_2\)AR gene transcription in normal airway epithelial cells treated with dexamethasone \textit{in vitro} (Aksoy, Mardini et al. 2002). The use of these drugs in combination may thus inhibit the desensitisation that can occur with prolonged beta-agonist use. In contrast, LABAs may augment the function of the glucocorticoid receptor by priming it for subsequent steroid binding and by increasing the translocation of the steroid/glucocorticoid receptor active complex from the cell cytosol to the nucleus (Johnson 2002).

The \(\beta_2\)-AR is a G protein-coupled membrane receptor that acts to raise intracellular cAMP levels via stimulation of adenylyl cyclase (Green 1996). Activation of the receptor on airway smooth muscle cells results in relaxation of the smooth muscle. The airway epithelium contains a high density of \(\beta_2\)-ARs (Carstairs, Nimmo et al. 1985), and the airway epithelium appears to be important in regulating airway tone. Overexpression of these receptors in the airway epithelium in a mouse model \textit{in vivo} resulted in reduced airway hyper-responsiveness (McGraw, Forbes et al. 2000), and it is postulated that this may be due to the release of vaso-active mediators by epithelial cells. Known epithelium-derived substances that relax airway smooth muscle include PGE\textsubscript{2} and nitric oxide (NO). Contractile factors produced by the bronchial epithelium include
leukotrienes, PGF$_{2\alpha}$, and endothelin-1. Airway epithelial cells produce a number of different cytokines and adhesion molecules that could affect smooth muscle responsiveness as well. Although beta agonists have been shown to modulate mediator release from a number of inflammatory cells, there is little direct evidence to date that activation of the $\beta_2$-AR in epithelial cells has similar effect (Barnes 1999).

Salmeterol has been shown to have a number of effects on cell mediated immune function, both alone and in combination with corticosteroids. The effect on innate immunity, however, has been largely unexplored. As an increasingly used component of the pharmacology of COPD, and given the reported effects on exacerbation frequency, I was interested to investigate the effect of the LABA, salmeterol, on aspects of innate immunity in respiratory epithelium. Could the immunomodulatory role of LABA extend to modulation of innate immunity molecules, in particular TLR2 and TLR4, thus altering the pro-inflammatory stimulus of bacterial products? Could the change in exacerbation rate be due to improved clearance of pathogens by enhanced induced expression of HBD2? What is the effect of LABA in combination with corticosteroid on these systems?
1.9 Cigarette smoke

Cigarette smoking clearly has been shown to be the major environmental risk factor predisposing to the development of COPD (Silverman and Speizer 1996). Occupational exposures to dust and fumes, air pollution, passive smoke exposure, childhood respiratory infections and diet may also be important, while alpha1-antitrypsin deficiency is the important pathogenic mechanism in a small minority of patients. The majority of COPD patients have a significant smoking history, yet only 10-20% of smokers will develop COPD (Burrows, Knudson et al. 1988). This points to the importance of some factor or factors specific to the subpopulation of smokers who develop COPD that is different from other smokers, and may be due to genetic polymorphisms in the various mechanisms by which cigarette smoke can cause airway inflammation and tissue destruction.

The relationship between cigarette smoke and airway inflammation is complex. Cigarette smoke is a complex mixture of chemical compounds, including free radicals and other oxidants that have the potential to induce tissue damage. Chronic exposure to cigarette smoke initiates a series of events that cause damage to the central airways, peripheral airways, and terminal airspaces, leading to physiologic and clinical abnormalities. In chronic bronchitis the inflammatory process is characterized by mucosal infiltration with neutrophils, macrophages, and lymphocytes, resulting in epithelial disruption, smooth muscle hypertrophy, and fibrosis. In emphysema, a combination of inflammatory cell recruitment in the terminal airways, protease-mediated damage of the extracellular matrix, and ineffective matrix mechanisms results in tissue breakdown (Sutherland and Martin 2003).
The neutrophil was the first cell implicated in the pathogenesis of COPD. The neutrophil produces proteolytic enzymes such as neutrophil elastase (NE), which can degrade lung parenchyma and cause emphysema. In 1983, Hunninghake and Crystal evaluated airway inflammatory cells in bronchoalveolar lavage fluid and lung biopsy specimens of non-smokers and smokers who did not have evidence of COPD. They reported that smokers had an increase in lavage fluid neutrophils and macrophages when compared with non-smokers and postulated that cigarette smoking induced macrophages to release neutrophil chemotactic factors (Hunninghake and Crystal 1983). In animal studies, exposure to cigarette smoke causes neutrophils to appear promptly in the airways (Hulbert, McLean et al. 1985), while biopsy studies in humans showed that the number of submucosal neutrophils in the airways correlated with the number of cigarettes smoked (Bosken, Hards et al. 1992).

Macrophages also play an important role in directly mediating tobacco smoke-induced lung destruction, in part through macrophage-derived matrix metalloproteases (MMPs). Using a mouse model, Churg et al demonstrated that both neutrophilic inflammation and activation of macrophage derived matrix metalloprotease-12 (MMP-12) were required for connective tissue breakdown, and that MMP-12 specifically mediates this effect by upregulation of TNFα, which in turn causes endothelial activation, neutrophil influx, and proteolytic matrix breakdown caused by neutrophil-derived proteases (Churg, Wang et al. 2003). Lymphocytic infiltration may also be important, and increased numbers of CD8+ T lymphocytes and increased ratio of CD8+/CD3+ lymphocytes have been reported in the large and small airways of smokers (O'Shaughnessy, Ansari et al. 1997; Lams, Sousa et al. 1998).
Cigarette smoke also impacts on the pro-inflammatory cytokine cascade. Increased levels of IL-8 in BAL fluid and stimulated release of IL-8 from alveolar macrophages have been reported in smokers (McCrea, Ensor et al. 1994; Morrison, Strieter et al. 1998). Indeed, Kuschner et al reported that concentrations of macrophages, neutrophils, IL-1β and IL-8 were elevated in the pulmonary microenvironment of smokers in a cigarette dose-dependent manner (Kuschner, D'Alessandro et al. 1996). Reduced levels of IL-1β in exhaled breath condensates (Garey, Neuhauser et al. 2004) and the counter-inflammatory IL-10 in sputum (Burgess, Nanson et al. 2002) have been observed following smoke exposure, and this has been suggested as a possible mechanism for increased susceptibility to infection in smokers. Park and colleagues reported a sustained increase in the concentrations of TNFα and IFNγ in the serum of smokers, suggesting a systemic inflammatory response (Park, Park et al. 2003), while increased expression of the adhesion molecule ICAM-1 may contribute to neutrophil chemotaxis in smokers (Floreani, Wyatt et al. 2003).

The mechanism by which cigarette smoke alters cytokine concentration is not clear, but it may involve activation of various transcription factors. Cigarette smoke has been shown to activate NF-κB through phosphorylation of IκB-α in a variety of cell lines including T cells (Jurkat) and respiratory cells (H1299) (Anto, Mukhopadhyay et al. 2002). Stimulation of bronchoalveolar cells from smokers with LPS resulted in activation of NF-κB and the MAP kinase p38 with faster kinetics than those of non-smokers (Mochida-Nishimura, Surewicz et al. 2001). Specific components of cigarette smoke, including nicotine (Crowley-Weber, Dvorakova et al. 2003) and acetaldehyde (Crowley-Weber, Dvorakova et al. 2003) have been shown to activate NF-κB in vitro. NF-κB plays a key role in
viral replication, carcinogenesis, anti-apoptosis, invasion, and metastasis, as well as inflammation and host defence, and activation of this and other transcription factors may be pivotal to the various pathogenic effects of cigarette smoke.

Given the many and various effects of cigarette smoke on leukocyte function, it is unsurprising that smoking is an independent risk factor for bacterial colonisation of the lower respiratory tract and infective exacerbations of COPD. Direct and passive smoking have long been recognised as independent risk factors for acute respiratory infection (Graham 1990). Looking retrospectively at a cohort of 3074 patients, Lipsky et al identified previous smoking as a risk factor for acquiring pneumococcal infection (OR 2.14), independent of having COPD (Lipsky, Boyko et al. 1986). Active smoking is also associated independently and very significantly with the isolation of *H. influenza* from the sputum of exacerbated COPD patients (OR 8.1) (Miravitlles, Espinosa et al. 1999).

Using a two-step BAL and semi-quantitative culture technique to correct for oropharyngeal contamination, Qvarfordt and colleagues found lower airway bacterial colonisation in 6 of 10 asymptomatic smokers and 7 of 35 patients with chronic bronchitis, whereas none of 10 non-smokers were colonised (Qvarfordt, Riise et al. 2000). Looking at patients with stable COPD, Zalacain et al reported smoking to be an independent risk factor for bacterial colonisation of the lower respiratory tract as determined by protected specimen brush technique, with an odds ratio of 3.17 (95%CI 2.5-8) (Zalacain, Sobradillo et al. 1999). Smoking has also been shown to facilitate bacterial colonisation of the tracheo-bronchial tree in a rat model (Ozlu, Cay et al. 1999).

The mechanism by which smoking facilitates bacterial colonisation has not been elucidated. Putative mechanisms include epithelial damage aiding bacterial
adherence, impaired mucociliary clearance, mucous hypersecretion and altered cell mediated immunity. The effect on the innate immune system has been largely unexplored. Beisswenger and colleagues exposed respiratory cells in culture (MM-39 epithelial cells and primary Human Bronchial Epithelial cell cultures), grown at an air-fluid interface, to cigarette smoke and found reduced expression of HBD2 in response to LPS (Beisswenger C 2003). It was postulated that this might be due to inhibition of NF-κB or AP-1. There is no published data regarding the effect of cigarette smoke on TLR expression and function.

In summary, bacterial colonisation is important in the pathogenesis and progression of COPD, and may be due at least in part to impaired host defence mechanisms in the respiratory epithelium. HBD2 is an inducible anti-microbial peptide with potent microbicidal activity against Gram-negative organisms. Induction of HBD2 by LPS is likely to involve signal transduction through TLR4. Altered expression of TLR4 could thus result in reduced ability to clear Gram-negative pathogens. Potential modulators of TLR4 expression and function in COPD include cigarette smoke, corticosteroids and long-acting beta-agonists, and increased airway inflammation itself in more severe disease.
1.10 Aims of the project:

1. To determine if induction of HBD2 in respiratory epithelial cells by LPS involves TLR4 signalling.

2. To determine the effect of corticosteroids, the long acting beta agonist salmeterol and cigarette smoke condensates on constitutive expression of TLR2/TLR4 and induced expression of HBD2 and IL-8.

3. To evaluate the expression of TLR2, TLR4 and HBD2 in the upper and lower respiratory tracts of the following patient subgroups: (i) stable patients with mild-moderate COPD (FEV₁ >1 litre, GOLD Stages 1 and 2A), (ii) stable patients with moderate to severe COPD (FEV₁ < 1 litre, GOLD Stage 2B and 3) (iii) normal non-smoking controls, (iv) smokers with normal lung function. IL-8 levels in nasal lavage fluid will be measured by ELISA as a surrogate of airway inflammation.
Chapter 2

Materials and Methods
2.1 Cell Culture and Agonists

2.1.1 Thawing of Cell Stocks

Cells were thawed as quickly as possible by placing the cryovial in a 37°C incubator until the last crystals of ice had disappeared, but no longer. The contents of the cryovial were transferred into 5 ml medium (pre-heated to 37°C) in a cell culture flask and incubated at 37°C in 5% CO₂ humidified atmosphere.

2.1.2 Trypsinisation and Passaging of Cells

Media from culture flasks was poured off and disposed of in waste containers. Five ml of trypsin was added for 5 minutes and cells were replaced in 37°C incubator. After sufficient time had elapsed, 5 ml fresh medium was added (pre-heated to 37°C) to rinse remaining cells off the bottom of the flask, and to neutralise the trypsin. Medium with suspended cells was transferred into sterile 15 ml conical tube. At this point, approximately 10μl of cells was transferred into the well of a 96 well plate for counting by the method of Trypan Blue exclusion and a haemocytometer. Cells were spun at 1500-rpm at room temperature for 5 minutes. According to the cell count, cells were resuspended in an appropriate volume of medium and transferred to flasks or cell culture plates as required.

2.1.3 Freezing of Cells for Long-term Storage

Cells were trypsinised and spun down as in passaging. Based on cell count, cells were resuspended in freezing mix (90% FCS, 10% DMSO) at approximately 1 x 10⁶ cells per ml and 1 ml was placed in each cryotube. The cryotubes were then
carefully labelled with permanent marker and placed at -80°C overnight before
being transferred to liquid nitrogen the following day.

2.1.4 Medium
For 500 ml of media add:
10% FCS (50 ml)
1% Penicillin-streptomycin (5 ml)
1% L-Glutamine (5 ml) to Dulbecco’s Modified Eagles Medium (Gibco BRL).

2.1.5 Culture and Stimulation of Bronchial Epithelial Cells
The human embryonic kidney cell line, HEK293, (ECACC-85120602) was
obtained from the European Collection of Cell Cultures. Cells were cultured at
37°C in 5% CO₂ in Eagle's minimal essential medium (EMEM, Biowhittaker)
supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine, 1%
penicillin/streptomycin, 1% NEAA (Gibco-BRL). The human alveolar type II cell
line A549 was obtained from Americam Type Culture Collection (Manassas,
VA). Cells were cultured at 37°C in 5% in F12 (Gibco-BRL), 10% FBS, 1%
penicillin/streptomycin. Prior to agonist treatment, cells were washed with serum-
free EMEM/F12 and placed under serum-free conditions or in serum containing
1% FCS for LPS stimulations.
Recombinant human TNFα and interleukin-1β were purchased from R & D
Systems, UK. Staph Aureus LTA, Pseudonomas Aeruginosa LPS and
Dexamethasone were purchased from Sigma (UK). Salmeterol and Fluticasone
were obtained from Glaxo Smith Kline Beecham, UK. (see Table 2.1)
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<th>Concentrations Used</th>
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<tr>
<td>Interleukin-1β (IL-1β)</td>
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<td>100ng/ml</td>
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<tr>
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<td>Lipopolysaccharide (LPS)</td>
<td>Sigma, UK</td>
<td>1-50μg/ml</td>
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<tr>
<td>Dexamethasone</td>
<td>Sigma, UK</td>
<td>10^{-10} – 10^{-6} M</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>GSK, UK</td>
<td>10^{-10} – 10^{-6} M</td>
</tr>
<tr>
<td>Fluticasone Propionate</td>
<td>GSK, UK</td>
<td>10^{-10} – 10^{-6} M</td>
</tr>
<tr>
<td>Cigarette smoke extract</td>
<td>Marlboro</td>
<td>10^{-4} – 1</td>
</tr>
</tbody>
</table>

Table 2.1 Agonists used in this study.

2.1.6: Preparation of cigarette smoke extracts:

Cigarette smoke extract (CSE) was prepared by a modification of a previously published method (Laurent, Janoff et al. 1983). Briefly, 2 filtered Marlboro Red cigarettes, each containing 0.8 mg of nicotine and 10 mg of tar according to the manufacturer's report, were bubbled through 20 ml serum free F-12 medium, pre-warmed to 37°C, by a mechanical vacuum pump. The extract was filtered through a 0.45μm pore filter (Millipore, Bedford, MA) to remove bacteria and particles, and serial dilutions made.
2.1.7 Preparation of Fluticasone, Salmeterol and Dexamethasone

Fluticasone propionate and salmeterol were obtained from Glaxo SmithKline, Glaxo Wellcome UK Ltd, Stanley Park West, Uxbridge, Middlesex UB11 1BT. A 10⁻²M solution of Fluticasone was prepared in dimethylacetamide (DMA), and further diluted using serum free Ham's F12/0.1% Tween to a stock solution of 10⁻⁶M (0.01% DMA). A solution of 0.01% DMA 0.1% Tween was used for control wells. A 10⁻²M solution of salmeterol was prepared in methanol and further diluted in serum free Ham's F12 to a stock solution of 10⁻⁶M (0.01% Methanol). Ham’s F12 0.01% methanol was used in control wells. Dexamethasone was purchased from Sigma-Aldrich, Tallaght, Dublin, Ireland, and reconstituted in Ham's F12.

2.1.8 Cell Viability

A549 cells (1 x 10⁴/ well) were plated in a 96 well plate in serum-free medium overnight, rinsed and placed in medium containing 1% FCS. Triplicate samples were left untreated or treated with dexamethasone (16 hours), Salmeterol (6 hours) or CSE (4 hours) at the indicated dilutions and cell viability was quantified using the Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay as recommended by the manufacturer.
2.2 General Methods

2.2.1 Bradford Protein Estimation Assay

0, 0.25, 0.5, 1.0, 2.0μg/ml of BSA (1 mg/ml) was pipetted into the wells of a 96-well plate. 5μl of unknown samples was pipetted into separate wells to the standards. 95μl of H₂O was pipetted in duplicate into the wells containing the unknown samples. Blanks of H₂O were included. 100μl of Bradford Reagent was added to all wells containing standard or sample. Absorbance was read at 570 nm, and a standard curve was constructed. Protein content was calculated from this standard curve, prepared using the known values obtained from BSA standards.

2.2.2 RNA Isolation

TRI reagent (Sigma) was used in the isolation of RNA and protein. This isolation technique is based on the principle of single-step liquid phase separation, resulting in the simultaneous isolation of RNA and protein. After adding chloroform and centrifuging, the mixture separated into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA and an organic phase containing proteins. Each component was then isolated after separating the phases. 7.5ml of TRI reagent was used to isolate RNA, DNA and protein from 5-8 X 10⁶ cells grown in a confluent monolayer in a T75 tissue culture flask (1ml of TRI reagent used per 10cm² of culture plate surface area).
2.2.3 Quantification of RNA

10μl of RNA was diluted in 490μl dH₂O and the A₂₆₀ value was read on a spectrophotometer at the multi-wavelength setting. The amount of RNA in micrograms was calculated using the following formula: A₂₆₀ X Dilation Factor (in this case 1/50) X 40μg/ml. Final preparation of RNA is free of DNA and protein and should have an RNA purity ratio (A₂₆₀/A₂₈₀) of greater than 1.7.

2.2.4 cDNA Synthesis

After cells were harvested in TRI reagent and RNA was extracted, this RNA was used to synthesise complementary DNA (cDNA) for use as template in RT-PCR reactions. RNA (1μg) was reverse transcribed into single-stranded cDNA at 42°C using 1st strand cDNA Synthesis Kit for RT-PCR (Roche, UK) with 1mM deoxynucleotide mix, 1.6μg Oligo-p(dT)₁₅ primer and 0.8μl Amv reverse Transcriptase in a 20μl volume as described in manufacturers protocol. In this method, AMV reverse transcriptase synthesized the new cDNA strand at a site determined by the primer used: at the 3'-end of the poly (A) -mRNA when Oligo-p (dT)₁₅ was used as a primer. The resulting first strand cDNA was then used as a template for PCR. The cDNA was stored at -80°C until required, avoiding multiple freeze-thaw exposures.

2.2.5 Design of PCR Primers

PCR primers were designed using published mRNA gene sequences for the molecules of interest, located using NCBI Entrez Nucleotide (NIH, Bethesda, MD)(Table 2.2). Certain criteria were considered in designing the primers. The overall purine versus pyrimidine composition was important. If possible, half or greater of the bases were G or C as this makes oligonucleotide binding to template
cDNA more stable. Sequences of four or more of the same base were avoided. Also, care was taken to ensure that the two primers to be used together did not contain significant regions of complementarity, or inverted repeats, which would result in hairpin loop formation. If the 3' ends are complementary, primer dimers may be the main PCR products obtained. All primers were designed using the Primer 3 software programme (Whitehead Institute/MIT Centre for Genome Research, Cambridge, MA) (http://www.basic.nwu.edu/biotools/Primer3) and synthesised by MWG Biotech.

2.2.7 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

1-3μl of each cDNA sample from reverse transcription reactions were amplified with 1.25U Taq DNA Polymerase, 1 x PCR Buffer and 10mM dNTPs (Promega) in a 50μl volume containing 100pmol of each of the appropriate forward and reverse primers (see Table 2.2). In semi-quantitative RT-PCR, the integrity of RNA extraction and cDNA synthesis was verified by PCR by measuring the amounts of the housekeeping gene, GAPDH, in each sample using 50 picomoles GAPDH-specific primers. PCR reaction mixtures contained 10X Reaction Buffer, 2.5 mM MgCl₂, 1.25 U Taq polymerase, and 0.2 mM each dNTP (Promega, WI). Thermocycling conditions for template cDNA were 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55/58°C for 30 sec, and 72°C for 30 sec, followed by a holding step of 4°C (see Table 2.2). Twenty cycles were used to amplify the more abundant GAPDH cDNA. A final extension step of 72°C for 10 min was followed by resolution of the various PCR products on a 1.5% TBE agarose gel containing 0.5μg/ml ethidium bromide (Sigma, Ireland).
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<th>Cycling conditions</th>
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<td>68-1050</td>
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<tr>
<td>F</td>
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<td>2688-2709</td>
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Table 2.2 Primers used in this study.
2.2.8 Agarose Gel Electrophoresis

Agarose gels (1.0 or 1.5% (w/v)) were heated for approximately 2 min in 1X TBE until dissolved. Ethidium bromide was then added carefully to give a final concentration of 0.1μg/ml. Gels were run at up to 100 volts using TBE running buffer (1X TBE). PCR products to be analysed were mixed with one-tenth volume of 10X glycerol loading dye and applied to the wells of a gel. Following electrophoresis for a suitable time period, the ethidium bromide-stained DNA was visualised on a UV transilluminator Image Master VDS-CI and software Total Lab v1.00 (Amersham) and photographed for future reference and semi-quantitative analysis.

2.2.9 Real Time PCR

TLR4 mRNA was quantified using commercially available SYBR Green assays. The SYBR Green fluorescent dye (excitation max 497nm, emission 520nm) binds to the minor grooves of the amplified DNA during the primer annealing and extension steps of each PCR cycle, resulting in a 1000-fold increase in fluorescence intensity. Fluorescence is dependent on the presence of dsDNA and is therefore determined by the specificity of the primers. Primers are designed according to the specifications of the reaction: the annealing temperature is restricted to 58–60°C, which corresponds to the optimal working conditions for the AmpliTaq Gold DNA polymerase enzyme (Perkin Elmer), and the length of the PCR product is set between 60 and 150 bp. TLR4 (NM_003266)* primers were F: GTGGAGCTGTACCGCCTT (2982-3002) and R: GCCCCAGGACACTGTCCCTCCTC (2697-2716) to give a product size of 65 base pairs. A standard curve using at least 4 samples of known concentration was included in each run, and the data analysed using the fit points method.
extrapolated to the respective standard curve. The results are expressed as the ratio of the mean of triplicate target gene cDNA measurements to the triplicate housekeeping gene (β-actin) measurement.
2.3 Transformation and Transfection

2.3.1 Transformation Reagents

Ampicillin

1g of ampicillin (Sigma) was dissolved in 10ml sterile distilled water. The solution was filter sterilized through a 0.22μm syringe filter, and was stored at -20°C in 1ml aliquots in sterile tubes.

LB broth

LB Broth powder (20g) (Sigma) was dissolved in 1000ml of sterile distilled water and autoclaved. The sterile broth was stored at room temperature or at 4°C.

LBAmp\(^{100}\) broth

LBAmp100 broth was prepared by adding 1μl ampicillin stock solution per ml of LB Broth, producing a final concentration of 100μg/ml.

LB Agar

LB Agar powder (35g)(Sigma) was dissolved in 1L of sterile distilled water and autoclaved. The sterile agar was stored at 4°C.

LB Amp agar plates

The LB agar prepared in (d) above was dissolved completely in a microwave and allowed to cool to below 50°C. Ampicillin stock solution (1μl) was added per ml of agar, producing a final concentration of 100μg/ml. LBAmp\(^{100}\) agar was poured into sterile 85mm petri dishes (25 -30ml per dish) under sterile conditions. The Petri dishes were covered immediately, and allowed to set. Prepared plates were stored for up to one month at 4°C.
2.3.2 Transformation of *E. coli*

Supercompetent *E. Coli* XL-1 Blue (Stratagene) cells were removed from -80°C storage and allowed to thaw on ice. A 1μl volume of the plasmid being transformed was transferred to a sterile eppendorf containing 20μl XL-1 Blue *E. Coli* cells and incubated on ice for 15 minutes. A control tube was also set up. This was followed by heat shock at 42°C for 2 minutes and immediate transfer to ice for 5 minutes. Ampicillin-free LB medium (1ml) was added to the tube, followed by rotary shaking at 220rpm and 37°C for one hour. After 1 hour, a 100μl volume of the transformation reaction was plated onto separate LBAmpl^100^ plates and incubated at 37°C overnight, together with the control plates.

2.3.3 Single Colony Purification

Following incubation the plates were checked for colony growth. Plasmid control plates should exhibit no colony growth compared to the transformed plasmid plate. The plate streaked with the plasmid of interest should exhibit a large number of colonies. Sterile 20ml tubes were prepared, each containing 5ml of LBAmpl^100^ broth. Using a sterile tip, one colony was picked from the transformation plate and transferred to the LBAmpl^100^ broth. Small isolated colonies were chosen for selection. A broth only control tube was also prepared. Each broth was incubated overnight in a shaking incubator at 37°C and 220rpm. Following incubation, the broths were examined and growth should be observed. The control broth was discarded. The Qiagen Plasmid Mini Kit plasmid extraction procedure was performed on 1.5ml of each successful broth, as described in the manufacturer’s instructions, if the amount of plasmid DNA required was small.
If relatively large amounts of plasmid DNA were required, the remaining successful broths were streaked out to a single colony on LBAmp$^{100}$ plates using a sterile wire loop, and the plates were incubated overnight at 37°C. Following overnight incubation, one small isolated colony was chosen from each plate, and transferred to a 50ml sterile tube containing 20ml of LBAmp$^{100}$ broth. At this stage, each colony on the streak plate is considered to have originated from one single cell. The broths were again incubated overnight at 37°C and 220rpm. Plasmid DNA was then extracted and purified using a commercial midi prep protocol (Qiagen Plasmid Midi Kit), as described in the manufacturer’s instructions.

2.3.4 Storage of Transformed E. Coli Cells

7% DMSO stocks of transformed E. Coli cells containing plasmids of interest were prepared from single colony broths. Each broth (1.86ml) was added to sterile DMSO (0.14ml) in a 2.0ml cryovial, preparing a cell suspension in 7% DMSO. The cryovial was placed in a secure container and stored at -80°C.

In addition, plates were prepared for short-term storage of transformed XL-1 Blue cells. A sterile wire loop was used to inoculate fresh LBAmp$^{100}$ plates with the appropriate plasmid colony. The plate was incubated at 37°C overnight, and was then stored at 4°C for up to a few weeks.

2.3.5 Optimisation of Transfection of A549 and HEK cells

Transfection of mammalian cells was performed using TransFast Tranfection Reagent (Promega). All reagent preparation and transfection procedures were carried out under sterile conditions in a Class II microbiological safety laminar flow hood cabinet. Uniform transfection efficiencies were achieved by initially
optimising transfection conditions using a constitutive luciferase expression vector, pGL3 control (Promega). Experiments were carried out to optimise TransFast and DNA concentrations in A549 and HEK cells.

2.3.6 HBD2 Reporter Gene Studies

A549 or HEK cells were seeded at 1 x 10^5 on 24-well plates 24 h before transfection. Transfections were performed with TransFast Transfection Reagent (Promega) in a 1:1 ratio according to the manufacturer’s instructions, using 200 ng of HBD2 promoter-linked luciferase reporter genes (Walsh, Greene et al. 2001). In combination with luciferase reporter genes, varying amounts of dominant negative or functionally active expression vectors were co-transfected into the cells. The total amount of DNA (400ng) introduced into the cells was kept constant by supplementation with the appropriate empty vectors. Transfections were incubated for 1 h at 37°C. Cells were then supplemented with additional growth medium (1 ml/well) for 24 h at 37°C before being left untreated or stimulated with the relevant agonist. In some experiments, after stimulation for the indicated time, 300ml of cell supernatants were recovered for analysis of IL-8 protein concentrations by ELISA. The cells were then lysed with 1 X Reporter Lysis Buffer (Promega), protein concentrations determined, and reporter gene activity was quantified by luminometry using the Promega luciferase assay system according to the manufacturer’s instructions. Reporter gene expression was expressed as light units per μg total protein. Data expressed as relative luciferase stimulation +/- standard error of the mean (Table 2.2).
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<th>Plasmid</th>
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<th>Description</th>
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<td>Reporter</td>
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Table 2.2 Plasmids used in this study.

2.3.7 Cell lysis

After 24 hours incubation of the transfected cells, the plates were transferred from the incubator to a class II laminar flow hood. Media was aspirated from each well. All wells were then rinsed with phosphate buffered saline (pH 7.4)(Gibco). Cell lysis and luciferase assays were performed using Reporter Lysis Buffer (Promega) according to the manufacturer’s instructions. Cell lysis was accomplished by the addition of an appropriate amount of 1X reporter lysis buffer (RLB) to completely cover each well, followed by incubation on a rocking table at room temperature for 30 minutes. Following lysis, the cell lysates were assayed for firefly luciferase activity (indicating reporter gene expression) as described below.
2.3.8 Luciferase Assays

Assays of firefly luciferase activities in the prepared cell lysates were performed using the Luciferase Reporter Assay (Promega) according to the manufacturers instructions. Luciferase Assay Buffer substrate was made up using kit reagents. 20μl cell lysate was added in duplicate to the wells of a 96 well plate, including a blank with 1X RLB. 100μl of luciferase substrate was then added and reporter gene activity was quantified by luminometry on a Wallac Victor² 1420 multilabel counter (Perkin Elmer, Finland). Protein concentrations of each sample were determined and reporter gene expression was expressed as light units per μg total protein. Data expressed as relative luciferase stimulation +/- standard error of the mean (SEM).
2.4 Protein Studies

2.4.1 Measurement of Receptor Surface Expression

For analysis of A549 cells by laser cytometry, 5 x 10^4 cells/well were seeded in 8 well chamber slides (Nunc). Slides were fixed in methanol (AnalaR) for 5 minutes and labelled with primary antibody (mouse anti TLR4 (Santa Cruz) 1:10 or goat anti-HBD-2 antibody (a gift from Dr Paul McCray, University of Iowa, Iowa City, IA, USA) 1:100 dilution) or isotype control (1:1000 dilution of 1 mg/ml stock of isotype mouse IgG2A or goat IgG) and incubated at 4°C in the dark for 30 min. Slides were washed three times in PBS and probed with 1:10 dilution of anti-mouse or anti-goat IgG fluorescein isothiocyanate (FITC, Dako, Glostrup, Denmark) at 4°C in the dark for 30 min. The washing step was repeated and cells were permeabilised in a 1:1 ratio of permeabilisation solution (Dako) and 0.2 μg/ml solution of propidium iodide (PI, Molecular Probes, Leiden, The Netherlands). Slides were washed in PBS and TLR4 or HBD-2 expression was quantified on a CompuCyte laser scanning cytometer (CompuCyte, Cambridge, MA, USA). Cellular fluorescence of at least 5 x 10^3 cells was measured by laser scanning cytometry. Fluorescence excitation was provided by a 488nm laser line. Orange (PE) and green (FITC) fluorescence were measured at 588 +/- 10nm or 530 +/- 20nm, respectively. The threshold contour was set on scatter or orange to detect all cells as appropriate. Artificially contoured debris was gated out based on contour size. Aggregated cells were gated out using an algorithm in the LSC software that finds and marks multiple cells. Individual TLR4 or HBD-2-expressing cells were identified and quantified using CompuCyte software on the basis of integrated green fluorescence reflecting binding of anti-mouse or anti-
2.4.2 IL-8 Protein Production

A549 or HEK cells were seeded at 1 x 10^5 on 24-well plates (16-mm diameter) 24 h before stimulation. Cells were left untreated or were stimulated with different doses of the various agonists for different time periods or with a positive control (TNFα). IL-8 protein concentrations in the cell supernatants were determined by enzyme-linked immunosorbent assay (ELISA)(R&D Systems, UK). Data expressed as relative IL-8 protein +/- standard error of the mean (SEM).

2.4.3 Preparation of membrane and cytoplasmic Subcellular Fractions

Cells were seeded at 1 x 10^6/ml on 6-well plates (34-mm diameter) 24 h before stimulation. Cells were washed and resuspended in 1 ml ice-cold PBS and kept on ice for 5 min. Cells were lifted from plates with a cell scraper and pelleted by centrifugation at 10,000 rpm for 5 min at 4°C. Supernatant was removed and the cell pellet resuspended in 100 μl hypotonic buffer A (5 mM Tris (pH 6.8), 2mM EDTA, and the protease inhibitors leupeptin 5μg/ml, pepstatin 0.7μg/ml, benzamidine 5μg/ml and PMSF 1mM)(Sigma, Ireland). Cellular components were separated by ultracentrifugation at 55000rpm x 20 minutes at 4°C. Supernatant, which constituted cytoplasmic and nuclear fractions, was removed and stored at -20°C. The pellet consisting of the membrane fraction was resuspended in hypotonic buffer B (20mM Tris HCl pH 6.8%, 150mM NaCl, 10mM EDTA, 1mM EGTA, 1% Triton X100 plus protease inhibitors as before) by forcing pellet through a 22G needle 5-8 times. Protein concentrations of
extracts were determined by the method of Bradford, and stored at -20°C until required for use.

2.4.4 Western Blot Reagents

10% Separating Gel

Buffer

Acrylamide - 3.3ml
Sterile distilled water (SDW) - 3.9ml
1.5 M Tris (pH 8.8) - 2.5ml
200μl
10% SDS - 200μl
10% APS (0.1g/ml) - 67μl
TEMED - 6.7μl

SDS Sample Treatment

1M Tris (pH 6.8) - 1ml
10% SDS - 2ml
0.1% Bromophenol Blue -
SDW - 5ml
Glycerol - 2ml
β-mercaptoethanol - 1ml

4% Stacking Gel

Acrylamide - 500μl
SDW - 2.25ml
0.5 M Tris (pH 6.8) - 950μl
10% SDS - 38μl
10% APS - 25μl
TEMED - 3.8μl

Running Buffer 5X

Tris - 7.5g
Glycine - 36g
SDS - 25g
Make up to 500ml in SDW
### Transfer Buffer 1X
- Tris - 2.42g
- Glycine - 11.26g
- 10% SDS - 1ml

Make up to 200ml in methanol,
Add 800ml SDW.

### Blocking Buffer
- I-Block - 0.2g
- PBS - 100ml
- 0.1% Tween 20 - 100µl

### Washing Buffer
- 1 sachet PBS
- 1 L SDW
- Tween 20 - 1ml

### Glycine Stripping Buffer
- Glycine - 1.5g
- 10% SDS - 1ml
- Tween-20 - 1ml

Make up to 100ml SDW, pH 2.2

### Substrate Solution per Blot
- LumiGlo Reagent A and Peroxide Reagent B - 100µl of each
- SDW - 1.8ml
2.4.5 Western Blot Protocol

Separating gel and stacking gel were prepared, according to the recipe (section 2.4.5). The protein concentration in the samples was measured according to the Bradford method. Hot plate was preheated to 94°C. Samples were made up to the same volume (~10μl) using SDW. Equal amounts of SDS sample treatment buffer were added to each sample and proteins were by heating at 95°C denatured for 5 min. 350 ml 1X running buffer (70ml 5X buffer and 280ml SDW) was gradually added to electrophoresis chamber. 10μl of molecular weight pre-stained protein marker (New England Biolabs) was used to estimate sample size. 20μl of each sample was added to wells. Gel was run at 30milliAmps per gel for approximately one hour, or until loading dye had run to the bottom of gel. In order to transfer the blot, a semi-dry transfer apparatus (Sigma) was used to transfer our proteins to nitrocellulose membrane. Transfer apparatus was run at 100mA, 500 V, 30 W for one hour. Our transferred membrane was removed from the transfer apparatus and stained with Ponceau S solution for a few minutes to detect protein and assess transfer efficiency. Nitrocellulose membrane was rinsed under the tap to remove Ponceau S, immersed in blocking buffer and left overnight on a shaking table at 4°C. TLR4 protein was detected by incubating the membrane with rabbit anti-TLR4 (sc-10741 Santa Cruz Biotechnology, diluted 1:200 in blocking buffer). Membrane was then washed with washing buffer 6 x 5 minutes on shaker. Secondary antibody, horse-radish peroxidase-conjugated anti-rabbit IgG (Tropix, MA), was prepared in blocking buffer and incubated with blot for 1 hour at RT, and washing procedure repeated. Development substrate (New England Biolabs) was prepared prior to use and pipetted directly onto blot placed on a plastic sheet. Membrane was incubated for one minute, after which excess
substrate was removed. The membrane was then sandwiched between a plastic folder and exposed to x-ray film for between 30 seconds-1 hour, depending on signal intensity. The blot was then developed in an AGFA CP1000 developer.

2.4.6 Immunohistochemistry

A549 cells were grown on chamber slides as before. Following treatments, cells were washed with PBS 0.5% BSA and fixed with paraformaldehyde 4%. After blocking with normal blocking serum, cells were incubated with goat anti-HBD2 1:100 x 30 minutes. HBD2 was detected using a biotinylated horseradish peroxidase staining system, goat ABC Staining System (Santa Cruz Biotechnology), according to the manufacturers instructions. Slides were photographed using a Nikon Eclipse E600 and Lucia ScMeas Version 4.51 software.

2.4.6 Cell signalling assays: Activation of MAP Kinase and NF-κB signalling pathways as indicated by p38 MAPK and IκBα phosphorylation respectively were assayed using SuperArray Cellular Activation of Signaling ELISAs (FE-003 and FE-005, SuperArray Bioscience Corporation, USA) according to the manufacturer's instructions. Cells (1.4 x 10⁴/well) were plated in a 96 well plate in serum-free medium overnight, rinsed and placed in medium containing 1% FCS. Triplicate samples were left untreated or treated as indicated. Following fixation in formaldehyde, total and phospho-versions of p38 MAPK and IκBα were quantified using pan-specific and phospho-specific antibodies, respectively. Relative cell numbers were quantified by cell staining and these values were used for normalisation. Target protein phosphorylation was determined by calculating phospho-protein:pan-protein ratios.
2.5 *In-Vivo Study*

2.5.1 Study Population:

Subjects were recruited from the inpatient and outpatient population of Beaumont Hospital and informed consent was obtained from each patient. Severe COPD was defined as FEV1 <50% predicted, and stable disease defined as no exacerbation in the preceding 3 months requiring anti-biotics or pulse dose steroids. An exacerbation was defined by the following: two consecutive days of either two or more of three major symptoms (increase in dyspnoea, increased sputum volume or sputum purulence) or one major symptom plus one or more of the following minor symptoms - increased nasal discharge, wheeze, sore throat, cough or fever (Anthonisen, Manfreda et al. 1987). Age and sex-matched control subjects were recruited from the general medical/surgical population of Beaumont Hospital following informed consent. Patients were excluded on the basis of pre-existing immunosuppression or concomitant pulmonary pathology.

2.5.2 Nasal Epithelial cell sampling:

Following informed consent, brushing was performed using a modification of the technique of Bridges et al (Bridges, Walker et al. 1991). Prior to cell collection, topical nasal decongestant and local anaesthetic were applied to the nose. The nose was carefully cleaned to remove crusts and mucous. Nasal epithelial cells were obtained by vigorous brushing of the medial wall of the inferior turbinate under direct vision using a Cervibrush + (CellPath plc). Cells were placed in chilled serum free nutrient mixture F-12 (HAMS). Part of the sample was prepared for cytocentrifuging to identify the cell types present by May-Grunwald-
Giemsa (Sigma, Ireland) staining. Samples were accepted for analysis if they contained at least 80% epithelial cells. Cells were washed three times in ice cold PBS prior to resuspension in TriReagent for RNA extraction.

2.5.3 Tracheobronchial cell sampling

Tracheobronchial epithelial cells were harvested as in the method of Kelsen et al (Kelsen, Mardini et al. 1992). Briefly, a flexible fiberoptic bronchoscope was inserted transnasally and the distal tip of the scope positioned in the lower trachea. A sleeved catheter cytology brush (Olympus BC-15C) was introduced via the sampling channel and the sleeve retracted. The brush was rubbed against the epithelial surface under direct vision, the brush retracted, and dissociated cells recovered by vortexing in ice-cold Ham’s F-12 nutrient medium. Brushing was repeated 4-6 times at other locations. Part of the sample was prepared for cytocentrifuging to identify the cell types present by May-Grunwald-Giemsa (Sigma, Ireland) staining. Samples were accepted for analysis if they contained at least 80% epithelial cells. Cells were washed three times in ice cold PBS prior to resuspension in TriReagent for RNA extraction.

2.5.4 Nasal Lavage

Nasal lavage was collected by instilling and recovering 10mls of normal saline, pre-warmed to 37°C, into the nose. BAL was filtered using a microcon filter and fluid was aliquoted and stored at -20°C for later analysis of cytokine concentration.
2.6 Statistical Analysis

Data were analysed with GraphPad Prism 3.0 software package (GraphPad Software, San Diego, CA). Results are expressed as mean +/- standard error of the mean (SEM) and were compared by Students one-tailed unpaired t test. Differences were considered significant when the P value was ≤ 0.05.
Respiratory epithelial cells require Toll-like receptor 4 for induction of Human β-defensin 2 by lipopolysaccharide
3.1 Introduction

In the first part of my study, I sought to investigate the transcriptional regulation of HBD2 in the lung as an important component of innate immune defence against Gram-negative bacteria. In particular, I wished to define a critical role for TLR4 in signalling production of HBD2 in response to LPS, thus indicating that downregulation of TLR4 may confer increased susceptibility to colonisation and/or infection by Gram-negative organisms.

I first validated the model system by characterising the cell lines used in the study in terms of responsiveness to LPS and expression of key components of LPS signaling, namely TLR4, CD-14, MD-2 and TIRAP/Mal as well as induced expression of HBD-2. This was particularly important in view of the conflicting data in the literature regarding the TLR4 expression and LPS responsiveness of A549 cells. IL-8 is known to be produced in response to LPS activation of TLR4, and IL-8 ELISA was employed as an easily quantifiable surrogate of LPS responsiveness. TLR4 gene expression was demonstrated by RTPCR. Because TLR4 signals LPS response from the membrane, it was important to demonstrate protein expression localised to the membrane rather than total cell TLR4 protein, and therefore sub-cellular membrane and cytosolic protein fractions were prepared for Western Blot analysis. I further confirmed membrane expression of TLR4 in A549 cells by Laser Scanning Cytometry, where slides were prepared without the use of a permeabilisation agent, thus allowing binding of anti-body to surface structures only. While HEK-293 cells have previously been reported to be TLR4 deficient, and were indeed unresponsive to LPS, in my hands I did find TLR4 mRNA expression, and thus went on to further characterise the cell line in
terms of accessory proteins important in LPS signaling and surface expression of TLR4 protein.

Demonstration of the critical role of TLR4 in signaling the induced expression of HBD-2 in response to LPS involved two complementary approaches. I first inhibited TLR4 signaling in A549 cells by use of a neutralising anti-TLR4 antibody and looked at the effect on LPS induced HBD-2 production. TLR4 signaling was also inhibited by overexpression of a non-functioning dominant negative TLR4 construct in A549 cells. Having investigated the effect of neutralising TLR4 in the A549 cells, I then looked at the effect of restoring functional TLR4 in the LPS-unresponsive HEK 293 cells by transfection of MD-2 plus a constitutively active TLR4 construct (CD4-Toll) or of MD-2 plus functional TLR4 followed with LPS stimulation. Because accessory proteins have been shown to confer specificity to TLR4 signaling, I went on to further define the signaling pathway by transfection of dominant negative constructs of MyD88 and Mal/TIRAP. Induced expression of HBD-2 was the output measure in all of these experiments.

HBD-2 gene transcription was determined firstly by RTPCR. Because of the limitations of this semi-quantitative analysis, further quantification was made using a HBD-2 promoter linked luciferase construct, such that HBD-2 gene transcription could be accurately measured by luciferase induced fluorescence of substrate. The defensins are small highly charged molecules making them extremely difficult to prepare for Western Blot or ELISA analysis. HBD-2 protein was therefore determined by immunohistochemistry and further quantified by Laser Scanning Cytometry.
3.2 Methods in brief

Methods are described in full in Chapter 2

Cell lines and culture: The human embryonic kidney cell line, HEK293, and the type II-like human lung epithelial cell line A549 were cultured as described. Prior to agonist treatment, cells were washed with serum-free EMEM/F12 and placed under serum-free conditions or in serum containing 1% FCS for LPS stimulation experiments, including control conditions.

Gene transcription: Gene transcription was determined by semi-quantitative RT-PCR using gene specific primers and conditions and in the case of HBD-2, was additionally quantified by luciferase activity following transfection of the HBD2 promoter-linked luciferase reporter plasmid.

Protein studies: Surface expression of TLR4 protein was determined by western blot analysis of membrane protein extracts from and by Laser Scanning Cytometry as described. HBD-2 protein was determined by Laser Scanning Cytometry and by Immunohistochemistry. IL-8 production was quantified by ELISA.

Transfection and reporter gene studies: Cells (1.5x10^5) were transfected with plasmid DNA (dominant negative (Δ) Mal (Mal P/H), ΔMyD88, ΔTLR4, MD2, wild type TLR4 or CD4-Toll plasmid) and/or 1 μg HBD2 promoter-linked luciferase reporter plasmid (Griffin, Taggart et al. 2003) using TransFast (Promega) according to the manufacturer's instructions. ΔMyD88 contains only a functional TIR domain and lacks the death domain required for downstream
signaling, while Mal P/H is a dominant negative version of Mal with a proline to histidine point mutation in box 2 of the TIR domain. ΔTLR4 lacks an intracytoplasmic signaling domain and CD4-Toll is a constitutively active chimera of the extracellular domain of CD4 fused to the transmembrane and cytosolic domains of TLR4 (Frantz, Kobzik et al. 1999).

Statistical analysis: Data were analyzed with GraphPad Prism 3.0 software package (GraphPad Software, San Diego, CA). Results are expressed as mean ± S.E. and were compared by Mann-Whitney test. Differences were considered significant when the P value was ≤ 0.05.
3.3 Results and Figures

A549 cells respond to LPS with production of IL-8 and up regulation of HBD2

Signaling via TLR4 by LPS activates NF-κB and up regulates a variety of pro-inflammatory genes, including IL-8. I investigated LPS responsiveness in A549 and HEK293 cells using IL-8 protein production as a surrogate of LPS responsiveness. Figure 3.1A shows that A549 cells dose-dependently induced IL-8 protein expression in response to stimulation with LPS to levels similar to those induced by IL-1β. In contrast HEK293 cells failed to induce IL-8 expression in response to LPS, but did respond to IL-1β or TNFα stimulation with increased IL-8 expression.

I next investigated the effect of LPS stimulation on HBD2 gene expression in both cell lines. Compared to untreated cells, LPS induced HBD2 expression at both 10 and 50 μg/ml in A549 cells (Figure 3.1B). Densitometric analysis quantified these increases to be 2- and 3.6-fold, respectively. IL-1β was used as a positive control and increased HBD2 expression over 30-fold. There was no response to LPS in the HEK293 cells however both IL-1β and TNFα did up regulate HBD2 by a factor of 1.8 and 1.5 respectively.
Figure 3.1

A

IL-8 ng/ml

A549

HEK 293

B

+ A549

HEK 293

cDNA Cont LPS 10 LPS 50 IL-1β Cont LPS 10 LPS 50 IL-1β
Figure 3.1. LPS-induced IL-8 and HBD2 expression in A549 and HEK293 cells.

(A) A549 or HEK293 cells (3 x 10^5/ml) were left untreated or stimulated for 4 h with LPS (10 or 50 μg/ml), IL-1β (100 ng/ml) or TNFα (10ng/ml). Levels of IL-8 in supernatants were measured by ELISA and values are expressed as ng/ml. Assays were performed in duplicate a minimum of three times. Values are expressed as mean +/- S.E. (n=3).

(B) Total RNA was extracted from A549 or HEK293 cells, reverse transcribed into cDNA and used as a template in PCR reactions using HBD2 gene-specific primers. Products were electrophoresed in 1.5% TBE agarose gels containing 0.5 μg/ml ethidium bromide and visualised under UV. Gels are representative of three independent experiments.
Airway epithelial cells express TLR4 on the cell surface.

I characterized the HEK293 cell line to determine whether it lacked a critical factor for LPS responsiveness. RTPCR revealed that the HEK293 cells, similar to the A549 cells, express TLR4, Mal, and CD14 mRNA but unlike the A549 cells do not express MD2 mRNA (Figure 3.2A). MD2 is a secreted protein who's interaction with LPS and CD-14 is necessary for the cellular response to LPS (Re and Strominger 2003)

I performed western immunoblotting of cytosolic and membrane fractions from HEK293 and A549 cells to detect TLR4 protein expression. Figure 3.2B shows that TLR4 was present in both fractions from the A549 cells but was not evident in membrane fractions isolated from HEK293 cells. Next I quantified cell surface expression of TLR4 on A549 by fluorescence microscopy. In accord with the findings of Monick et al (Monick, Yarovinsky et al. 2003) but in contrast to Guillot, (Guillot, Medjane et al. 2004) I detected TLR4 on the surface of A549 cells (Figure 3.2C).
Figure 3.2

A

TLR4
481 bp

TIRAP
237 bp

CD-14
197 bp

MD2
212 bp

GAPDH
211 bp

B

TLR4

Cyt Mem Cyt Mem

HEK A549

C

Isotype

TLR4
Figure 3.2. Characterisation of A549 and HEK293 cell lines

(A) Total RNA was extracted from 1 x 10⁶ HEK293 and A549 cells, reverse transcribed into cDNA and used as a template in PCR reactions using TLR4, Mal, CD14, MD2 and GAPDH gene-specific primers. Products were electrophoresed in 1.5% TBE agarose gels containing 0.5 μg/ml ethidium bromide and visualised under UV. Duplicate lanes represent two independent experiments.

(B) Western blot analysis of membrane (mem) and cytosolic (cyt) extracts (10μg) from A549 and HEK293 cells probed with an anti-TLR4 antibody. Data are representative of three separate experiments.

(C) For fluorescence microscopy, A549 cells (2 x 10⁴) were grown in chamber slides, Fc-blocked and labelled with anti-TLR4 (clear) or isotype control antibodies (solid) and fluorophore-conjugated detection antibodies. TLR4 expression was quantified by laser scanning cytometry.
LPS-induced HBD2 gene and protein expression in A549 cells requires TLR4

Next the role of TLR4 in LPS-induced regulation of HBD2 (Figure 3.3A) in A549 cells was investigated. Compared to untreated cells (lane 1), LPS increased HBD2 expression at 24 h (lane 5). This effect was blocked by pre-treatment with a TLR4 neutralizing antibody (lane 6). An isotype control antibody had no effect (data not shown). A similar effect was demonstrated at protein level by immunohistochemistry (Figure 3.3B), with increased HBD2 production following stimulation of A549 cells with LPS, and inhibition of this effect by pre-treatment with TLR4 neutralizing antibody. IL-1β was used as a positive control. I further quantified this effect using laser scanning cytometry. Figure 3.3C shows that A549 cells express basal levels of HBD2 compared to isotype control-treated cells (see inset). Stimulation with LPS increased HBD2 protein production above control (P<0.02). This effect is inhibited by pretreatment with the TLR4 blocking antibody (P<0.02).
Figure 3.3

A

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B

Control - LPS 4 hours LPS 24 hours

Control IL-1β 100nmol/ml

LPS 10µg/ml Anti-TLR4 mAB + LPS 10µg/ml

C

D

Isotype

HBD2

89
Figure 3.3. LPS-induced HBD2 expression in A549 cells is abrogated by TLR4 blocking antibody.

A459 cells were grown to confluence in 6 well plates (A) or chamber slides (B-D) and incubated with an isotype control or anti-TLR4 neutralising antibody (5 μg/ml, 30 min) then,

(A) left untreated or stimulated with LPS (10 μg) for 4 or 24 hours. Total RNA was extracted, reverse transcribed into cDNA and used as a template in PCR reactions using HBD2 gene-specific primers. Products were electrophoresed in 1.5% TBE agarose gels containing 0.5 μg/ml ethidium bromide and visualised under UV. Gels are representative of three independent experiments.

(B) left untreated or stimulated with LPS (10 μg/ml) or IL-1β (100 ng/ml), as a positive control, for 24 h, fixed in 4 % paraformaldehyde and immunostained using goat anti- antibody as described in the methods. Brown staining indicates HBD2.

(C) left untreated or stimulated with LPS (10 μg) for 24 hours, Fc-blocked and labelled with anti-HBD2 (solid) or isotype control antibodies (clear) and fluorophore-conjugated detection antibodies. HBD2 expression was quantified by laser scanning cytometry, as described, and data from three experiments is presented. HBD2 expression is expressed as Mean Channel Fluorescence (MCF) ± SEM. (* P<0.05 vs control, † P<0.05 vs control + LPS).

(D) Representative graph of HBD2 expression above isotype control in unstimulated A549 cells.
Dominant negative TLR4 inhibits LPS-induced HBD2 expression in A549 cells

In order to further demonstrate the functional role of TLR4 in LPS-induced HBD2 expression, I examined the effect of functionally ablating TLR4 by over expression of a functionally inactive ΔTLR4 construct in A549 cells. Because the upregulation of HBD2 by LPS as measured by semi-quantitative RTPCR was small, albeit statistically significant, I further quantified the effect by luciferase activity of a co-transfected HBD2 promoter-linked luciferase construct (Figure 3.4). LPS increased both HBD2 mRNA expression (Figure 3.4A) (P<0.05) and HBD2 promoter activity (Figure 3.4B) (P<0.03) however over expression of ΔTLR4 significantly inhibited both effects (P<0.05 and 0.03 compared to LPS-treated empty vector-transfected cells, respectively).
Figure 3.4

**A**

**B**

HBD2 luciferase activity

- Control
- +LPS
- ΔTLR4 Control
- ΔTLR4 + LPS

HBD2/GAPDH

- Control
- +LPS
- ΔTLR4 control
- ΔTLR4 + LPS
Figure 3.4 Dominant Negative TLR4 inhibits LPS-induced HBD2 expression in A549 cells

A549 cells (1.5 x 10^5) were transfected with pcDNA3 (empty vector) or a ΔTLR4 expression plasmid. 24 h post transfection, cells were left untreated or stimulated with LPS (10 μg/ml) for 24 h.

(A) Total RNA was extracted, reverse transcribed into cDNA and used as a template in semi-quantitative PCR reactions using HBD2 and GAPDH gene-specific primers. HBD2 expression was given an arbitrary value of 1 in control cells. Data are expressed as mean +/- S.E. and are obtained from three experiments. HBD2 assigned a value of 1. (* P < 0.05 vs control, † P < 0.05 vs control - LPS).

(B) Duplicate experiments were performed using a HBD2 promoter-linked luciferase reporter plasmid. Cells were lysed and reporter gene activity was quantified by luminometry (* P < 0.05). Data are expressed as relative luciferase activity (n=3). (* P < 0.05 vs control, † P < 0.05 vs control + LPS).
TLR4/MD2 transgene expression confers LPS-responsiveness on HEK293 cells

Having established the inhibitory effect of neutralizing TLR4 in A549 cells by receptor blockade and over-expression of a dominant negative construct, I next determined whether HEK293 cells could be rendered responsive to LPS by expression of functional TLR4 and MD2. Expression of CD4/Toll (a constitutively active TLR4 chimera (Frantz, Kobzik et al. 1999)) and MD2 resulted in significant induction of the HBD2 promoter (Figure 3.5A).

Stimulation of HEK293 cells with LPS has no effect on HBD2 promoter activity (Figure 3.5B). However transfection with MD2 and TLR4 transgenes resulted in significant up regulation of HBD2 promoter activity (P<0.005 compared to LPS-treated empty vector-transfected cells), an effect that was further significantly augmented by stimulation with LPS (P<0.05 compared to untreated MD2/TLR4-transfected cells).
Figure 3.5. TLR4/MD2 transgene expression confers LPS-responsiveness on HEK293 cells.

HEK293 cells (1.5 x 10⁵) were cotransfected with MD-2 and CD4/Toll (A) or full-length TLR4 (B) expression plasmids and a HBD2 promoter-linked luciferase reporter gene. Equal amounts of the corresponding empty vector were transfected into control cells such that the difference in total transfected DNA is negligible. 24 h post transfection, cells were left untreated or stimulated with LPS (10 µg/ml) then lysed and reporter gene activity was quantified by luminometry. Data are expressed as relative luciferase activity. Assays were performed in duplicate and are representative of at least three separate experiments.
Expression of dominant negative MyD88 and Mal constructs inhibits LPS-stimulated HBD2 expression.

Having demonstrated that LPS up regulates HBD2 expression via TLR4, I went on to elucidate the pathway by which the signal is transduced to the nucleus. Figure 6 shows that transfection of dominant negative constructs of the TIR domain containing adaptor proteins MyD88 and Mal alone or in combination dose-dependently inhibited HBD2 expression in response to LPS. Technical restrictions of performing transfection experiments in chamber slides precluded measurement of HBD2 protein in this part of the study. I would intuitively expect a similar qualitative effect in HBD2 mRNA and protein expression, as demonstrated in Figure 3.
Figure 3.6. ΔMyD88 and ΔMAL inhibit LPS-induced HBD2 expression in A549 cells.

A549 cells (1.5 x 10^5) were transfected with pCDNA3.1 or pDC304 (empty vectors), ΔMyD88 or Mal P/H expression plasmids as indicated. Regarding amounts of transfected DNA, ΔMyD88 + and ++ represent 100 and 200 ng of dominant negative MyD88 plasmid DNA respectively while Mal + and ++ represent 50 and 100 ng of dominant negative Mal plasmid DNA respectively. 24 h post transfection, cells were stimulated with LPS (10 μg/ml) for 24 h. HBD2 expression was measured by semi-quantitative RTPCR. Expression in LPS-treated cells was ascribed a value of 100 %. Data shown are mean+/- S.E. (n=3). (* P<0.05, **P<0.01, ***P<0.005 vs control + LPS)
3.4 Discussion

Induced expression of HBD2 in response to infective and pro-inflammatory stimuli represents an immediate and dynamic response by the host epithelium to potential infection, and the mechanism by which this occurs has been the subject of much recent investigation. Here I provide evidence for a critical role for TLR4 in LPS-induced HBD2 expression in airway epithelial cells. The data show that A549 cells respond to LPS with increased HBD2 gene and protein expression and that these effects can be blocked by a TLR4 neutralizing antibody or transfection with functionally inactive TLR4, MyD88 or Mal transgenes. I further implicate TLR4 in LPS-induced HBD2 expression by demonstrating that expression of functional TLR4 and MD2 (a co-factor which is unique to and necessary for TLR4 activity) in HEK293 cells confers LPS responsiveness to these cells, which can lead to HBD2 induction.

There is some discrepancy in the literature regarding surface expression of TLR4 in A549 cells. Guillot and colleagues reported that TLR4 is not expressed on the surface of A549 cells, but is compartmentalized to the intracellular compartment (Guillot, Medjane et al. 2004), while Monick et al. demonstrated low level surface expression on the same cells (Monick, Yarovinsky et al. 2003). My data showed low-level surface expression of TLR4 by LSC, and TLR4 was also detectable in membrane fractions by Western blotting. Further evidence of surface expression comes from the ability to block the receptor with anti-TLR4 monoclonal antibody. Surface expression of TLR4 has been demonstrated on other respiratory epithelial cells including human bronchial epithelial cells (Devaney, Greene et al. 2003) and human airway cells in primary culture, where it was found in a more basolateral distribution. The findings of higher levels of TLR4 in the cytoplasmic fraction is also relevant, as internalisation of LPS in
A549 cells has been reported as early as 4 hours after LPS challenge (Lee, Del Sorbo et al. 2004), and this internalisation modulated expression of ICAM-1 and TNF. It remains unclear whether LPS-TLR4 co-localisation intracellularly activates the same signaling cascade as at the cell membrane.

Similar controversy exists regarding the LPS responsiveness of A549 cells. Previous studies have suggested that A549 cells were hyporesponsive to LPS, at doses of up to 100μg/ml (Keicho, Elliott et al. 1997; Krakauer 2002; Tsutsumi-Ishii and Nagaoka 2003). This was not the case in our study. A549 cells responded to 10μg/ml LPS with significant up regulation of both HBD2 and IL-8. One potential reason for this difference is the type of LPS used. Previous studies used E. coli LPS. P. aeruginosa is an important respiratory pathogen, particularly in patients with lung diseases such as cystic fibrosis (CF) (Parad, Gerard et al. 1999). E. coli, in contrast, is more important in the gastrointestinal and genitourinary tract, and as an important cause of septic shock (Kaper, Nataro et al. 2004). Mucoid strains of Pseudomonas have been demonstrated to induce HBD2 in respiratory epithelia including A549 cells (Harder, Meyer-Hoffert et al. 2000). For these reasons, I used Pseudomonas LPS in my study. CD-14 is a glycoprotein which, together with TLR4 and LPS Binding Protein (LBP), forms the LPS signaling complex, and exists in membrane bound and soluble (in serum) forms. Soluble CD14 is required for LPS signaling in A549 cells (Schulz, Farkas et al. 2002), and it is therefore important that LPS stimulation is performed in the presence of serum, as in my study.

Different types of LPS differ in their ability to stimulate cells. Structural differences in LPS, most commonly in the O-polysaccharide chain (Erridge, Bennett-Guerrero et al. 2002), may result in different biological properties. E. coli
LPS is highly toxic in its ability to propagate the systemic inflammatory response syndrome, principally through activation of monocytes. *P. aeruginosa* LPS differs from *E. coli* LPS both in the O-polysaccharide side chain and in the Lipid A component, and stimulates significantly less endotoxic effect (Kulshin, Zahringer et al. 1991), but induces sustained airway inflammation in a number of chronic lung diseases including CF and diffuse panbronchiolitis (Lyczak, Cannon et al. 2000). *Pseudomonas* LPS has been shown to be significantly more potent than LPS from a number of different strains of *E. coli* in its ability to stimulate IL-8 and granulocyte colony-stimulating factor (G-CSF) from respiratory epithelial cells, including A549 cells (Koyama, Sato et al. 2000). The reason for this is not clear, but structural differences may determine its processing by the TLR4/MD2 complex, either directly or through its interaction with the host plasma membrane (Wurfel and Wright 1997; Viriyakosol, Tobias et al. 2001). Basolateral expression of TLR4 has been reported in pulmonary epithelial cells (Muir, Soong et al. 2003), and this is particularly interesting given that *Pseudomonas* elastase has been shown to increase epithelial permeability by its effect on tight junctions (Azghani, Miller et al. 2000), thereby potentially increasing access of *Pseudomonas* LPS to the basally expressed TLR4.

Previous work by Becker et al. (Becker, Diamond et al. 2000) in primary human tracheobronchial cells shows a clear increase in HBD2 protein in response to LPS by western blotting, although another recent study in primary airway epithelial cells reports low expression of MD2 limiting LPS responsiveness (Jia, Kline et al. 2004). MD2 was induced in response to pro-inflammatory cytokines and bacterial products (Jia, Kline et al. 2004), while MD2 expression in A549 cells is enhanced along with TLR4 following infection with RSV (Monick, Yarovinsky et al. 2003). While differences speak to the limitations of using a
cultured cell model, as well as to the variable responses of cultured primary cells, they also reinforce the critical importance of both TLR4 and MD2 in the signaling pathway. A549 cells clearly expressed MD2 and TLR4 and responded to Pseudomonas LPS in my hands. LPS stimulation resulted in a 5-fold increase in HBD2 promoter linked activity. The increase in HBD2 protein, though statistically significant, was small in absolute terms raising questions regarding the physiological significance of LPS-induced epithelial derived HBD2 production.

Indirect activation of epithelial cells by proinflammatory cytokines released by stimulated alveolar macrophages may be more important at lower concentrations of LPS (Tsutsumi-Ishii and Nagaoka 2003), while direct stimulation of the epithelial cells may become important when bacterial load is high, where TLR4 and MD2 expression is enhanced, or following internalisation of LPS. The kinetics of the epithelial response in this and other studies (Becker, Diamond et al. 2000; Griffin, Taggart et al. 2003) may also be relevant, with direct stimulation of the epithelial cells providing a slower but potentially more sustained release of HBD2 than that induced by inflammatory mediators. Indeed, recent studies of cellular cross-talk between epithelial cells and mononuclear cells suggests co-localisation of these cells may result in a more pronounced immune response in vivo (Lee, Del Sorbo et al. 2004). Further work using primary cell culture and co-culture with immune cells mimicking physiologic conditions is required to clarify the relative contributions of these cells to HBD2 production in vivo.
The mechanism by which LPS induces HBD2 expression in respiratory epithelium has not been previously reported. Indirect evidence for involvement of a TLR came first from Diamond and colleagues (Diamond, Russell et al. 1996) in 1996, who described CD-14 dependent LPS induced production of an antimicrobial peptide in bovine tracheal epithelium, and later from Becker and colleagues, who showed that HBD2 up regulation in response to LPS in human tracheobronchial cells was CD14 dependent (33). The GPI-linked CD14 receptor lacks a cytosolic domain and must interact with another receptor to transduce its signal to the nucleus (Haziot, Chen et al. 1988). Transcriptional regulation of HBD2 in response to LPS has also been shown to involve NF-κB (Tsutsumi-Ishii and Nagaoka 2002), but the signaling pathway upstream of NF-κB has not been elucidated. Until recently, TLRs appeared to share a common signaling pathway downstream of their TIR domain. It is now known that individual TLRs utilize different adaptor proteins for signaling, thus conferring biological specificity in their response to activation by individual ligands (O'Neill, Dunne et al. 2003; Takeda and Akira 2004). MyD88 is involved in signaling from all TLRs with the exception of TLR3, whilst Mal is known to have a role in TLR2 and TLR4 intracellular signaling. My data implicate both MyD88 and Mal in LPS-induced HBD2 expression and clearly demonstrates the critical role of TLR4 in LPS induction of HBD2, thus defining another role for TLR4 in pulmonary host defense. Along with the previously well-defined functions of TLR4 in induction of a large number of cytokines, chemokines and adhesion molecules that activate phagocytosis and the adaptive immune responses, activation of TLR4 in the epithelium results in a direct microbicidal response via production of a potent anti-microbial peptide.
Modulation of TLR4 expression in respiratory epithelium may critically affect the production of HBD2. Risk factors for respiratory tract infections include increased age and smoking (Graham 1990), both of which may affect TLR4 expression. TLR4 expression in macrophages is reduced in aged mice, who have a blunted cytokine response to LPS (Renshaw, Rockwell et al. 2002). Cigarette smoke has also been demonstrated to reduce LPS responsiveness in alveolar macrophages (Ohta, Yamashita et al. 1998). As LPS is an active component of cigarette smoke (Hasday, Bascom et al. 1999), and down regulation of TLR4 expression by LPS in cigarette smoke, otherwise known as LPS tolerance, may result in impaired HBD2 production in response to Gram-negative pathogens, facilitating colonization and infection. This is the subject of ongoing work in our laboratory. Similarly, therapies for septic shock aimed at inhibiting LPS signaling by blockade of the TLR4 receptor (Iwami, Matsuguchi et al. 2000) may result in increased susceptibility to nosocomial Gram-negative pneumonia through impaired HBD2 induction.

HBD2 is an important component of host defense in the lung. This study defines a critical role for TLR4 in induced expression of HBD2 in response to LPS and highlights the potential effect of modulation of TLR4 and the accessory proteins MyD88 and Mal expression on pulmonary production of this potent antimicrobial peptide. It also provides important information regarding the cellular responses of A549 cells, a cultured cell model which is widely used in studies of host defense.
Chapter 4

Modulation of TLR4 and HBD2 expression by corticosteroids, salmeterol and cigarette smoke
4.1 Introduction

COPD is a disease characterised by chronic airway inflammation and intermittent infective exacerbations. Inhaled corticosteroids and beta-agonists are therapies that are regularly prescribed in COPD, resulting in symptomatic relief and reduced exacerbation frequency, while cigarette smoke has been identified as the primary pathogenic determinant of this disease. In this chapter, I examine the effect of these compounds on TLR4 expression and LPS responsiveness, thus defining novel effects of these compounds on innate immune response to Gram-negative pathogens.

I first looked at the effect of Fluticasone Propionate as a potent corticosteroid, which is frequently used as an inhaled medication in COPD. The hydrophobic properties of this compound, which make it ideal for use as a dry powder medication, mean that it is difficult to use in cell culture, and I therefore used dexamethasone for further corticosteroid experiments. Gene transcription was determined by RTPCR, and expression of TLR2 and HBD2 was determined for comparison, and surface expression of TLR4 protein by Western Blotting of the membrane fractions. Functional effect was demonstrated by IL-8 production (measured by ELISA) and HBD2 gene transcription (by RTPCR) in response to LPS.

I next looked at the effect of salmeterol on TLR4 expression. While it appeared with RTPCR that there was an upregulation of TLR4 at the highest dose of salmeterol used, the lack of a dose response effect made me question this effect and I therefore went on to quantify this effect using Real Time PCR, also taking the opportunity to confirm the effect of dexamethasone on TLR4 gene transcription. TLR4 surface protein expression was again determined by Western
blotting of the membrane fraction, and because it showed a different effect to that of the mRNA, was further confirmed by Laser Scanning Cytometry. Further examination of TLR4 protein in total cell lysates and cytosolic fractions indicated that the salmeterol induced effect on TLR4 membrane expression was mediated by a post-translational transport effect. That this was a beta-adrenoreceptor mediated effect was demonstrated by use of the specific beta2-blocker Butoxamine to abrogate the effect.

LABAs are most frequently prescribed in combination with corticosteroids and therefore the effect of the two drugs used in combination on TLR4 gene transcription by Real Time PCR and protein by Western blotting was determined. I next looked at the effect of cigarette smoke on the respiratory epithelium. Cigarette smoke extracts were prepared by bubbling smoke through culture media and serial dilutions were applied to A549 cells. TLR4 gene transcription was determined by RTPCR and protein by Western Blotting. Because of the potential multiple toxic effects of cigarette smoke, a cell viability assay was performed to ensure that any observed effects were not merely the result of cell necrosis. Functional effect was determined by IL-8 ELISA, acknowledging the competing effects of LPS in the cigarette smoke as well as direct cell toxicity.

The roles of the transcription factors NK-κB and p38 MAPK in mediating the observed effects on TLR4 expression was investigated by measuring activation of these pathways. Commercially available ELISA kits allowed measurement of the proportion of phosphorylated to total Iκ-Bα and p38 respectively.

Finally, I examined the expression of TLR4 mRNA, along with TLR2 and HBD-2, in the nasal mucosa of non-smokers and smokers to determine if this correlated with the observed *in vitro* effect.
4.2 Methods in Brief:

All methods are described in full in Chapter 2.

Cell lines and treatments: In-vitro experiments were performed using human alveolar epithelial A549 cells. Prior to agonist treatment, cells were washed with serum-free F12 and placed under serum-free conditions or in serum containing 1% FCS for LPS stimulations. Stock solutions of Fluticasone, Salmeterol and Dexamethasone were prepared as described, and fresh dilutions were prepared for each experiment. Cigarette smoke extract (CSE) was freshly prepared for each experiment, filtered to remove bacteria and particles, and serial dilutions were made.

Gene Transcription studies: Gene transcription was analysed by semi-quantitative RTPCR using gene-specific primers for TLR2, TLR4 and HBD2 and corrected to relative GAPDH expression. Further quantification of TLR4 mRNA was performed where indicated in the results by Real Time PCR using different Primers for TLR4 and Actin, optimised for the specifications of the reaction.

Protein Studies: Expression and sub-cellular localisation of TLR4 was determined by preparation and western Blot analysis of membrane, cytosolic and total cell extracts from A549 cells. Membrane localisation of TLR4 was further analysed by Laser Scanning mcroscopy of A549 cells grown in chamber slides. IL-8 production as a surrogate of LPS responsiveness was determined by sandwich ELISA.
Cell viability: A549 cells (1 x 10⁴/well) were plated in a 96 well plate in serum-free medium overnight, rinsed and placed in medium containing 1% FCS. Triplicate samples were left untreated or treated with dexamethasone (16 hours), Salmeterol (6 hours) or CSE (4 hours) at the indicated dilutions and cell viability was quantified using the Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay as recommended by the manufacturer.

Cell signalling assays: The effects of dexamethasone (10⁻⁷M), salmeterol (10⁻⁶M) or CSE (10⁻¹ dilution) on p38 MAPK and IκBα phosphorylation were assayed using SuperArray Cellular Activation of Signaling ELISAs according to the manufacturer’s instructions.

Study population: Outpatients attending for upper GI endoscopies were recruited for nasal brush sampling following approval of study protocol and consent forms by the Beaumont Hospital Ethics Committee. Subjects were excluded on the basis of pre-existing immunosuppression, pulmonary or nasal pathology, including current or recent (within 6 weeks) upper or lower respiratory tract infection and reported normal functional status.

Nasal Epithelial cell sampling: Following informed consent, brushing was performed using a modification of the technique of Bridges et al (Bridges, Walker et al. 1991). Prior to cell collection, topical nasal decongestant and local anaesthetic were applied to the nose. The nose was carefully cleaned to remove crusts and mucous. Nasal epithelial cells were obtained by vigorous brushing of the medial wall of the inferior turbinate under direct vision using a Cervibrush + (CellPath plc). Cells were placed in chilled serum free nutrient mixture F-12
(HAMS). Part of the sample was prepared for cytocentrifugation to identify the cell types present by May-Grunwald-Giemsa (Sigma, Ireland) staining. Samples were accepted for analysis if they contained at least 80% epithelial cells. Cells were washed three times in ice cold PBS prior to resuspension in TriReagent (Sigma, Ireland) for RNA extraction.

Statistical analysis: Data were analyzed with GraphPad Prism 3.0 software package (GraphPad Software, San Diego, CA). Results are expressed as mean ± S.E. and were compared by Mann-Whitney test. Differences were considered significant when the P value was ≤ 0.05.
4.3 Results and Figures

Corticosteroids downregulate TLR4 expression and LPS responsiveness in respiratory epithelial cells.

We first examined the effect of Fluticasone on expression of mRNA of TLR2, TLR4 and HBD2 by rtPCR in the respiratory epithelial cell line A549 grown in culture (Figure 4.1). A dose dependent downregulation of TLR4 compared to the housekeeping gene GAPDH was observed with an Inhibitory Concentration (IC) 50 between $10^{-9}$ and $10^{-8}$ M, while expression of TLR2 and HBD2 was unchanged.

Fluticasone is a hydrophobic compound ideally suited for use as a dry powder inhalation, but it's poor solubility makes it unpredictable for use in cell culture. I therefore assessed whether the observed effect was a class effect of corticosteroids, using the more soluble compound dexamethasone (Figure 4.2). Again, a dose dependent downregulation of TLR4 mRNA and protein was observed, and consistent with the increased potency of fluticasone which has approximately 8 times the binding affinity of dexamethasone, a higher dose of dexamethasone was required to achieve a comparable effect (IC$_{50}$ between $10^{-8}$ and $10^{-7}$ M).
Figure 4.1

HBD2

TLR2

TLR4

GAPDH

Fluticasone  Cont  $10^{-9}$ M  $10^{-8}$ M  $10^{-7}$ M  $10^{-6}$ M
A549 cells (3 x 10^5) were seeded onto 6-well plates and grown to confluence. Cells were washed, placed in serum free medium and were left untreated or incubated with fluticasone propionate over the dose ranges 10^{-9} to 10^{-6} Molar for 16 hours. Total RNA was extracted, reverse transcribed into cDNA and used as a template in PCR reactions using HBD2, TLR2, TLR4 and GAPDH gene-specific primers. Products were electrophoresed in 1.5% TBE agarose gels containing 0.5 μg/ml ethidium bromide and visualised under UV. Gels are representative of three independent experiments.
Figure 4.2

A

TLR4 mRNA

GAPDH mRNA

Dexamethasone

Cont $10^{-10}$ $10^{-9}$ $10^{-8}$ $10^{-7}$ $10^{-6}$

B

TLR4 Protein

Actin Protein

Dexamethasone

Cont $10^{-10}$ $10^{-9}$ $10^{-8}$ $10^{-7}$ $10^{-6}$
Figure 4.2 Dexamethasone downregulates TLR4 gene and protein expression.

A549 cells (3 x 10^5) were seeded onto 6-well plates and grown to confluence. Cells were washed, placed in low-serum (1% FCS) medium and were left untreated or incubated with dexamethasone at dose of 10^-9 to 10^-6 Molar for 16 hours.

A. Total RNA was extracted, reverse transcribed into cDNA and used as a template in PCR reactions using TLR4 gene-specific primers. Products were electrophoresed in 1.5% TBE agarose gels containing 0.5μg/ml ethidium bromide and visualised under UV. Gels are representative of three independent experiments.

B. Western blot analysis of membrane extracts (10μg) from A549 cells probed with an anti-TLR4 antibody. Data are representative of three separate experiments.
Acknowledging the semi-quantitative nature of this analysis, for which Real Time PCR would give superior quantification of mRNA upregulation, I was anxious to demonstrate a quantifiable functional effect. I therefore went on to stimulate the cells with the TLR4 ligand LPS. Stimulation of A549 cells with LPS 10μg/ml for 24 hours results in a significant induction of IL-8, as measured by ELISA of the cell culture supernatant (P<0.05) (Figure 4.3A). Pre-treatment of the cells with dexamethasone results in a dose-dependent abrogation of this effect, reaching statistical significant at a dose of 10^{-7} M (P<0.05). A similar effect is seen on the induced expression of HBD2 mRNA in response to LPS (Figure 4.3B).
Figure 4.3

A

![Bar chart showing IL-8 pg/ml](image)

- control
- control + LPS
- Dex 10^{-10}
- Dex 10^{-9}
- Dex 10^{-8}
- Dex 10^{-7}
- Dex 10^{-6}

B

- **HBD2 mRNA**

- **Dexamethasone**
  - Cont
  - 10^{-10}
  - 10^{-9}
  - 10^{-8}
  - 10^{-7}
  - 10^{-6}
Figure 4.3 Downregulation of TLR4 by Dexamethasone is associated with relative hypo-responsiveness to LPS

A549 cells were grown and incubated with dexamethasone as described in Figure 4.2. Following treatment with dexamethasone, cells were stimulated with LPS 10μg/ml for a further 24 hours.

(A) Levels of IL-8 in supernatants were measured by ELISA and values are expressed as pg/ml. Assays were performed in duplicate a minimum of three times. Values are expressed as mean± S.E. (n=3).

(B) Total RNA was extracted, reverse transcribed into cDNA and used as a template in PCR reactions using HBD2 gene-specific primers. Products were electrophoresed in 1.5% TBE agarose gels containing 0.5μg/ml ethidium bromide and visualised under UV. Gels are representative of three independent experiments.
Membrane expression of TLR4 is upregulated by the long-acting beta-agonist Salmeterol via specific β-agonist effect.

I next examined the effect of the long acting beta agonist salmeterol on expression of TLR4 mRNA by RT-PCR over a dose range of $10^{-9}$ and $10^{-6}$ M (Figure 4.4A). Cells were incubated with the drug for 6 hours. Semi-quantitative analysis suggested a small increase in TLR4 expression over control at the highest dose of $10^{-6}$ M (Figure 4.4A), however the lack of a dose response cast doubt on the functional relevance of this observation. I therefore went on to quantify this effect by Real Time RT-PCR and found no significant effect of Salmeterol $10^{-6}$ M on TLR4 gene expression (Figure 4.4B). Analysis of protein expression in total cell lysates similarly showed no significant change in total TLR4 (TLR4t) expression (Figure 4.4C lower panel), however levels in cytosolic extracts (TLR4c) were decreased (Figure 4.4C upper panel). A concomitant increase in membrane expression was evident at doses of $10^{-7}$ M and $10^{-6}$ M. (Figure 4.4D), an effect which was confirmed by Laser Scanning Cytometry (Figure 4.4E). Pre-treatment of cells with the beta-blocker Butoxamine abrogated the effect of Salmeterol $10^{-6}$ M on membrane expression of TLR4, indicating a specific beta-adrenoreceptor mediated effect (Figure 4.4D). Taken together these data indicate that salmeterol induces a post-translational transport effect on TLR4.
Figure 4.4

A

TLR4

GAPDH

Salmeterol Cont 10⁻⁹M 10⁻⁸M 10⁻⁷M 10⁻⁶M

Figure 4.4

B

TLR4/actin

control Sal 10⁻⁶

Figure 4.4

C

TLR4c

Actin c

TLR4t

Actin t

Sal Cont 10⁻¹⁰M 10⁻⁹M 10⁻⁸M 10⁻⁷M 10⁻⁶M
Figure 4.4 Salmeterol upregulates TLR4 membrane protein expression in respiratory epithelial cells

A549 cells (3 x 10⁵) were seeded onto 6-well plates and grown to confluence. Cells were washed, placed in serum free medium and were left untreated or incubated with salmeterol over the dose ranges 10⁻⁹ to 10⁻⁶ M for 6 hours. Beta-agonist effect was examined by pre-treatment of cells with Butoxamine 0.5 M x 30 minutes prior to salmeterol treatment.

A. Total RNA was extracted, reverse transcribed into cDNA and used as a template in PCR reactions using HBD2, TLR4 and GAPDH gene-specific primers. Products were electrophoresed in 1.5% TBE agarose gels containing 0.5μg/ml ethidium bromide and visualised under UV. Gels are representative of three independent experiments.

B. Real time PCR was performed as described in the methods. Data is expressed as mean +/- SEM and represents 7 independent experiments with TLR4/actin given an arbitrary value of 1 in control cells.

C. Western blot analysis of total cell extracts (t) and cytosolic extracts (c) (10μg) from A549 cells probed with an anti-TLR4 or anti-Actin antibody. Data are representative of three independent experiments.
Median channel fluorescence

Figure 4.4
D. Western blot analysis of membrane extracts (10μg) from A549 cells probed with an anti-TLR4 or anti-Actin antibody. Densitometry was performed and corrected for corresponding β-actin density. Data are expressed as mean +/- S.E. and are obtained from three experiments. (* P=0.05 compared to control)

E. A549 cells were incubated with an isotype control (clear) or anti-TLR4 (solid) antibody and fluorophore-conjugated detection antibodies. HBD2 expression was quantified by laser scanning cytometry, as described and data from three experiments is presented. HBD2 expression is expressed as Median Channel Fluorescence (MCF) ± SEM. (* P<0.05 vs. control, ** P<0.005 vs. control).
Salmeterol reverses the inhibitory effect of dexamethasone on TLR4 mRNA and protein

Because inhaled long acting beta agonists are most often prescribed in combination with inhaled corticosteroids, I examined the effect of these compounds used in combination. The lowest dose of dexamethasone at which a functionally significant downregulation of TLR4 was observed, namely $10^{-7} \text{ M}$, was used in combination with the dose of salmeterol required to produce upregulation of the same receptor, namely $10^{-6} \text{ M}$. TLR4 gene expression was determined by Real Time PCR (Figure 4.5A). Again, treatment with salmeterol alone caused no significant change in TLR4 expression above untreated cells, while dexamethasone down regulated TLR4 expression. At the mRNA level, the dexamethasone effect persists when the two compounds are used in combination, resulting in significant downregulation in TLR4 mRNA expression compared to untreated cells. Looking at membrane protein expression however, salmeterol reverses the effect of dexamethasone on TLR4 expression resulting in no net change in TLR4 membrane expression with the two drugs used in combination (Figure 4.5B). Cell viability was not affected by either drug (data not shown).
Figure 4.5

A

B
Figure 4.5. Salmeterol reverses the inhibitory effect of dexamethasone on TLR4 membrane protein expression despite downregulation of mRNA.

A549 cells \((3 \times 10^5)\) were seeded onto 6-well plates and grown to confluence. Cells were washed, placed in serum free medium and were left untreated or incubated with \(10^{-6}\text{M}\) dexamethasone (Dex), \(10^{-7}\text{M}\) salmeterol (Sal) or both drugs in combination \((\text{Sal} + \text{Dex})\) for 16 hours. Numbers indicate Molar doses of drug. Following treatment, total RNA was extracted, reverse transcribed into cDNA and used as a template in PCR reactions using TLR4 and GAPDH gene-specific primers.

A. Real-time PCR analysis of TLR4 mRNA expression as a factor of \(\beta\)-actin expression. TLR4 expression was given an arbitrary value of 1 in control cells. Data are expressed as mean +/- S.E. and are obtained from three experiments. \((* \ P=0.05 \text{ compared to control})\)

B. Western blot analysis of membrane extracts \((10\mu g)\) from A549 cells probed with an anti-TLR4 or anti-Actin antibody. Densitometry was performed and corrected for corresponding \(\beta\)-actin density. Data are expressed as mean +/- S.E. and are obtained from three experiments. \((* \ P=0.05 \text{ compared to control})\)
Cigarette smoke condensates down regulate TLR4 expression in respiratory epithelium in-vitro

I next examined the effect of cigarette smoke on expression of TLR4 in respiratory epithelium in vitro. Cigarette smoke extracts were freshly prepared for each experiment and airway epithelial cells were stimulated with serial dilutions of the condensates for 4 hours. There was a dose dependant downregulation in TLR4 mRNA (Figure 5A) and protein (Figure 5B) following exposure to the cigarette smoke extracts. To ensure that the effect was not caused by direct toxicity of the cigarette smoke, a viability assay was performed which demonstrated 50% reduction in viability with undiluted CSE, but no toxic effect following dilution of the extracts (Figure 5C). I went on to examine functional effect by IL-8 ELISA. Concordant with the reduced expression of TLR4, the respiratory epithelial cells have reduced secretion of IL-8 following treatment with CSE, both with and without additional LPS (Figure 5D). Failure of the cells to produce any IL-8 following exposure to undiluted CSE may be a result of the direct toxic effects demonstrated in the viability assay.
Figure 4.6

A

![Bar graph showing TLR4/GAPDH expression levels.](image)

B

![Western blot images for TLR4 and Actin.](image)

C

![Viability graph.](image)

D

![IL-8 graph.](image)
Figure 4.6 Cigarette smoke downregulates TLR4 gene and protein expression in A549 cells resulting in relative hyporesponsiveness to LPS.

A549 cells (3 x 10^5) were seeded onto 6-well plates and grown to confluence. Cells were washed, placed in serum free medium or cigarette smoke condensates for 4 hours. Cigarette smoke condensates were prepared as described in the methods and numbers correspond to serial dilutions of the initial cigarette smoke extract.

A. Following treatment, total RNA was extracted, reverse transcribed into cDNA and used as a template for semi-quantitative PCR reactions using TLR4 and GAPDH gene-specific primers. TLR4 expression was given an arbitrary value of 1 in control cells. Data are expressed as mean +/- S.E. and are obtained from three experiments. (* P<0.05 compared to control)

B. Western blot analysis of membrane extracts (10μg) from A549 cells probed with an anti-TLR4 antibody. Data are representative of three separate experiments. (CSE † cigarette smoke extract)

C. Viability assay of A549 cells following treatment with CSE. Data are representative of three separate experiments.

D. A549 cells (3 x 10^5) were seeded onto 6-well plates and grown to confluence. Cells were washed, placed in low-serum (1% FCS) medium and were left untreated or incubated with serial dilutions of CSE x 4 hours. Following treatment with dexamethasone, cells were stimulated with LPS 10μg/ml for a further 24 hours. Levels of IL-8 in supernatants were measured by ELISA and values are expressed as pg/ml. Assays were performed in duplicate a minimum of three times. Values are expressed as mean +/- S.E. (n=3). (* signifies P≤0.05 of observed effect vs. control, † signifies P≤0.05 of observed effect vs. control plus LPS).
Roles of p38 MAPK and NFκB in Dexamethasone, Salmeterol- and CSE-induced changes in TLR4 expression.

In order to address the mechanism by which dexamethasone, salmeterol and CSE may be inducing their effects on TLR4 gene expression I evaluated their ability to phosphorylate p38 or IκBα and hence activate p38 or NFκB, respectively. Figure 6A shows that dexamethasone, salmeterol and CSE can all inhibit phosphorylation of p38 whilst Figure 6B shows that only salmeterol enhances IκBα phosphorylation.
Figure 4.7

A

Relative $\text{I\kappa B}\alpha$ activation

B

Relative p38 activation

- c
- Dex
- Sal
- CSE

- c
- Dex
- Sal
- CSE
Figure 4.7. Effect of dexamethasone, salmeterol and CSE on p38 and IκBα phosphorylation.

Cells (1.4 x 10^4/well) were left untreated or treated with dexamethasone (10^{-7} M), salmeterol (10^{-6} M) or CSE (10^{-1} dilution). Following fixation in formaldehyde, total and phospho-versions of (A) p38 MAPK and (B) IκBα were quantified using pan-specific and phospho-specific antibodies, respectively from SuperArray's Cellular Activation of Signaling ELISAs. Relative cell numbers were quantified by cell staining and these values were used for normalisation. Target protein phosphorylation was determined by calculating phospho-protein:pan-protein ratios. Assays were performed in triplicate (* ≤ 0.05 vs. control).
TLR4 expression is down regulated in the nasal epithelium of smokers in-vivo.

To determine if this phenomenon translated into a real effect in vivo, I examined expression of TLR4 along with TLR2 and HBD2 in the nasal epithelium of healthy smokers and age matched controls (Figure 8). Semi-quantitative analysis of mRNA expression revealed a very significant reduction in TLR4 expression in the nasal mucosa of smokers compared to controls (P<0.005). There was no significant reduction in expression of TLR2 (P=0.28) or HBD2 (P=0.20).
**Figure 4.8**

**A**

<table>
<thead>
<tr>
<th></th>
<th>Non Smoker</th>
<th>Smoker</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Age, yr Mean and range</td>
<td>55.2 (44-66)</td>
<td>46.2 (24-62)</td>
<td>0.5</td>
</tr>
<tr>
<td>Gender, % male</td>
<td>44.4</td>
<td>25</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**B**

(i) [Image of non-smoker sample]

(ii) [Image of smoker sample]

**C**

![Graphs of TLR4/GAPDH and TLR2/GAPDH](image)

- TLR4/GAPDH: Non-Smoker vs Smoker, P=0.28
- TLR2/GAPDH: Non-Smoker vs Smoker, p=0.20
Figure 4.8 TLR4 mRNA expression is down-regulated in the nasal mucosa of smokers in vivo.

Outpatients attending for upper GI endoscopy were recruited for nasal brush sampling. Subjects were excluded on the basis of pre-existing immunosuppression, pulmonary or nasal pathology, including current or recent (within 6 weeks) upper or lower respiratory tract infection.

(A) Table showing demographics of the study population. There was no significant difference between the study groups.

(B) Following informed consent, nasal epithelial cells were harvested by brush technique as described in the methods. Cytospins were prepared from each specimen and stained with Diff-Quick to determine cell populations. (i) shows a uniform population of nasal epithelial cells. At higher magnification stained with trypan blue (ii), we see columnar epithelial cells with cilia at the apices. Only samples containing >80% epithelial cells were accepted for further analysis. Cells were washed three times in ice cold PBS prior to resuspension in TriReagent for RNA extraction.

(C) Total RNA was reverse transcribed into cDNA and used as a template for semi-quantitative PCR reactions using TLR4, TLR2, HBD2 and GAPDH gene-specific primers. (** P <0.005)
4.4 Discussion

TLR4 is the predominant pattern recognition receptor for LPS and mediates a significant proportion of the innate immune response to Gram-negative organisms. Expression of TLR4 on respiratory epithelium allows rapid and direct activation of host defense by potential pathogens, resulting in induction of inflammatory mediators and anti-microbial peptides, including HBD2. This may be important in preventing bacterial colonization and infection. Consistent with previous reports (Becker, Diamond et al. 2000; Armstrong, Medford et al. 2004; Homma, Kato et al. 2004), I demonstrate that respiratory epithelial cells express TLR4 and respond to LPS with upregulation of IL-8.

Glucocorticoids have been previously reported to modulate lung responses to infection. Pre-incubation of organ cultures with fluticasone resulted in significantly reduced inflammatory response to *Pseudomonas aeruginosa* (Dowling, Johnson et al. 1999). There have been no previous reports about the effect of corticosteroids on TLR4 expression in epithelial cells. I have previously shown that LPS-induced HBD2 production in respiratory epithelial cells is TLR4 dependent (MacRedmond, Greene et al. 2005). Consistent with our data, Tomita et al demonstrated reduced LPS-induced HBD2 expression in an airway epithelial cell line following pre-treatment with dexamethasone (Tomita, Nagase et al. 2002), but the effect on the LPS receptor, TLR4, was not explored. Here I provide evidence for a mechanism whereby corticosteroids could impair host defence against Gram-negative bacteria by downregulation of TLR4 expression.

Glucocorticoids have a broad spectrum of molecular targets on which they exert their anti-inflammatory effect. These include genomic DNA, histone-modifying
enzymes, transcription factors, and signalling cascades activated by a wide range of stimuli (Adcock and Ito 2000). A recent study showed TLR2 expression can be enhanced in respiratory epithelium by treatment with dexamethasone in combination with the pro-inflammatory cytokines TNFα and IL-1β (Homma, Kato et al. 2004), while another study showed enhancement of dexamethasone-induced TLR2 expression by non-typeable *H. influenzae* (NTHi) in human airway epithelial cells (Imasato, Desbois-Mouthon et al. 2002). The proposed mechanism in the latter study involved specific up-regulation of the MAPK phosphatase-1 (MKP-1) that, in turn, leads to dephosphorylation and inactivation of p38 MAPK, a negative regulator of TLR2 expression. A p38 MAPK inhibitor had no effect in the former study however, suggesting a different mechanism, possibly via a putative Glucocorticoid Response Element (GRE) in the TLR2 promoter. Our data demonstrates that downregulation of TLR4 gene transcription is associated with downregulation of p38 activation (Figure 6), suggesting that in contrast to TLR2, p38 may be a positive regulator of TLR4 expression. Although NF-κB is reported to be inhibited by corticosteroids, I observed no change in NF-κB activation, suggesting it is not responsible for the observed effect (Pfahl 1993; Homma, Kato et al. 2004).

Long acting beta-2 agonists such as salmeterol relax bronchial smooth muscle causing bronchodilatation, and are a widely used and effective treatment in obstructive airways disease. In addition to bronchoconstriction, both COPD and asthma are characterised by airways inflammation, albeit of different types. Accumulating evidence in recent years indicates that LABAs have anti-inflammatory properties, which augment their known bronchodilator activity, with multiple effects on activation, mediator release, recruitment and survival of inflammatory cells (Johnson 2002). Beta₂-adrenergic receptors are expressed in
respiratory epithelium, but the immunomodulatory effect of LABAs on these cells has been largely unexplored.

Here I demonstrate that the LABA salmeterol treatment had no effect on TLR4 gene transcription. While salmeterol had a similar effect on p38 activation as dexamethasone, the failure to downregulate TLR4 may result from competing effects of NF-κB activation which is upregulated by salmeterol, although this did not reach statistical significance. Salmeterol did however induce membrane expression of TLR4 from the cytoplasmic/nuclear compartment independent of any effect on gene transcription or total protein expression. A similar post-translational effect has been described in nasal epithelium of patients with allergic rhinitis compared to healthy subjects (Fransson, Adner et al. 2005), while nuclear localisation of TLR4 has been confirmed in bronchial epithelium (Janardhan, McIsaac et al. 2006). TLR4 has been shown to cycle rapidly between the Golgi and the membrane, with signal transduction occurring only at the membrane (Latz, Visintin et al. 2002). Little is known about the mechanism of this translocation or indeed transport from the nucleus. Our data, demonstrating a beta-receptor mediated effect on post-translational TLR4 transport suggests a potential role for cAMP-dependent protein kinases in this process. Other potential mechanisms include cytoskeletal effects secondary to changes in intracellular calcium, while the accessory protein MD-2 has also been implicated in stable expression of TLR4 at the membrane (Nagai, Akashi et al. 2002), (Sin, McAlister et al. 2003).

LABAs and inhaled corticosteroids are frequently prescribed in combination for both asthma and COPD. The complimentary and synergistic activity of LABAs and corticosteroids in clinical practice may be explained by their physiological
effects, but it is increasingly clear that they may also interact at a molecular level (Johnson 2002). Corticosteroids may augment the effect of LABAs by binding to GREs in the promoter region of the β2-receptor gene resulting in increased gene transcription, and an increase in β2-receptor density (Mak, Nishikawa et al. 1995; Baraniuk, Ali et al. 1997). LABAs in turn may augment corticosteroid function by a number of mechanisms, including induced nuclear translocation of the glucocorticoid receptor (Eickelberg, Roth et al. 1999) and enhanced activity against transcription factors such as NF-κB (Pang and Knox 2000). Given the opposing effects of the two drugs on TLR4 expression, their net additive effect masks any potential synergistic effect in this study. The abrogation of steroid induced downregulation of TLR4 membrane protein expression, and consequent susceptibility to Gram-negative infection, by addition of salmeterol may represent another important advantage of co-prescription of these compounds.

Cigarette smoking has been clearly implicated as the major environmental risk factor predisposing to the development of COPD (Silverman and Speizer 1996). Cigarette smoke has multiple effects on leukocyte function resulting in mucosal inflammation, which over time contributes to the epithelial disruption, smooth muscle hypertrophy, and fibrosis seen in this disease (Sutherland and Martin 2003). Smoking is also an independent risk factor for bacterial colonisation of the lower respiratory tract (Zalacain, Sobradillo et al. 1999; Qvarfordt, Riise et al. 2000), acute respiratory infection (Graham 1990), and infective exacerbations of COPD (Miravitlles, Espinosa et al. 1999). In a recent study, mice exposed to cigarette smoke demonstrated a reduced rate of clearance of Pseudomonas compared to sham exposed mice, resulting in increased airway inflammation. (Drannik, Pouladi et al. 2004). While these effects may be due in part to previously documented effects on cell-mediated immunity, little is known about
the effect of smoking on innate immune function. Our data demonstrates that cigarette smoke extracts downregulate TLR4 expression and LPS responsiveness in respiratory epithelium \textit{in vitro} and is consistent with data from Beisswenger and colleagues who demonstrated reduced HBD2 production in response to LPS in respiratory epithelial cells following exposure to cigarette smoke (Beisswenger C 2003).

A potential limitation of our study is the use of A549 cells, a cell line derived from lung carcinoma, and whose responses may be different to those of primary cultured epithelial cells. While methods have been established to isolate primary cultured human lung epithelial cells, the purity of these preparations is difficult to control and responses may vary. A549 cells are widely used in lung cell biology and are a valid model to generate new hypotheses. As with any model system, questions regarding physiological relevance remain. It was therefore exciting that our findings could be reproduced in vivo. Even with this small sample size, a clear reduction in TLR4 expression in the nasal mucosa of smokers was demonstrated. If this effect is replicated in the lower respiratory tract, it may result in impaired innate immune response to LPS and contribute to the increased susceptibility to bacterial colonisation and infection in the lungs of smokers.

We explored several potential mechanisms of CSE-induced downregulation of TLR4. Consistent with the data of Laan and colleagues, I did not find any change in NF-κB activity following treatment with CSE (Laan, Bozinovski et al. 2004), however as with dexamethasone, the downregulation of p38 activity correlated with decreased TLR4 gene transcription, suggesting this as a potential mechanism, and again consistent with their observation of reduced LPS-induced AP-1 activation (Laan, Bozinovski et al. 2004). TNFα is a negative regulator of
TLR4 expression in intestinal epithelium (Abreu, Thomas et al. 2003) and is thought to be important in the pathogenesis of COPD (Churg, Dai et al. 2002). I did not find elevated levels of TNFα in the supernatants of cells stimulated with cigarette smoke extracts as measured by ELISA (data not shown). Moreover a recent study reports no increase in TNFα in exhaled breath condensates of young healthy smokers compared to non-smoking controls (Garey, Neuhauser et al. 2004), suggesting that TNFα is not an important component of the mechanism involved here.

The respiratory epithelium is in constant dynamic interaction with the environment, and is uniquely directly exposed to airborne pathogens and toxins, as well as aerosolised drugs. The TLRs perform a pivotal role in host defence, and this study demonstrates that TLR4 expression in respiratory epithelium is modulated both by drugs used to treat common respiratory disorders and by cigarette smoke, the major pathogenic determinant of COPD, and implicates p38 as a potential positive regulator of TLR4 gene transcription. Demonstration of a novel β-adrenoreceptor mediated TLR4 transport effect indicates new avenues for exploration of the regulation of expression of this important receptor. A greater understanding of the mechanism of these effects may improve our understanding of the pathogenesis of airways disease, and direct future therapies aimed at augmenting host defence.
Chapter 5

Expression of TLR2, TLR4 and HBD2 in Nasal and Tracheo-bronchial Mucosa in COPD in vivo.
5.1 Introduction

Chronic Obstructive Pulmonary disease is complicated by infective exacerbations which result in significant morbidity and mortality and which may contribute to progression of lung function decline, while bacterial colonisation may contribute to chronic airway inflammation in these patients. In the final part of my study, I sought to determine whether the expression of TLR2, TLR4 and HBD2 in nasal epithelium of COPD patients differed from control subjects (both smokers and non-smokers) and between patient groups in terms of disease severity and whether these differences correlated with the reported infection and airway colonisation patterns in these patients. Ethics committee approval was granted by the Ethics Committee of Beaumont Hospital prior to commencement of this part of the study.

Nasal epithelial cells were collected by a brush technique, and RNA was extracted following confirmation of sample purity by cytospin and DiffQuick staining. Expression of TLR4, TLR2 and HBD2 mRNA was determined by semi-quantitative RT-PCR, corrected to the housekeeping gene GAPDH. Unfortunately, the quantification of protein expression from brush samples was not possible due to contamination of the sample with mucus despite various efforts to eliminate this.

Tracheo-bronchial brush samples were also collected from a subgroup of patients undergoing bronchoscopy for independent indications and TLR4 expression in these samples was correlated to expression of TLR4 in the nasal mucosa of the same subject to validate the use of nasal mucosa as representative of respiratory epithelium for study of innate immune function. IL-8 levels in nasal lavage specimens were used as a surrogate of airway inflammation, measured by standard ELISA.
5.2 Methods in Brief

Methods are described in detail in Chapter 2

*Patient population:* COPD patients were recruited from the Respiratory outpatient clinic in Beaumont Hospital, and from patients attending for bronchoscopy for indications independent of this study. The diagnosis of COPD was based on consistent clinical and lung function abnormalities. Control subjects were recruited from the outpatient population attending for GI endoscopy at Beaumont Hospital. At the time of sample collection, all subjects were free of acute intercurrent nasal or respiratory infection.

*Collection of epithelial cell samples:* Nasal epithelial cells were collected by brushing of the nasal mucosa with a cytology brush following application of local anaesthetic and cleaning the area of mucous and crusts. A similar method was employed for tracheobronchial sampling via a fibreoptic bronchoscope under direct vision. Cells were shaken into cold F-12 medium and transported on ice to the laboratory. Cell population was examined by cytopsin and DiffQuick staining to determine that the samples contained at least 80% epithelial cells. RNA was extracted using Trireagent.

*Gene transcription analysis* Gene transcription was determined by semi-quantitative RTPCR using the housekeeping gene GAPDH as the denominator.
Nasal lavage analysis Nasal lavage was collected by instillation and collection of warmed saline into the nasal passages. Lavage was spun down to remove cellular debris and supernatant stored for batch analysis. IL-8 protein was determined by standard ELISA.
5.3 Patient consent form

CONSENT FORM TO PARTICIPATE IN A CLINICAL RESEARCH STUDY

You are being asked to consent to participate in a study involving patients with chronic obstructive airways disease and chest infections. It is important that you understand several general principles that apply to all that participate in this study:

i) taking part is voluntary and you will not be paid

ii) personal benefit will not result from taking part in this study, however the knowledge gained may benefit others in the future

iii) you may withdraw from the study at any time and withdrawal from the study will have no bearing on the medical care received

iv) once taken, all samples will be made anonymous, and we will not be able to differentiate the results of their analysis from others in the study.

Chest infections cause frequent and serious complications for people with Chronic Obstructive Airways Disease (COAD, chronic bronchitis and emphysema), resulting in worsening breathlessness, wheeziness and often requiring hospital admission. The types of bacteria causing chest infections in patients with severe COAD are different to those that commonly effect healthy individuals, and frequently cause more serious illness. The reason for this change are not fully understood but one factor may be a special protein, called beta-defensin 2, made by the lining cells of the nose, throat and lungs which can kill these harmful bacteria. This study will investigate whether patients with severe COAD have less of this anti-bacterial protein therefore making them more susceptible to particular types of harmful infections.
In order to do this, we wish to take samples of the epithelial cells lining the nose from patients with severe COPD. We will be looking at some patients whose disease is currently stable, and others during an episode of infection. Collection of nasal cells involves pre-treating the nose with a local anaesthetic and brushing the inside of the nose with a special brush. This takes approximately 1 minute. The procedure may cause some slight discomfort and watering of the eye, and very occasionally there is slight bleeding. Participation in the study will not affect any clinical decisions regarding your medical care. After collection of samples, all further analyses will be done in a “test tube”. The samples are labelled so that the identity of the patient is not known. Furthermore, you will not be informed of the results.

Prior to brushing the nose, you may be asked for a simple blood test to check your blood clotting capacity. This will not be necessary if this test has been done within the previous six months and results were satisfactory. Blood (2 x 3.5 ml tubes) will be collected from the forearm using the vacutainer system, exactly as per a standard blood test. There is a small risk of bruising associated with collecting blood.

If you have any questions regarding taking part in this study, you should contact either of the principal investigators, Dr Ruth MacRedmond (Tel 8093801) or Prof. Shane O’Neill (Tel 8093516), in the Department of Respiratory Medicine, Beaumont Hospital, Dublin 9. We suggest you retain a copy of this document for your later reference and personal records.
Consent for Patient:

I have read the explanation of this study and have been given the opportunity to discuss it and ask questions. I hereby consent to participate in the study.

__________________________________    _________________________
Signature of Patient                  Date

__________________________________    _________________________
Signature of Investigator              Date
5.4 Results and Figures

Demographics of patient population

The demographics of the study population are shown in Figure 5.1. There is no significant difference in the characteristics of the COPD subgroups in terms of age, gender or medication use. All COPD subjects were using inhaled LABA and corticosteroids. COPD patients were on average a decade older than the control subjects. There was difficulty in recruiting a population of “normal” older smokers, that is, smokers who had no history of respiratory disease and normal FEV1. The main objective of the study was to observe differences in between COPD patients of different degrees of severity. Observed differences with control groups represent a “real world” differences between typical subjects with this condition and healthy control subjects.
Figure 5.1

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>F:M</th>
<th>Current smokers</th>
<th>FEV1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Non-smoker</td>
<td>55.2 (44-66)</td>
<td>5:4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Control smoker</td>
<td>46.25 (24-62)</td>
<td>3:1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>COPD FEV1 &gt;1</td>
<td>68.78 (44-75)</td>
<td>5:7</td>
<td>8</td>
<td>1.45 +/- 0.37</td>
</tr>
<tr>
<td>COPD FEV1 &lt;1</td>
<td>65.4 (55-75)</td>
<td>6:4</td>
<td>1</td>
<td>0.77 +/- 0.12</td>
</tr>
</tbody>
</table>

Demographics of patient populations.
Expression of TLR2, TLR4 and HBD2 is comparable between smokers and non-smokers in COPD.

Looking first at the COPD group, there are no significant differences between mRNA expression of TLR4, TLR2 or HBD2 in nasal epithelium between smokers and non-smokers in either the severe (FEV1 <1L) or less severe (FEV1 >1L) groups. Smokers and non-smokers were therefore grouped together for further analysis.
Figure 5.2 Expression of TLR2, TLR4 and HBD2 are not significantly different between smokers and non-smokers.

Following informed consent, nasal epithelial cells were harvested by brush technique from patients with COPD, as described in the methods. Cytospins were prepared from each specimen and stained with Diff-Quick to determine cell populations. Only samples containing >80% epithelial cells were accepted for further analysis. Cells were washed three times in ice cold PBS prior to resuspension in TriReagent for RNA extraction. Total RNA was reverse transcribed into cDNA and used as a template for semi-quantitative PCR reactions using TLR4, TLR2, HBD2 and GAPDH gene-specific primers. Results were grouped according to severity of disease and current smoking status.
Expression of TLR4 and HBD2 is upregulated in COPD, and decreased in more severe disease.

We next compared expression of TLR4, TLR2 and HBD2 in the two COPD groups and normal controls. Differences between smokers and non-smokers in the control group have been discussed previously. There was significant upregulation of TLR4 expression in mild to moderate COPD compared to controls (P < 0.005), while severe disease was associated with a significant reduction in TLR4 expression compared to less severe disease (P < 0.05). There was no difference in TLR2 expression between the study groups. Changes in HBD2 expression mirrored those of TLR4, with significant upregulation in mild-moderate COPD compared to controls (P < 0.005), and reduced expression in severe COPD compared to mild-moderate disease (P < 0.05). HBD2 expression in severe COPD was statistically similar to normal controls. (P = 0.17).
Figure 5.3

A

B

C

HBD2/GAPDH

TLR2/GAPDH

TLR4/GAPDH

Normal NS

Normal S

FEV1 >1L

FEV1 <1L

**

*

Figure 5.3
Figure 5.3 TLR4 and HBD2 expression is reduced in severe COPD

Following informed consent, nasal epithelial cells were harvested by brush technique from normal controls and patients with COPD, as described in the methods. Cytospins were prepared from each specimen and stained with Diff-Quick to determine cell populations. Only samples containing >80% epithelial cells were accepted for further analysis. Cells were washed three times in ice cold PBS prior to resuspension in TriReagent for RNA extraction. Total RNA was reverse transcribed into cDNA and used as a template for semi-quantitative PCR reactions using TLR4, TLR2, HBD2 and GAPDH gene-specific primers.
Nasal expression of TLR4 correlated with tracheo-bronchial expression in vivo

In order to see if nasal expression of TLR4 could be extrapolated to expression in the lower respiratory tract, a subgroup of COPD patients underwent bronchoscopy and brush sampling of the tracheo-bronchial epithelium as well as nasal brushing. Data from each of nine subjects is presented in bar chart (Figure 5.4A), showing comparable levels of TLR4 expression at the two sites, and linear regression analysis demonstrates good correlation between upper and lower respiratory tract expression of TLR4 mRNA (Figure 5.4B).
Figure 5.4

A

B

TLR4/GAPDH

Nasal
Tracheal

\[ r^2 = 0.76 \]
Figure 5.4 Nasal expression of TLR4 correlates with tracheo-bronchial expression in COPD patients in vivo.

Following informed consent, nasal and tracheal epithelial cells were harvested from stable COPD patients by brushing technique as described in the methods. Cytospins were prepared from each specimen and stained with Diff-Quick to determine cell populations. Only samples containing >80% epithelial cells were accepted for further analysis. Cells were washed three times in ice cold PBS prior to resuspension in TriReagent for RNA extraction. Total RNA was reverse transcribed into cDNA and used as a template for semi-quantitative PCR reactions using TLR4, and GAPDH gene-specific primers.

(A) Semi-quantitative measurement of TLR4 expression in nasal and tracheo-bronchial epithelium for each of nine individuals.

(B) Linear regression of tracheal vs nasal expression of TLR4
IL-8 levels are increased in nasal lavage in COPD.

We next looked at IL-8 levels in nasal lavage as a surrogate of airway inflammation (Figure 5.5). Consistent with reports of increased IL-8 levels in induced sputum and BAL in COPD (Keatings, Collins et al. 1996; Soler, Ewig et al. 1999; Rutgers, Timens et al. 2000), I demonstrate increased IL-8 in the nasal lavage from these patients compared to controls. Somewhat surprisingly, the levels of IL-8 were higher in the nasal mucosa of patients with mild-moderate COPD compared to severe disease.
Figure 5.5 IL-8 levels in nasal lavage

Nasal lavage specimens were collected from controls and COPD patients. 10mls warmed 0.9% saline was instilled into the nose and forcibly expelled into a 50ml tube. The recovered fluid was centrifuged and the supernatant filtered prior to storage at -80°C for batch analysis. IL-8 ELISA was performed according to manufacturers instructions (R&D Systems).
5.5 Discussion

The hypothesis of this study was that the observed increase in colonisation with and exacerbations due to Gram-negative pathogens in severe COPD might be associated with changes expression of TLR4, the principal pattern recognition receptor for LPS, in respiratory epithelium. Using a brush sampling technique, I demonstrate that levels of TLR4 expression in nasal epithelium of subjects with severe COPD are significantly reduced compared to less severe disease. HBD2 expression, which is induced by LPS via TLR4, is similarly down regulated in severe COPD. No changes were observed in the expression of TLR2, which principally signals the response to Gram-positive PRRs. I further demonstrate excellent correlation between upper and lower respiratory tract in terms of TLR4 expression, while levels of the inflammatory cytokine IL-8 confirm nasal inflammation similar to that observed in the lower respiratory tract in COPD.

The first interesting finding of this study was that TLR4 expression in nasal mucosa was increased in subjects with mild-moderate COPD compared to normal controls. A similar upregulation in TLR4 expression has been observed in the intestinal mucosa of patients with both active and inactive inflammatory bowel disease (Cario and Podolsky 2000). The fact that TLR2 expression is not changed suggests that this is not a non-specific response to airway inflammation.

Modulation of TLR4 expression in macrophages and peripheral blood mononuclear cells by various cytokines, including IL-2 and IL-4 (Mita, Dobashi et al. 2002), MIF (Roger, David et al. 2001) and IFN-γ (Bosisio, Polentarutti et al. 2002) has been described. There is little existing data regarding the transcriptional regulation of TLRs in epithelial cells, although IFN-γ and TNFα have been shown
to modulate TLR4 expression and function in human intestinal epithelium (Abreu, Thomas et al. 2003; Suzuki, Hisamatsu et al. 2003). The inflammatory milieu in the airways in COPD includes many potential modulators of TLR4. These include cytokines, acute phase reactants such as HSP60 (Ohashi, Burkart et al. 2000) and Fibronectin (Okamura, Watari et al. 2001) which are endogenous ligands of TLR4, proteases including neutrophil elastase (NE) which both activates and downregulates TLR4 in bronchial epithelium (Devaney, Greene et al. 2003), and anti-proteases such as secretory leukoprotease inhibitor (SLPI) which has been shown to inhibit TLR2 and TLR4 function in monocytes (Taggart, Greene et al. 2002; Greene, McElvaney et al. 2004), and microbial organisms.

Chronic or repeated exposure of mononuclear cells to LPS is known to result in a blunted cytokine response to LPS, a phenomenon known as endotoxin tolerance. Mechanisms include downregulation of TLR4 expression (Nomura, Akashi et al. 2000), although inhibition of TLR4 signalling by effects on IRAK and NF-κB may be more important (Medvedev, Lentschat et al. 2002; Dobrovolskaia, Medvedev et al. 2003). TLR4 expression on human gingival fibroblasts is downregulated by Porphyromonas gingivalis LPS (Wang, Oido-Mori et al. 2001). Whether the reduced expression of TLR4 expression in severe COPD is an adaptive response to increased exposure to Gram-negative pathogens, an attempt to attenuate ongoing LPS induced airway inflammation, or pre-exists and thus promotes colonisation is not clear.

HBD2 levels were also increased in COPD patients. Dauletbaev and colleagues found increased HBD2 in the nasal mucosa of normal subjects with an acute cold, and levels correlated well with levels of the inflammatory cytokine IL-8.
Interestingly, HBD2 levels were not elevated, and did not correlate with IL-8, in cystic fibrosis patients (Dauletbaev, Gropp et al. 2002), suggesting another dysregulated response to airway inflammation in these patients. Whether the observed increase in COPD patients is due to the increase in TLR4 expression, rendering the epithelium more responsive to LPS, to an increase in inflammatory inducers of HBD2 such as TNFα and IL-1β (Chung 2001), or to other effects on HBD2 transcription is not clear. Increased expression of HBD2 may represent an appropriate immune response whereby the host attempts to defend the inflamed and disrupted epithelium from infection. This response is lost as COPD progresses to more severe disease, and Gram-negative pathogens become increasingly important players in infective exacerbations. Indeed, airway colonisation with Pseudomonas, which is a prominent feature of cystic fibrosis, is infrequent in COPD, but is significantly more common in more severe disease (Lieberman 2003).

The link between endotoxin and atopic asthma is well established though incompletely understood. Epidemiological studies indicate that that early exposure to LPS may protect against development of asthma (Gereda, Leung et al. 2000; Braun-Fahrländer, Riedler et al. 2002), and the proposed mechanism is dendritic cell induced polarisation of T-helper cell maturation towards a Th1 phenotype (Schnare, Barton et al. 2001). Both IL-12 dependent (Schnare, Barton et al. 2001) and independent (Kuipers, Hijdra et al. 2003) mechanisms have been described. The relationship is far from simple, however, and the timing (Langenkamp, Messi et al. 2000) and dose (Eisenbarth, Piggott et al. 2002) of LPS exposure may be crucial in determining ultimate Th cell polarisation,
explaining some of the apparently conflicting epidemiological data in the literature. Genetic studies support the association. In a study of school-age children in Sweden, the TLR4 (Asp299Gly) polymorphism was associated with a 4-fold higher prevalence of asthma in school-aged children (adjusted odds ratio 4.5, 95% CI 1.1-17.4) but not to allergic rhinoconjunctivitis (Fageras Bottcher, Hmani-Aifa et al. 2004). A relationship between COPD and LPS/TLR4 has not been established. In a recent study of chronic smokers, the presence of the Asp299Gly TLR4 polymorphism had no significant impact on lung function (measured as forced expiratory volume in 1 second (FEV₁) before and after bronchodilator challenge) (Sabroe, Whyte et al. 2004). Our data identifies for the first time a potential role for the TLR4 in COPD, but whether the observed changes in TLR4 expression are secondary to airway inflammation or are significant to the pathogenesis will require further study.

Studies in asthma indicate that the TLRs may have other roles in airways disease beyond that of host defence. Using a mouse model, Bachar and colleagues demonstrated that TLR2, TLR3 and TLR4 are expressed in the mouse tracheal smooth muscle. Co-stimulation of these receptors with LPS and Poly -I-C results in NF-kappa B- and JNK-mediated transcription of B(1) and B(2) receptor, inducing hyper-responsiveness to bradykinin. Furthermore, this effect was attenuated by dexamethasone (Bachar, Adner et al. 2004). If this effect is replicated in humans, it indicates a mechanism by which respiratory tract infection may cause wheeze, and this effect may be accentuated by increased TLR4 expression in COPD.
Air pollution is recognised as a player in the pathogenesis of COPD (Silverman and Speizer 1996), and is an important precipitant of acute exacerbations. Indeed, the introduction of the ban on bituminous coal in Dublin and the subsequent reduction in airborne particulate matter, was associated with a significant reduction in the number of respiratory deaths, many of which could be attributable to COPD (Clancy, Goodman et al. 2002). There is mounting evidence that air pollution can adversely effect host defence, with NO₂ in particular documented to impair muco-ciliary clearance (Carson, Collier et al. 1993; Helleday, Huberman et al. 1995) and alter the number and function of mast cells, T-cell subsets and alveolar macrophages (Sandstrom, Helleday et al. 1992; Kienast, Knorst et al. 1996). I have shown that cigarette smoke, which also contains NO₂, downregulates TLR4 expression and thus innate immune defences may be similarly affected by environmental air pollution. Also interesting is a recent report of TLR4 mediated cytokine responses to particulate matter in alveolar macrophages, implicating microbial components as important players in stimulating an inflammatory response to air pollutants (Becker, Fenton et al. 2002). Increased expression of TLR4 in mild-moderate COPD may potentiate this inflammatory response, although the functional effect of this upregulation in vivo has not yet been evaluated.

The relationship between COPD exacerbations and viruses may also involve interesting interactions with the TLRs. 30-50% of exacerbations of COPD are temporally related to symptoms of the “common cold” (Seemungal, Donaldson et al. 2000), and two recent studies using PCR techniques reported respiratory virus infection, most commonly RSV, in >40% of acute exacerbations of COPD (Bandi, Jakubowycz et al. 2003; Rohde, Wiehege et al. 2003). While TLR3 is the
important TLR signalling inflammatory responses to viral ssRNA, it has recently been shown that RSV upregulates TLR4 expression and enhances LPS responsiveness in respiratory epithelium (Monick, Yarovinsky et al. 2003). Viral infection in severe COPD may in fact “sensitise” the airways to the inflammatory effects of inhaled or colonising Gram-negative bacteria by upregulation of TLR4.

Nasal brush sampling was used for the majority of this study due to the relative ease and high tolerance for this procedure compared to fibreoptic bronchoscopy and brushing of the lower respiratory tract. It was hoped that the respiratory epithelium of the nose would be involved in the same inflammatory processes involving the lower respiratory tract in COPD. This theory is borne out by a recent study of nasal and bronchial biopsies in COPD patients, which showed similar inflammatory changes of squamous metaplasia and neutrophilic inflammation at both sites (Vachier, Vignola et al. 2004). The finding of a high degree of correlation between TLR4 expression in the upper and lower respiratory tract in our study is consistent with this homogeneity throughout the respiratory epithelium, as is the finding of high IL-8 levels in nasal lavage. This allows us not only to extrapolate our findings to the lower respiratory tract, but reinforces the suitability of the nose as a valid and easily accessible in-vivo model for the study of immune processes in COPD.

This study provides evidence for the first time of altered innate immune characteristics in the respiratory tract epithelium of patients with severe COPD, which corresponds to the known increase in Gram-negative colonisation and infection in these patients. Furthermore, I found that there was good correlation between nasal and tracheo-bronchial epithelial expression of TLR4 in COPD.
patients, establishing nasal epithelial sampling as a valid and useful model for the study of innate immunity in respiratory epithelium in vivo. An appreciation of the changes in host defence in COPD enhances our understanding of the pathophysiology of this complex disease. Future therapies aimed at augmenting host defence may result in multiple benefits in terms of airway inflammation, exacerbation rates and disease progression.
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References


(1987). "Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986." Am Rev Respir Dis 136(1): 225-44.


Bandi, V., M. Jakubowycz, et al. (2003). "Infectious exacerbations of chronic obstructive pulmonary disease associated with respiratory viruses and non-


intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex."


