TOLL-LIKE RECEPTOR INTERACTIONS AND THEIR CONTRIBUTION TO AIRWAY INFLAMMATION

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Abstract

Toll-like receptors (TLRs) are key components of the innate immune system involved in surveillance and early detection of infection, as well as coordination of the subsequent adaptive immune response. Pulmonary immune responses are initiated by TLRs expressed on airway epithelial cells. In this thesis, primary normal human bronchial epithelial (NHBE) cells, immortalized human bronchial epithelial (HBE) cells, a glandular lung adenocarcinoma cell line (Calu-3) and an alveolar epithelial adenocarcinoma cell line expressing NF-κB (A549/NF-κB-luc) were analyzed for expression of mRNAs for TLRs, co-receptors, adaptor proteins and non-TLR pathogen recognition receptors (PRRs). Quantitative RT-PCR analysis in Calu-3 and NHBE/HBE cells revealed mRNA expression of only TLRs 1-6, with TLR4/TLR6 having the lowest abundance and TLR3 exhibiting the highest level of expression. Conversely, in A549/NF-κB-luc cells mRNA expression of all TLRs 1-10 was detectable; with TLR5 mRNA expressed at the highest level.

Poly i/c, a synthetic viral dsRNA analogue, elicited the greatest effect on expression of TLR subtypes. The effect was far more pronounced in NHBE/HBE cells as compared to Calu-3 and A549-NF-κB-luc cell lines. Poly i/c increased TLR2 mRNA expression by over sixty-fold and TLR3 by eleven-fold in NHBE cells, whereas TLR5 was significantly reduced. TLR2 protein was also enhanced by Poly i/c, but not by the TLR2 ligand, PAM_3CSK_4. Additionally, Poly i/c enhanced mRNA expression of adaptor molecules (MyD88, TIRAP, and TRIF)

and co-receptors (Dectin-1, CD14) involved in TLR2-signaling. In contrast, mRNA and protein expression of co-receptor CD36 was significantly reduced by Poly i/c. Overall, Poly i/c activation of NHBE cells differentially regulated expression of multiple TLRs, non-TLR receptors, adaptor proteins and co-receptors.

ELISA analysis of apical and basolateral solutions from Poly i/c-stimulated NHBE monolayers revealed significantly higher levels of IL-6 and GM-CSF. After priming with Poly i/c, an increase in IL-6 secretion was observed in cells stimulated with PAM_3CSK_4 and with Alternaria extract, a fungal allergen known to signal partly through TLR2. However, IL-6 secretion was not stimulated by other TLR2 ligands, zymosan or lipothechoic acid (LTA). Pretreatment with anti-TLR2 blocking antibody inhibited the PAM_3CSK_4-induced increase in IL-6 secretion after Poly i/c exposure. Up-regulation of TLR2 following exposure to dsRNA enhanced functional responses of the airway epithelium to certain (PAM_3CSK_4), but not all (zymosan, LTA) TLR2 ligands. In TLR3-deficient cells, induction of TLR2 mRNA expression by Poly i/c was reduced by thirteen-fold, indicating that Poly i/c-dependent TLR2 induction was TLR3-mediated. Furthermore, Poly i/c-dependent IL-6 secretion was abrogated by 83% in TLR3 knock-down cells. Following priming of NHBE cells with Poly i/c and subsequently with TLR5 ligand flagellin, no reduction in IL-6, GM-CSF, RANTES and IFN-β transcripts was observed. Similarly, IL-6 and GM-CSF protein secretion did not match Poly i/c-dependant down-regulation of TLR5. Using

A549/NFκB-luc cells and wild-type (flagellated) and flagella-mutated *Burkholderia (B.) cenocepacia*, intact flagella were found to be critical for NF-κB activation and enhanced secretion of IL-8.

To elucidate the *in vivo* actions of viral infection on the magnitude of TLR2 expression, and subsequent innate immune response to TLR2 activation, the effects of Poly i/c challenge on the inflammatory response of mouse airways to a selective TLR2 ligand (PAM_3CSK_4) and a common fungal allergen (*Alternaria alternata)* were investigated**.** Poly i/c enhanced mRNA and protein expression of TLR2 in the lung. It also elicited a marked induction of Th1 cytokines. However, priming with Poly i/c abrogated Alternaria-mediated Th2 cytokine production. Inflammatory cell recruitment into the airways, predominantly with neutrophils, was also observed. Taken together, both the *in vitro* and the *in vivo* studies show that activation of TLR3 by Poly i/c alters the inflammatory response of the airways to other microbial ligands, thereby modulating the severity of inflammation. This effect is partly due to changes in the expression of other TLR receptors and their associated co-receptors and adaptor proteins.

TABLE OF CONTENTS

List of Figures

Chapter I

Chapter II

Chapter III

List of Tables

Abbreviations

Adenosine A_2A receptor (A_2AR)
Airway epithelial cells (AECs)
Airway smooth muscle cells (ASMCs)
Alveolar epithelial adenocarcinoma cell line expressing NF-κB (A549/NF-κB-luc)
Aspergillus (A.) fumigatus
Bacterial lipoproteins (BLP)
Bronchoalveolar lavage (BAL)
Bronchoalveolar lavage fluid (BALF)
Bicinchoninic acid (BCA)
Bovine serum albumin (BSA)
Buffer used for lysis of cells and tissues before RNA isolation (Buffer RLT)
Burkholderia (B.) cenocepacia
Caspase recruitment domain-containing protein 9 (CARD9)
CARDIF (CARD adaptor inducing IFN-β)
CD (clusters of differentiation)
Chronic Obstructive Pulmonary Disease (COPD),
C_T (cycle threshold)
Cystic Fibrosis (CF)
Double-stranded ribonucleic acid (dsRNA)
Dipeptidyl-peptidase 10 (DPP10)
Dulbecco’s Modified Eagle’s Medium (DMEM)
Ethylenediaminetetraacetic acid (EDTA)
Enzyme-linked immunosorbent assay (ELISA)
Epithelial-derived neutrophil attractant (ENA)
E-twenty six (ETS) is families of transcription factors unique to metazoans
Figure (Fig.)
FliI is membrane ATPase underlying the assembly of the bacterial flagellum
Fluorescence-activated cell sorting (FACS)
Granulocyte colony-stimulating factor (G-CSF)
Glandular lung adenocarcinoma cell line (Calu-3)
Glycosylphosphatidylinositol (GPI)

Granulocyte macrophage colony-stimulating factor (GM-CSF)
G protein-coupled receptor (GPCR)
G protein-coupled receptor 154 (GPRA/GPR154)
Green Fluorescent Protein (GFP)
Growth-related oncogene-α (GRO-α),
Hank's balanced saline solution (HBSS)
Heat shock protein (Hsp)
Human bronchial epithelial cells, transformed by SV40 T-antigen (BEAS-2B cells)
Human leukocyte antigen G (HLA-G)
Interferon beta (IFN-β)
Immunoglobulin E (IgE)
Immunoglobulin G conjugated to horseradish peroxidase (IgG-HRP)
IκB kinase kinase (IKK) complex
IL (Interleukin)
IL-1R-associated protein kinase 1 (IRAK-1)
Immortalized human bronchial epithelial (HBE) cells (HBEC)
Influenza A virus (IAV)
Institutional Animal Care and Use Committee (IACUC)
Interferon (IFN) regulatory factor 3 (IRF3)
Interleukin (IL)
Intestinal epithelial cell line 6 (IEC-6)
IPS-1 (IFN-β promoter stimulator protein 1)
Leucine-rich repeat (LRR) domain
Leukotriene B4 (LTB4),
Lipopeptides (LP)
Lipopolysaccharide (LPS)
Lipothechoic acid (LTA)
LPS-binding proteins (LBP)
Luria broth (LB)
Radioimmunoprecipitation assay (RIPA) buffer

MAVS (Mitochondrial antiviral signalling protein)
Monocyte chemotactic protein-1 (MCP-1)
Monocyte chemotactic protein-2 (MCP-2)
Mean fluorescence intensity (MFI)
Melanoma differentiation-associated gene 5 (MDA5),
Mitogen-activated protein

kinases (MAPKs)

Messenger ribonucleic acid (mRNA)

Mycoplasmal lipopeptide 2 (MALP-2)

Myeloid differentiating factor-2 (MD-2)

Myeloid differentiation primary response gene 88 (MyD88)

MyD88 adapter-like (Mal)

Nuclear factor kappa B (NF-κB)

Normal human bronchial epithelial (NHBE) cells

N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-[R]-Cys-[S]-Ser-[S]-Lys$_4$ Trihydrochloride (PAM$_3$CSK$_4$), a synthetic bacterial lipopeptide

Outer-surface protein A (OspA)

PAR2 activating peptide (PAR2-AP)

Pathogen recognition receptors (PRRs)

Pathogen-associated molecular patterns (PAMPs)

Protocadherin 1 (PCDH-1)

Phosphate-buffered saline (PBS)

Phycoerythrin (PE)

Polyinosinic polycytidylic acid (Poly i/c, PIC, P)

Polyvinylidene difluoride membrane (PVDF)

Protease activated receptors (PARs)

Protein kinase R (PKR)

Purinergic (P2Y) receptors

Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR)

Regulated on Activation Normal T Cell Expressed and Secreted (RANTES)

Reactive oxygen species (ROS)

Respiratory syncitial virus (RSV)

Respiratory viral infections (RVIs)

Retinoic acid inducible gene I (RIG-I),

Rhinovirus (RV)

Retinoic acid inducible gene I-like-receptors (RLRs)

Scavenger receptors (SR)

SEM (Standard error of mean)

MicroRNA-adapted short hairpin ribonucleic acid (shRNAmir)

Small airway epithelial cells (SAEC)

Serine protease

inhibitor Kazal-type 5 (SPINK5)

Sarcoma (Src) and Spleen tyrosine kinase (Syk) kinases

Signal Transducers and Activators of Transcription (Stat)-1

Sterile and armadillo motifs (SARM)

TAK-1 binding proteins 1 and 2 (TBP-1 and TBP-2)

T helper (Th) 1

T helper (Th) 2

TIR-containing adapter molecule 1 (TICAM-1),

TIR-containing adapter molecule 2 (TICAM-2)

Toll and interleukin-1 receptor (TIR) domain

Toll/IL-1 receptor (TIR) domain-containing adapter protein (TIRAP),

Toll-like receptors (TLRs)

Transforming growth factor-β-activated kinase (TAK-1)

TIR-domain-containing adapter-inducing interferon-β (TRIF)

TRIF-related adapter molecule (TRAM)

Tumor necrosis factor (TNF)

Tumor necrosis factor receptor-associated factor 6 (TRAF6)

Vascular endothelial growth factor (VEGF)

Virus-induced signaling adaptor (VISA)

Wild type (WT)

Chapter 1: Literature Review

1. 1. Background and significance of inflammatory airway diseases

Airway inflammatory diseases including allergic rhinitis, asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) are prevalent with complex etiology (Hele DJ and Belvisi MG 2003). These chronic airway diseases, particularly asthma, COPD, and CF have different causes but share some common features in pathologic changes and clinical syndrome. They are characterized by chronic mucosal and airway inflammation with an increase in leukocyte infiltration and a variety of inflammatory mediators (Xiao W et al. 2005). The pathologic processes in these diseases also involve airway obstruction, at least in part because of mucin overproduction. Furthermore, tissue remodeling and reduction in expiratory air flow rates are other manifestations of airway inflammation (Xiao W et al. 2005, Patel, Brett and Holtzmann 2009). Identifying the different inflammatory/immune reactions and relating them to clinical phenotypes of the various airways diseases is of paramount importance in designing therapies against these diseases.

Annually, asthma affects about 17 million Americans (6.4 % of the total U.S. population) and claims about 5000 lives/yr (NIAID 2001). Twelve million Americans are diagnosed every year with COPD and at least equal numbers have symptoms but are not diagnosed. COPD is the fourth leading cause of death in America, behind heart diseases, cancer and cerebrovascular disease (stroke) and is projected to become the third leading cause of death by the year 2020 in the US and worldwide (Hurd S 2000).

Although this steady increase in morbidity from asthma and COPD presents a large economic burden from high health care costs and loss of productivity, the reasons for the striking increase in these diseases are still unclear. The pathophysiological mechanisms involved in their development are not completely understood; however, the principal mechanism involves an underlying inflammatory component (Douwes J et al. 2002). A common feature of lung inflammation is the development of an inflammatory–immune response that involves activation of many cell types including epithelial cells and other resident (macrophages) or recruited immune cells (neutrophils, eosinophils, monocytes and lymphocytes). The airway epithelium represents an important initial site of contact with airway pathogens leading to activation of innate immune responses that involve release of cytokines and chemokines with the subsequent recruitment of immune cells to the site of infection. A thorough understanding of the inflammatory processes inherent in these diseases should provide opportunities for innovative drug development and treatment of the underlying inflammation that characterizes their pathology.

1. 2. Role of airway epithelium in inflammatory airway diseases

The lung, with a surface area of 150 m^2, ventilates 10,000-20,000 liters (20 kg) of air daily. Thus, the lung is exposed to potentially harmful particles including allergens, and infectious agents (viruses, bacteria and fungi) (Bals R and Hiemstra PS 2004). Airway epithelial cells (AECs) are the first line of defense against these harmful intruders, serving as a passive physical barrier and providing mucociliary clearance (Abraham CM et al. 2007, Mall MA 2008, Wang Y et al. 2008). AECs are a polarized pseudostratified layer of cells with distinct luminal and basolateral membranes composed of different phospholipids which contain distinct receptors and transport proteins.

Airway epithelial cells are not simply a passive barrier to infectious agents as previously thought, but are now well recognized as important active orchestrators of airway inflammatory disease (Kunzelmann K and McMorran B 2004, Holgate ST 2004). AECs contribute to the inflammatory milieu by producing biologically active compounds, including lipid mediators, growth factors, costimulatory molecules, complement, acute phase proteins, endothelin, and a variety of cytokines/chemokines that are important in the pathogenesis of airway disorders (Bals R and Hiemstra PS 2004, Cook DN et al. 2004, Hertz CJ et al. 2001, Qureshi ST and Medzhtov R 2003, Schleimer RP 2004). The secretions of these cells also modulate the recruitment and activation of inflammatory cells, which in turn, stimulate the adaptive immune response. AECs also secrete chemical products that directly kill or inhibit replication of pathogens and also take part in airway mucosal immunity and remodeling, which are critical in resolving chronic airway inflammatory processes (Polito AJ and Proud D 1998, Takizawa H 1998, Holgate ST 2004, 2007, Simmons C and Farrar J 2008).

Studies have shown that impaired barrier function of AECs is fundamental to the origin and development of inflammatory airway disease (Holgate ST 2004).

For example, recent studies found novel asthma susceptible genes (e.g. DPP10, GPRA, HLA-G, ETS-2, ETS-3, PCDH-1, filaggrin and SPINK5) to be preferentially expressed in the airway epithelium (Holgate ST et al. 2007). In another study, Shornick and co-workers provided the most definitive evidence for the direct role of barrier function afforded by airway epithelial cells in innate immunity against viral infection. They showed that a prominent early event in respiratory viral infection using a mouse paramyxovirus (Sendai virus) model is activation of the IFN-signaling protein Stat-1 in airway epithelial cells (Shornick LP et al. 2008).

The inflammatory and innate immune responses of the airway epithelium depend on the engagement of epithelial cell surface, or cytosolic molecules, collectively known as pattern recognition receptors (PRRs). These PRRs detect specific microbial products referred to as pathogen-associated molecular patterns (PAMPs) (Polito AJ and Proud D 1998, Muir A et al. 2004, Kunzelmann K and McMorran B 2004). The best characterized PRRs are Toll-like receptors (TLRs). TLRs represent a major class of PRRs expressed by airway epithelial cells that initiate and sustain airway inflammation in diseases such as asthma, CF and COPD (Muir A et al. 2004, Murray CS 2004, Tan WC 2005, Wilkinson TM et al. 2006, Zeldin DC et al. 2006). It has now been recognized that the profound immune and inflammatory response triggered in these inflammatory airway diseases is dependent upon complex patterns of TLR cross-talk. Cross-talk of these receptors occur between TLRs within each cell type (e.g. airway epithelial cells) and/or among co-operative TLR responses between cells (epithelial cells, endothelial cells smooth muscle cells in the airway) as well as recruited professional immune cells (neutrophils, eosinophils, monocytes and lymphocytes) (Parker LC et al. 2007). An understanding of the mechanisms by which the innate immune system contributes to the development and regulation of airway inflammation should provide novel approaches for the diagnosis and treatment of airway inflammatory diseases.

1. 3. Toll-like receptor (TLR) structure and mechanisms of post-receptor signaling

Mammalian TLRs were originally discovered on the basis of their homology with TOLL; a receptor first identified as crucial for the dorso-ventral orientation of the developing fruit fly, Drosophila melanogaster, and later shown to confer immunity against fungal infections (Akira S et al. 2003, Lemaitre B 1996, Medzhitov R and Janeway CA Jr 1997). So far, 13 of these PRRs, TLR1 through TLR13, have been identified—10 in human and 12 in mice (Beutler B 2004). TLRs are type I transmembrane proteins with an extracellular domain which consists of a 19-25 leucine-rich repeat (LRR) domain and is involved in the recognition of a variety of pathogens. Showing a high degree of similarity to the interleukin (IL)-1 receptor family, TLRs also have a conserved cytoplasmic sequence of about 200 amino acids known as the Toll and interleukin-1 receptor (TIR) domain, which is crucial for receptor signaling (Akira S 2003, Akira S and Takeda K 2004). The role of the TIR domain as the initiator of TLR signaling pathway was first revealed in the C3H/HeJ mouse strain that had a point mutation that resulted in an amino acid change of the proline residue at position

712 to histidine (Poltorak A et al. 1998, Hoshino K et al. 1999). With exception of TLR3, this proline residue in the TIR domain is conserved among all TLRs, and its substitution to histidine causes a dominant negative effect on TLR-mediated signaling (Hoshino K et al. 1999, Underhill DM et al. 1999). This highly conserved proline residue within the TIR domain of TLR3 is uniquely replaced with alanine.

TLRs recognize and mediate intracellular signals for a wide range of microbial components: TLR1 (in association with TLR2) for tri-acyl lipopeptides; TLR2 for bacterial lipoproteins (BLP) and peptidoglycans from Gram-positive bacteria; TLR3 for double-stranded RNA; TLR4 for bacterial lipopolysaccharide (LPS); TLR5 for flagellin; TLR6 (in association with TLR2) for di-acyl lipopeptides; TLR7 and TLR8 for single-stranded RNA and TLR9 for non-methylated CpG DNA. TLR7 as well as human TLR8 (excluding mouse TLR8) recognize several synthetic immune response modifier compounds that are structurally related to nucleic acids, including imidazoquinolines and resiquimod. Viral envelope glycoproteins, glycolipids, capsid and nuclear proteins are also sensed by cell surface receptors, including TLR2 (Measles Virus H-protein) (Bieback K et al. 2002), TLR2/CD14 (Human Cytomegalo Virus) (Compton T et al. 2003), TLR4 (Mouse mammary tumor virus) (Rassa JC et al. 2002), TLR4/CD14 (RSV F-protein) (Kurt-Jones EA et al. 2000). Besides exogenous ligands such as PAMPs, TLRs are also able to recognize endogenous ligands including damage-associated molecular patterns and extracellular matrix molecules such as hyaluronan, heat-shock proteins (Hsps), and fibronectin (Gomariz RP et al. 2007). Moreover, TLR3 has been shown to recognize endogenous ligands such as mRNA released from necrotic cells (Kariko et al. 2004) suggesting that necrotic cell death at inflammation sites may contribute to activation of TLR3.

Diverse molecular and biochemical events are initiated during activation of TLRs with their specific ligands (O’Neil NA 2006, Kawai T and Akira S 2006). After ligation of TLRs by their ligands, downstream responses are tailored through a family of four TIR-domain-containing adapter molecules, comprising myeloid differentiation primary response gene 88 (MyD88), MyD88 adapter-like (Mal) also known as Toll/IL-1 receptor (TIR) domain-containing adapter protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) also known as TIR-containing adapter molecule 1 (TICAM-1), and TRIF-related adapter molecule (TRAM) also known as TIR-containing adapter molecule 2 (TICAM-2) (McGettrick AF and O'Neill 2004, Parker LC et al. 2007). A new member of the same family of adaptor protein, sterile and armadillo motifs (SARM), has also been described, but its signaling role has not yet been clearly identified (O’Neil NA 2006).

It has been clearly established that TIR domain-containing adaptors provide specificity for individual TLR-mediated signaling pathways (Takeda K and Akira S 2004). Following ligation of TLRs with their cognate ligands, docking platforms that aid in the recruitment of TIR domain-containing adaptor proteins are formed within the intra-cytoplasmic TIR domains of TLRs (Rallabhandi P et al. 2008). The only TLR that is capable of recruiting all adaptor proteins through TIR-TIR interaction is TLR4, the first mammalian homologue of Drosophila Toll to be discovered (Medzhitov R et al. 1997, Rallabhandi P et al. 2008). All TLRs, except TLR3, share a TIR domain containing common adaptor molecule, MyD88 (Akira S 2003). Besides MyD88, Mal/TIRAP is required for TLR2- and TLR4-dependent downstream signaling; while TRIF/TICAM-1 is essential for TLR3 as well as the MyD88-independent TLR4 signaling (Akira S 2003). TRAM appears to act as a bridging adapter connecting TLR4 and TRIF (McGettrick AF and O'Neill LA 2006).

For most TLR subtypes, MyD88 is recruited to the receptor complex after ligand binding, followed by IL-1R-associated protein kinase 1 (IRAK-1), IRAK-4 and tumor necrosis factor receptor-associated factor 6 (TRAF6). IRAK-1 and TRAF6 then dissociate from this complex and associate with another complex composed of transforming growth factor-β-activated kinase (TAK-1) and TAK-1 binding proteins 1 and 2 (TBP-1 and TBP-2). TAK-1 is then stimulated, which in turn activates the IκB kinase kinase (IKK) complex. IKK-mediated phosphorylation of IκB leads to its ubiquitination and degradation, thereby unmasking the nuclear localization domain of Nuclear Factor kappa B (NF-κB) that translocates into the nucleus. Mal also works together with MyD88 leading to early activation of transcription factors, NF-κB and mitogen-activated protein kinases (MAPKs) (Palsson-McDermott EM and O'Neill LA 2004), whereas TRIF and TRAM activate another transcription factor, IFN regulatory factor 3 (IRF3) (Oshiumi H et al. 2003, Fitzgerald KA et al. 2003, Yamamoto M et al. 2003). Early activation of NF-κB and MAPKs leads to induction of cytokines such as TNF, IL-1, and IL-6, whereas activation of IRF-3 leads to the production of cytokines such as IFN-β and RANTES. IRF3 has also been shown to be involved in the late activation of NF-κB and MAPKs in TLR4 signaling leading to the induction of TNF (Covert MW et al. 2005, Werner SL et al. 2005). Furthermore, engagement of selected TLRs, including TLR3, TLR7, TLR8, and TLR9 by various viral components as identified in rhinovirus (RV), RSV, and Influenza virus also result in the production of type I interferon (IFN) as an antiviral response (Mallia P and Johnston SL 2006).

1. 4. Innate immune function of the epithelium: Receptor and cellular interactions

Inflammatory airway diseases cannot be described by any one single causative agent, but rather by the interaction of multiple factors. Exposure of target cells to a single entity can modulate their sensitivity to another agent. A variety of studies indicate that primary respiratory viral infections intensify chronic bacterial inflammatory airway diseases. For example, respiratory viral infections (RVIs) often exacerbate chronic inflammatory airway diseases such as asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) and often precede bacterial pneumonia (LeVine AM et al. 2001, Murray CS et al. 2004, Singh AM and Busse WW 2006, Stark JM 2006, Tan WC 2005, Zeldin DC 2006). Viral replication also impairs specific anti-bacterial defense mechanisms and enhances bacterial adherence, changing inflammatory responses and the clinical features of COPD and CF (Muir A et al. 2004, van Ewijk BE et al. 2005, Wilkinson TMA et al. 2006). Viral predisposition to secondary bacterial infections has also been demonstrated in murine models of sequential pneumonia. Mice exposed to influenza virus or to respiratory syncytial virus (RSV) show enhanced inflammatory responses, and increased bacterial burden following *Streptococcus pneumoniae* exposure, compared to control mice (LeVine AM et al. 2001). In another study, mice exposed to RSV and then challenged with *Streptococcus pneumoniae*, *Staphylococcus aureus* or *Pseudomonas aeruginosa* also exhibited decreased bacterial clearance (Stark JM et al. 2006).

Viral infections also amplify sensitization to allergen, aggravating allergic inflammatory airway responses. This is shown in increased airway responsiveness to methacholine and pulmonary eosinophilic and neutrophilic inflammation of mice sensitized to ovalbumin subsequent to RSV infection. This response is characterized by the predominant production of Th-2-type cytokines (Stark JM et al. 2006). Besides viral infections, a TLR4 agonist, lipopolysaccharides (LPS), has also been shown to have variable effects on allergic airway inflammation in the mouse, depending on the dose and duration of allergen exposure (Eisenbarth SC et al. 2002, Hollingsworth JW et al. 2006).

A growing body of evidence suggests that the progression of chronic inflammatory lung disease is associated with impairment of the innate immune response (Basu S et al. 2004, Fransson M et al. 2005, Muir A et al. 2004, Proud D and Chow CW 2004, Schleimer RP 2004). Various investigators have demonstrated potent amplification of inflammation induced by TLR agonists via cooperative response between distinct cell types; for instance alveolar macrophages and bronchial epithelial cells (Fujii T et al. 2004), airway smooth muscle cells (ASMCs) and mononuclear leukocytes (Peng Q et al. 2004; Morris GE et al. 2005), eosinophils and monocytes (Meerschaert J et al. 2000), PBMC and neutrophils (Prince LR et al. 2004). Exposure of the whole lung to TLR agonists in vivo, such as dsRNA, was also shown to have a proinflammatory effect on multiple cell types, including AECs, ASMCs, macrophages and other cells. Accordingly, exacerbation of airway inflammatory diseases will likely involve simultaneous or subsequent activation of TLRs or other non-TLR PRRs by multiple microbial pathogens in diverse cell types (Fransson M et al. 2005, Proud D and Chow CW 2006, Schleimer RP 2004). This combined activation of different receptors in distinct or multiple cell types can result in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity (Trinchieri G, and Sher A 2007). Deciphering the multiple receptor interactions that detect various microbial components that are synergistically involved in inflammatory airway diseases is crucial for understanding of the role of TLRs in host resistance to infection.

Certain TLRs function as homodimers or heterodimers. TLR2 activity is enhanced by heterodimerization with TLR1 or TLR6, while TLR4 appears to form homodimers (Lee H-K, Dunzendorfer S, and Tobias PS 2004). Important functional interactions of TLRs with non-TLR molecules are also well known, for example, TLR4 interacts with MD-2 and CD14 to sense bacterial LPS (Lee H-K, Dunzendorfer S, and Tobias PS 2004, Mukhopadhyay S et al. 2004). Also, TLR2 has been shown to functionally interact with structurally unrelated receptors like Dectin-1 to recognize fungal products (Tekeda K and Akira S 2005). In a model of asthma induced by *Aspergillus (A.) fumigatus*, IL-18 production was shown to be up-regulated and to interfere with the development of the disease. Depletion of IL-18 exacerbates hyper-responsiveness, which correlates with a decrease in TLR2 expression and retention of the fungus suggesting that TLR2 plays a role in the amelioration of *A. fumigatus*-mediated asthma (Blease K et al. 2001).

Synergistic effects between virus- and allergen-induced airway inflammations have also been proposed (Murray CS, Simpson A and Custovic A 2004), but no definitive molecular mechanism has been described to explain the interactions between viruses and allergens and their respective receptors. Up-regulation of TLR2, 3 and 4 has been reported in the nasal mucosa of patients with allergic rhinitis, indicating the role of multiple TLRs in allergic airway inflammation (Fransson M et al. 2005). TLR2, TLR4 and TLR6 have been implicated in activation of epithelial cells by fungal products (Marr KA et al. 2003). Infection of epithelial cells with RSV has been shown to sensitize cells to dsRNA by up-regulating the expression of its cognate receptor, TLR3 (Groskrutz DJ et al. 2006). Additionally, RSV was shown to increase expression of TLR4 and to increase the sensitivity of the airway epithelium to both LPS and viral motifs, including the F protein of RSV (Jafri HS et al. 2004).

Certain TLR4-polymorphisms were shown to produce impaired innate immune responses to RSV and LPS and are associated in an increased risk of severe RSV bronchiolitis (Tal G et al. 2004). Previous studies in primary small airway epithelial cells (SAECs), airway smooth muscle cells (ASMCs) and thyroid follicular cells showed that activation of TLR3 using ; a synthetic viral double stranded RNA (dsRNA) analogue, polyinosinic polycytidylic acid (Poly i/c) increases mRNA expression of TLR2, TLR3 and suppresses mRNA expression of TLR5, suggesting that viral infection can differentially alter the epithelial sensitivity and response to fungal allergens, bacteria, and other viruses (Hewson CA et al. 2005, Ritter M,et al. 2005 Sukkar MB et al. 2006, Yamazaki K et al. 2007). Abundant expression of TLR3 mRNA was also observed in ex vivo nasal mucosal samples from normal human subjects and rhinovirus challenge resulted in increased mRNA expression of TLR2, TLR3, TLR4, TLR7 and TLR8, but reduced mRNA expression of TLR5 (Avila PC et al. 2005). A recent microarray study examining the transcriptional profile of the lung and bronchial lymph nodes from mice infected with RSV demonstrated that TLR2 and TLR3 were among the set of upregulated genes (Jenssen R et al. 2007).

Viral pathogen-associated molecular patterns trigger multiple signalling cascades through TLR-dependent and TLR-independent pathways, leading to kinase activation through TRAF family members. Following entry by receptor-mediated fusion or endocytosis and subsequent uncoating, transcription and replication of the viral genome nucleic acid can lead to the production of viral RNA with dsRNA character in the infected cell that is sensed by TLR3. Besides TLR3, dsRNA is also recognized by non-TLR cytosolic sensors, including retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), Protein kinase R as well as 2’-5’-oligoadenylate synthetases, which initiates antiviral signaling (Gitlin L et al. 2006, Hornung V et al. 2006, Yoneyama M et al. 2007, Vercammen E et al. 2008**)**. TLR3 and the RIG-I/MDA5 RNA helicases differ in their cellular localizations, ligand specificities, and downstream signaling pathways, which suggests that host cells have multiple defense mechanisms against viral infection (Vercammen E et al. 2008). RIG-I and MDA5 detect viral RNA and activate downstream events by employing an adaptor protein. This adaptor protein was discovered by four independent groups in 2005 and given four different names: IPS-1 (IFN-β promoter stimulator protein 1) (Kawai T et al. 2005), MAVS (mitochondrial antiviral signalling protein) (Seth RB et al. 2005), VISA (virus-induced signalling adaptor) (Xu LG et al. 2005) and Cardif (CARD adaptor inducing IFN-β) (Meylan E et al. 2005). The signaling pathway involves this adaptor protein that interacts with RIG-I and MDA5 via the CARD-like domain, followed by the activation of IRF3 and IRF7 via TBK1- and IKKi-dependent phosphorylation. IPS-1 also activates NF-kB via FADD/RIP1-dependent pathways (Kawai T & Akira S 2006).

Non-traditional PRRs, including scavenger receptors (SR), mannose receptors and β-glucan receptors, recognize ligands directly on microbial surfaces and mediate the engulfment of particulates (Peiser L et al. 2002, Martinez-Pomares L et al. 2001, Herre J et al. 2004). Macrophages also express Fc and complement receptors, which recognize antibody and complement coated particles, respectively (Daeron M 1997, Carroll MC 1998). Apart from PRRs, innate immune cells express surface antigens including Integrins, immunoglobulin superfamily proteins, glycosylphosphatidylinositol (GPI)-anchored proteins and G-protein coupled receptors, which control migration, adhesion, activation or suppression of the cells involved in innate immunity (Hamm HE 1998, Loertscher R and Lavery P 2002, Barclay AN 2003).

Surface antigens, TLRs and other non-TLR PRRs could interact with one another to increase and diversify the recognition and overall handling of microbial infection by the innate immune system. Furthermore, cells of the innate immune system also communicate with one another through soluble mediators like cytokines and chemokines through either cis- or trans-cellular interactions, at the cell-surface or intracellular which can either be contact-dependent or contact-independent (Mukhopadhyay S et al. 2004). Such interactions could augment, inhibit or synergize with the functions of either participating partner (Mukhopadhyay S et al. 2004). Although the majority of the existing data are suggestive of a collaborative interaction between multiple TLRs and other non-TLR PRRs to regulate pulmonary innate immune responses, no studies have elucidated the functional consequence of altered TLR2 and TLR5 expression as a result of TLR3 activation in airway epithelial cells. Therefore, this thesis investigated the functional consequences of changes in TLR2 and TLR5 expression resulting from Poly i/c exposure.

1. 5. TLR regulation of host-defense molecules and their role in airway diseases

When TLRs recognize microbial components, pathways are activated that trigger expression of a variety of genes including those encoding cytokines, chemokines and antimicrobial proteins (Polito AJ and Proud D 1998, Martin TR 2000, Ritter M et al. 2005, Strieter RM et al. 2002). These mediators are essential for mounting innate immune responses against pathogen dissemination and also initiating the development of antigen-specific acquired immunity.

Several investigators have documented distinct patterns of cytokine release from airway epithelial cells from individuals with chronic inflammatory diseases compared with healthy controls (Lordan JL et al. 2002). In the lung, the cytokines and chemokines produced by the activation of TLRs expressed on airway epithelial cells play a critical role in regulating local inflammatory processes (Krakauer T 2002). Although this is the case, the role of TLRs as mediators in chronic airway inflammatory diseases has not been completely elucidated.

When an individual simultaneously suffers from a chronic inflammatory disease (e.g. asthma, COPD, CF) and a viral infection (RV, RSV, Influenza), TLR-activity is altered in a complex way. For example, when asthmatic patients had experimental rhinovirus infection, their sputum showed increased levels of Interleukin (IL)-6, IL-8, and a chemokine known as regulated upon activation, normal T cell expressed and secreted (RANTES) (Grunberg K et al. 1997). In viral exacerbation of asthma, the adaptive immune response is associated with a T-helper (Th) type 2 cytokine profile (IL-4, IL-5, and IL-13). In contrast, in viral exacerbation of COPD, the central components of the increased inflammation are proinflammatory cytokines and neutrophil chemokines including tumor necrosis factor (TNF)-α, IL-8, RANTES, growth-related oncogene-α (GRO-α), and leukotriene B4 (LTB4), epithelial-derived neutrophil attractant (ENA) (Mallia P and Johnston SL 2006). Viral exacerbation of chronic inflammatory airway diseases also induces typical antiviral responses such as type I interferons.

The effector function of innate immunity is also served by other mediators of host defense including the complement cascade, superoxides, nitric oxides, prostaglandins, acute phase proteins, and antimicrobial peptides, which have the ability to render the invading organisms non-pathogenic (Bals R 2002, Sha Q et al. 2004). Antimicrobial peptides such as collectins, lysozyme, lactoferrin, secretory leukocyte protease inhibitor and defensins are expressed in human airways and are involved in the host defense with direct antimicrobial and mediator function (Bals R 2002). Besides localized containment and destruction of microbial agent, it has been recently suggested that antimicrobial peptides also participate in signaling the existence of injury or infection to the adaptive immune system (Salzet M 2002).

In many chronic inflammatory airway diseases including asthma, COPD, chronic rhinosinusitis, and allergic bronchopulmonary aspergillosis, the mechanisms regulating these immune responses are impaired (Martin TR 2000, Schleimer PR 2004). Exacerbations of inflammatory airway diseases are usually triggered by infections of the respiratory tract by viruses, bacteria and fungi (Greenberg SB et al. 2000, Zalacain R et al. 1999, Cohen L and Castro M 2003, Schleimer PR 2004), apparently due to a biased release of inflammatory mediators from activated immune and epithelial cells (Tulic MK et al. 2005, Ritter M et al. 2006). It has been generally recognized that the primary trigger of asthma exacerbation in young children, particularly in the western world, is the lower rate of respiratory infections in their early life (Johnston SL et al. 1995, Liu AH and Redmon AH Jr 2001). Alternatively, recent observations indicate that exposure to fungal agents, either single-handedly or in combination with viral and bacterial infections, exacerbate allergic asthma (Cohen L and Castro M 2003, Bellocchio S et al. 2004, Ritter M et al. 2005) or COPD (Zalacain R et al. 1999, Greenberg SB et al. 2000).

The dynamics and integral role of concomitant infections on the pathophysiology of asthma, COPD or other chronic inflammatory airway disease has yet to be sufficiently characterized. In this thesis, the issue was approached by examining the cytokine profiles of surface bronchial (NHBE), carcinomic human alveolar basal epithelial (A549) cells and carcinomic serous glandular epithelial cells (Calu-3) in response to viral dsRNA analogue; i.e. Poly i/c, bacterial triacylated lipopeptide; i.e. PAM_3CSK_4; LPS, flagellin or flagellated bacteria and fungal components (zymosan, Alternaria alternata extract), as a single entity or in combination. The focal point of the study was assessment of the activation of various TLRs by these diverse microbial components on the cytokine and chemokine secretion profiles. The fact that the future therapies for asthma, COPD or other chronic inflammatory airway diseases will likely involve specific targeting of cytokine and chemokine receptors, rather than global immunosuppression, enhances the potential applications for knowledge derived from this approach.

1. 6. Significance of the study

Respiratory viral infections (RVIs) predispose the airway to secondary bacterial complications and allergen-induced inflammation (LeVine AM et al. 2001, Murray CS et al. 2004. Singh AM and Busse WW 2006, Stark JM et al. 2006, Tan WC 2005, Zeldin DC et al. 2006). RVIs elicit acute exacerbations of chronic airway inflammatory diseases however; the mechanisms that underlie these exacerbations are poorly understood. Complex immune and inflammatory interactions that result from exposure of the host to various TLR ligands have been linked to the development of such chronic airway diseases and this link requires further study (Basu S, Fenton MJ 2004, van Ewijk BE et al. 2005).

The innate immune system has both recognition and effector functions. The recognition function of the airway epithelium is mainly served by Toll-like-receptors (TLRs). TLRs allow for discrimination between arrays of viral, bacterial, and fungal products from host cells (Qureshi ST and Medzhitov R 2003, Martin TR and Frevert CW 2005). In this thesis, expression and functional characterization of TLRs are denoted as coming from NHBE, A549 and Calu-3 cells. NHBE cells, being surface epithelial cells, are regularly exposed to microbial agents. Calu-3 cells, on the other hand, exhibit characteristics of the submucosal glandular epithelium, which significantly contribute to the physical and chemical nature of airway surface fluid (ASF). This ASF is vital for the local host defense of the pulmonary system. A549, an alveolar epithelial carcinoma cell line, which originated from type II pneumocytes and employed by various investigators as established models for studying the role of flagellated bacteria in inflicting human pulmonary diseases, were also used in experiments that involve the flagellated bacterium, Burkholderia cenocepacia and its genetically modified strains (flagella-deprived and flagella-complemented mutant strain). The interactions among various receptor subtypes, particularly TLR2, TLR3, TLR4 and TLR5 that are known to sense viral, bacterial, and fungal components were also investigated. This study focused on characterizing the effects of TLR3 activation on the expression and function of TLR2 and TLR5. This knowledge of cross-talk among TLRs describes the molecular basis of how exposure of the host to one microbial agent predisposes it to subsequent infection with other microbes (e.g. bacteria, fungi).

Despite the advances made in the treatments of asthma, COPD and CF, acute exacerbations remain a major cause of morbidity and mortality (Mallia P and Johnston SL 2006). As our understanding of the interactions of the diverse PRRs on the development of inflammatory airway disease improves, novel interventions designed to modulate the host response to these exposures can be developed and implemented. To that end, one remarkable feature of the TLR family of receptors is that despite the diversity of chemical structures they respond to, significant specificity in agonist-receptor recognition still exists among receptor subtypes. This specificity may likely be the key to development of TLR-selective agonists and antagonists that may be used to modulate TLR activity as part of therapeutic interventions to alleviate chronic inflammation.

1. 7. Objectives, central hypothesis and specific aims

Evidence suggests that individual members of the TLR family and other non-TLR receptors both physically or functionally interact with each other to determine the subsequent nature and outcome of the immune response to a particular pathogen (Mukhopadhyay S et al. 2004). The data on the possible involvement of TLRs in inflammatory airway diseases are very limited and the contribution of TLRs, particularly of the epithelial cells, to the development and/or maintenance of these diseases remains poorly understood. Only a few studies have analyzed modulation of TLR expression by various microbial components, mostly at the mRNA level without analyzing whether modulation of mRNA expression level translates to protein expression and changes in innate immune function of the epithelium. Therefore, systematic identification and characterization of the effects of diverse microbial components on the expression of the various TLRs and non-TLR PRRs at mRNA and protein levels, deciphering their interaction and assessing functional consequences of these interactions will be required to advance our understanding of the role of TLRs in human inflammatory airway diseases.

Objectives: TLR3 and members of the cytoplasmic helicase family of proteins (including RIG-I and MDA5) recognize viral replicative intermediate, dsRNA, or its synthetic analog, Poly i/c (Matsukura S et al. 2007, Sasai M et al. 2006). Although the roles for TLR3, RIG-I and MDA5 in the recognition of several viruses have been investigated, the functional relationships between these dsRNA sensors and other pathogen recognition receptors remain to be determined. Therefore, the experiments presented in this thesis focus on examining the mechanisms of dsRNA-dependent regulation of TLR and non-TLR PRRs in airway epithelial cells and the consequences of these interactions on innate immune function. In agreement with a few previous studies in various cell systems, our data show that stimulation of NHBE cells with Poly i/c increases mRNA and protein expression of TLR2, RIG-I and MDA5 and decreases mRNA expression of TLR5. These observations serve as the basis for the central hypothesis of the thesis:

Central Hypothesis: Double stranded RNA-dependent stimulation of airway epithelial cells alters their sensitivity to TLR2 and TLR5 stimuli and modulates the inflammatory response of the airway epithelium to allergen and bacterial challenge. To address this hypothesis, three specific aims were developed:

Specific Aim 1: Identification and characterization of Toll like receptors (TLRs), associated co-receptors and down-stream signaling molecules expressed in human airway epithelial cells and investigation of the effects of specific TLR ligands on the expression of various TLR and non-TLR receptors.

Specific Aim 2: Investigation of the effects of TLR activation on cytokine secretion and evaluation of the consequences of altered TLR2 and TLR5 expression induced by dsRNA (Poly i/c) on cytokines/chemokines and antimicrobial protein secretion by human airway epithelial cells.

Specific Aim 3: Investigate the in vivo effects of dsRNA (Poly i/c) on airway inflammatory responses to TLR2 selective ligand (PAM_3CSK_4) and Alternaria alternata to assess effects of enhanced TLR2 expression following TLR3 activation.

Chapter 2: Toll like receptors (TLRs) and associated co-receptors and down-stream signaling molecules as well as non-TLR pathogen recognition receptors expressed in human airway epithelial cells and their modulation by specific TLR ligands

2. 1. Introduction

Previous studies have demonstrated the presence of TLR1-6 mRNAs in airway epithelial cells (Fransson M et al. 2005, Mayer AK et al. 2007). Molecular patterns of the pathogens introduced into the airway show some specificity to these receptors. The sensing of these microbial patterns by the receptors leads to epithelial activation, which has been observed in inflammatory airway diseases (Fransson M et al. 2005, Proud D and Chow C-W 2006, Schleimer RP 2004). It has also been recognized that exacerbation of airway inflammatory diseases is likely to involve simultaneous or subsequent activation of TLRs by multiple microbial pathogens (Fransson M et al. 2005, Proud and Chow 2006, Schleimer 2004). TLRs recognize distinct sets of PAMPs either alone, by homodimerzation, or by forming heterodimers with other members of the TLR family (Mukhopadhyay S et al. 2004). For instance, TLR2 activity is enhanced by heterodimerization with TLR1 or TLR6, while TLR4 appears to form homodimers (Lee H-K et al. 2004).

In addition to the interactions of individual members of the TLR family, evidences suggest that TLRs and other non-TLR PRRs either physically or functionally interact with each other. The cumulative effects of these interactions instruct the nature and outcome of the immune response to a particular pathogen (Mukhopadhyay S et al. 2004). Collaboration between TLRs and other immune receptors has been best understood from the identification of different members of the LPS signaling complex including TLR4, CD14, LPS-binding proteins (LBP), and myeloid differentiating factor-2 (MD-2) (Lee H-K et al. 2004, Mukhopadhyay S et al. 2004). These findings redefined the knowledge that the receptors for LPS are multipartite and the cellular responses to physiological levels of LPS are dependent on LBP, TLR4, MD-2 and/or CD14 expression. It was initially shown in 1990 by Wright and coworkers that CD14, which is incapable of signaling by itself, is an important component of LPS receptor complexes. Furthermore, a plasma protein (LBP) was known to engage LPS and to facilitate its interactions with CD14 (Wright et al. 1990). Then in 1995, with the discovery of TLR4 as a membrane spanning component of the receptor complex, a small exteriorized protein known as MD-2 was found to be an essential component of the complex. At the time, it was believed that all LPS molecules were sequentially engaged by LBP and CD14; ultimately leading to the formation of a complex that contains LPS, CD14, MD-2 and TLR4. Conversely studies in mice showed that the TLR4/MD-2 receptor complex can distinguish between smooth and rough LPS and was evidently shown to function in two distinct modes. In the absence of CD14, lipid A and rough but not the smooth LPS act directly on TLR4 resulting in the activation of the MyD88-dependent but not the MyD88-independent pathway. On the other hand, in the presence of CD14, both rough and smooth LPS resulted in the activation of both the MyD88-dependent and MyD88-independent

(TRIF-dependent) pathways (Jiang Z et al. 2005). Overall, besides TLR4, other non-TLR proteins including LBP, MD-2 and CD14 are required for activation of the MyD88-independent signaling pathways by all LPS chemotypes, whereas CD14 is not required for MyD88 dependent signaling.

Of all TLRs, TLR2 has a unique ability to heterodimerize with other TLRs (TLR1 and TLR6) and also to interact with adaptor proteins and non-TLR receptors including CD14, Dectin-1, and CD36. CD14 was shown to mediate signaling with TLR2/TLR1 or TLR2/TLR6 ligands by interacting with the complex in the same way it interacts with the TLR4/MD-2 complex. It has been shown that lipoproteins or lipopeptides (LP) binding is facilitated by CD14 followed by molecular association with TLR2/TLR1 heterodimers as demonstrated specifically at the lower concentration of a synthetic lipopeptide, PAM_3CSK_4 (Manukyan M et al. 2005). Likewise, recognition of the protozoan GPI-anchors required CD14 in concert with TLR2 (Campos MAS et al. 2001). A classic member of the non-TLR PRRs, Dectin-1, has been shown in macrophages to synergize with TLR2 to recognize fungal wall-derived β-glucans, and generate TNF in responses to fungal pathogens (Perera PY et al. 2001, Brown GD et al. 2003, Gantner BN et al. 2003, Brown GD and Gordon S 2001, Rogers NC et al. 2005¸ Brown GD 2006). Although Dectin-1 displays a cell type-specific ability to directly induce cytokine production, it has been demonstrated in both macrophages and dendritic cells (DCs) that Dectin-1 can interact with other MyD88-coupled TLRs (TLR-2, TLR-4,TLR-5, TLR7, TLR-9), resulting in the synergistic induction of multiple cytokines including TNF, IL-10, IL-6 and IL-23

(Dennehy KM et al. 2008, Ferwerda G 2008, Reid DM et al. 2009). The scavenger receptor, CD36, was also revealed to function as a co-receptor acting in conjunction with the TLR2/TLR6 heterodimer to recognize diacylglycerides including bacterial LTA and mycoplasmal lipopeptide (MALP-2) (Hoebe K et al. 2005). Stuart LM et al. (2005) reinforced this concept by demonstrating that CD36 is required to internalize *Staphylococcus aureus* and LTA and deliver these ligands to their cognate receptors (TLR2/TLR6) by functioning as an accessory receptor at the cell surface. Their data showed that CD36 can act as a phagocytic receptor able to recognize *Staphylococcus aureus* and its cell wall component LTA (Stuart LM et al. 2005).

The ability of TLR2 to team up with other TLR and non-TLR receptors enhances the ability of TLR2 to bind a broad repertoire of structurally diverse ligands (Ozinsky A et al. 2000). For example, TLR2 participates in the recognition of peptidoglycan and lipothechoic acid (LTA) of Gram-positive bacteria, leptospiral lipopolysaccharide (LPS), lipoarabinomannan of mycobacteria, lipopeptides of Gram-positive bacteria, mycobacteria, spirochetes and mycoplasma, outer-surface protein A (OspA) of Borrelia burgdorferi, and several synthetic di- and tri-acylated lipopeptides, molecular components of fungus (β-glucan, zymosan) and of protozoa (glycosylphosphatidylinositol-GPI anchors or their fragments (Werts et al. 2001, Alexopoulou et al. 2002, Means et al. 1999a,b, Takeuchi et al. 2002). Depending on which of the receptors/adaptor molecules are expressed by a given host cell, TLR2 ligands can stimulate a diverse array of effects in different cells, reflecting on the range of the elicited host responses (Ozinsky A et al. 2000, Burke JM et al. 2007).

A few recent findings also implied a role of protease activated receptors (PARs) as an additional level of the innate immune defense, apart from the classical PRRs. The possible connection between PAR2- and TLR4-mediated signaling pathways was elucidated in endothelial cells by demonstrating concurrent activation of PAR2 and TLR4 by PAR2 activating peptide (PAR2-AP) and LPS, respectively, both of which amplify nuclear factor kappa B (NF-κB) activation and interleukin-6 (IL-6) production (Chi L et al. 2001). Inflammatory signaling was also shown to be synergistically augmented in airway epithelial cells via activation of PAR2 and TLR4 by raising PAR expression level and IL-8 release (Ostrowska E et al. 2007). In contrast, there has been a report that the inflammatory response induced by Aspergillus infection involving TLR4 produced suppression of PAR2 signaling (Moretti S et al. 2008). Thus, signaling cross-talk between PAR2 and TLR4 has the potential to augment or diminish an ongoing inflammatory response when both receptors are accessible. It has recently been revealed that TLR4 augmented GPCR-mediated signaling through an alternative PAR2 receptor via an MyD88-dependent mechanism suggesting that PAR2 and TLR4 interact functionally at the level of intracellular adapter utilization for the activation of NF-κB (Rallabhandi P et al. 2008).

Furthermore, a unique paradigm of receptor synergism between several members of the TLR family and the adenosine A_2A receptor (A_2AR), a G-protein coupled purinergic receptor with adenosine as endogenous ligand, has also been reported. In murine peritoneal macrophages treated with different TLR agonists, TNF-α secretion was stimulated (Pinhal-Enfield G et al. 2003), but, in the presence of A_2AR agonists, TLR2, 4, 7, 9, and not TLR3 and 5, both of which failed to induce TNF-α secretion. Instead, significant stimulation of a potent stimulus for angiogenesis known as vascular endothelial growth factor (VEGF) was observed. Simultaneous down-regulation of TNF-α and up-regulation of VEGF appears to function as an angiogenic switch shifting macrophages from an inflammatory to an angiogenic phenotype (Pinhal-Enfield G et al. 2003). It was therefore suggested that, synergism between TLR and A_2AR agonists may initiate a repair mechanism in infected or inflamed tissues. However, VEGF-induced angiogenesis may also enhance tumorigenesis, thus suggesting a potential contribution to tumor pathology (Pinhal-Enfield G et al. 2003).

The general consensus is that viral/microbial infections and/or their products may have divergent effects on the development of allergy and asthma (Herz U et al. 2000). Epidemiological and clinical observations have provided compelling evidence that suggests a link between the relative lack of infectious diseases and the increase in allergic disorders (Erb KJ 1999). According to this theory referred as the 'hygiene hypothesis', viral and bacterial infections, particularly during prenatal period, prevent the induction of allergen specific Th2 cells because they establish Th1-biased immunity (Herz U et al. 2000). On the contrary, several studies have suggested that viral/bacterial infections exacerbate allergic diseases, including bronchial asthma and airway hyper-responsiveness. Sensitization to fungal antigens during asthmatic reactions is exacerbated by viral respiratory infections (Proud and Chow 2006; Beasley et al. 1988; Gern 2004; Groskreutz et al. 2006; Kauffman and van der Heide 2003; Mallia and Johnston 2006; Tan 2005). A synergistic effect between virus and allergen-induced airway inflammation has been proposed to explain why viral infections are the primary and the most common pathogens associated with exacerbation of both asthma and COPD (Groskrutz DJ et al. 2006, Murray et al. 2004, Proud D and Chow C-W 2006), but no definitive molecular mechanism has been described.

TLR2, TLR4 and TLR6 have been implicated in activation of epithelial cells by fungal allergens (Mallia and Johnston 2006) and any modulation of these receptors by viral infections may alter the sensitivity of the host to fungal allergens that are sensed by these receptors. Likewise, *in vitro* studies involving treatment of primary small airway epithelial cells and airway smooth muscle cells with a synthetic dsRNA (Poly i/c) increased mRNA expression of TLR2, TLR3 and suppressed mRNA expression of TLR5, suggesting that viral infection can differentially alter the epithelial sensitivity and response to fungal allergens, bacteria, and other viruses (Ritter et al. 2005, Sukkar et al. 2006). Concordantly, up-regulation of TLR2, 3 and 4 has been reported in the nasal mucosa of patients with allergic rhinitis, indicating a role for multiple TLRs in allergic airway inflammation (Fransson et al. 2005). The importance of viral activation of TLR in differentially altering sensitization of epithelial cells to microbial components and/or allergens may also be inferred from the high level mRNA expression of TLR3 in ex vivo nasal mucosal samples from healthy human subjects that resulted in increased mRNA expression of TLR2, TLR3, TLR4, TLR6, TLR7 and TLR8, but reduced mRNA expression of TLR5 when challenged with rhino viruses (RV) (Avila et al. 2005).

However, at this time, little is known about the effect of viral dsRNA dependent activation of dsRNA sensing receptors (TLR3, RIG-I and MDA5) on the expression of other TLR and non-TLR subtypes in airway epithelial cells. Therefore, in this study both basal mRNA expression and the effects of a synthetic viral dsRNA analogue, Poly i/c on the expression of several TLRs and their associated co-receptors, adaptor proteins was investigated. Furthermore, the basal and Poly i/c-induced expression of other non-TLR PRRs including RNA helicases, protease-activated receptors (PARs) and purinergic (P2Y) receptors in airway epithelial cells were also examined.

2. 2. Rationale and objectives for the experiments

The expression of TLRs is typically thought to be a feature of immune cells. However, cell types from other tissues including epithelial cells have been shown to express TLRs which contribute to protection against invading pathogens or allergens. It has also been recognized that TLRs interact with non-TLR PRRs including RIG-I, MDA5, PAR and P2Y receptors. Thus assessing the constitutive as well as induced expression of these non-TLR receptors in airway epithelial cells expands the understanding of receptor interaction in airway inflammatory diseases.

Therefore, the first objective of the study is to determine which TLRs, TLR-coreceptors (CD14, CD36, Dectin-1) or adaptor proteins (MyD88, TIRAP, TRIF) involved in TLR-signaling as well as non-TLR PRRs (RIG-I, MDA5, PAR and P2Y receptors) are expressed in airway epithelial cells. The second objective was to determine the effects of specific TLR-ligands on mRNA and protein expression for selected TLRs, TLR co-receptors, adaptor proteins and non-TLR PRRs in airway epithelial cells.

2. 3. Materials and Methods

Materials:

Zymosan, a fungal product from *Saccahromyces cerevisiae*, and lipoteichoic acid (LTA) from *Staphylococcus aureus* (activators of TLR2/TLR6 heterologue) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyinosinic polycytidylic acid (Poly i/c), a synthetic analog of double-stranded RNA (dsRNA) (a known activator of TLR3, RIG-I, and MDA5) and N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-[R]-Cys-[S]-Ser-[S]-Lys_4 trihydrochloride (PAM_3CSK_4), a synthetic bacterial lipopeptide (activator of TLR2/TLR1 heterologue), Lipopolysaccharide (LPS), a TLR4 actvator, and flagellin, a TLR5 activator were purchased from InvivoGen (San Diego, CA, USA). Extract of Alternaria alternata, a fungus that can induce acute asthmatic episodes, was prepared from cultures of *Alternaria alternata* following the protocol described by Inouei Y et al. 2005. For each experiment, all reagents were freshly prepared using the solvents recommended by the manifacturers.

Cell culture:

Calu-3 cells were originally purchased from the American Type Culture Collection (ATCC, Rockville, MD) and were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (Hyclone, Logan, UT) and 50 μg/mL penicillin and streptomycin (Sigma/Aldrich) at 37°C in a 90% humidified and 5% CO_2 incubator. These cells are originally derived from adenocarcinoma cells and possess properties similar to those of airway gland serous cells (Shen BQ et al. 1994).

NHBE cells were obtained from Clonetics (Walkersville, MD, USA). These cells were life-span extended by transfection with the catalytic subunit of the human telomerase gene (hTERT) as previously described (Palmer et al. 2006). Low passage (p10-p20) life-span extended cells grown in monolayer culture under air-interface conditions were also used in this study. The cells were cultured in media supplemented with defined growth factors and retinoic acid (0.1 μg/L) contained within the SingleQuot kit of bronchial epithelial cell growth medium (BEGM) provided by Clonetics. Cells were maintained in humidified incubators at 37°C and 5% CO2.

Immortalized human bronchial epithelial (HBE) cells (HBEC) and A549/NF-κB-luc cell lines are kind gift from Dr. Fekadu Kassie (Masonic Cancer Center, University of Minnesota). HBEC were immortalized by the introduction of genes encoding cyclin-dependent kinase-4 and human telomerase reverse transcriptase (Ramirez RD et al. 2004). The NF-κB reporter stable cell line

A549/NF-κB-luc, which maintains a chromosomal integration of a luciferase reporter construct regulated by multiple copies of the NFκB response element, was originally developed by Panomics, Inc (Redwood City, CA) and is designed for monitoring the activity of NF-κB transcription factor in cell-based assays. A549/NF-κB-luc cell lines have been used extensively for the study of NFκB signaling due to its robust response and are useful cell models for studying the role of NFκB in lung carcinogenesis and development of chronic airway inflammatory diseases, particularly of cystic fibrosis. HBE cells were grown in the same NHBE growth media while A549/NF-κB-luc cell were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (Hyclone, Logan, UT) and 50 µg/mL penicillin and streptomycin (Sigma/Aldrich) at 37°C in a 90% humidified incubator and 5% CO_2.

Experimental conditions and sample collection for RNA/protein determination:

Calu-3 and NHBE cells were grown on Transwell filters in their respective media for seven days and then maintained under air interface conditions for three days. Cells on filters were then washed with basal medium and treated with buffer RLT (Qiagen, Valencia, CA, USA) used for lysis of cells for RNA isolation. For Western blot identification of different proteins, cells were lysed with cell lysis buffer containing protease inhibitor cocktail, both from Sigma-Aldrich (St. Louis, MO, USA).

HBE cells and A549/NF-κB-luc cell lines on 96-well, 6-well, T-25 or T-75 culture flasks at similar cell density and treatments with various TLR-ligands were performed for 24 hrs and the appropriate samples were collected depending on the test to be performed (real time PCR or Western blotting) as described for other cell types.

Quantitative RT-PCR (QRT-PCR):

Total RNA was extracted from the lysed cells using RNeasy kit and treated with DNase I (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Single stranded cDNA was prepared with 500 or 1000 ng of RNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers. QRT-PCR was performed using SYBR green detection protocols from Strategene (Wilmington, DE, USA). Primer sets for the target genes were developed using Primer 3 software or taken from published work and all sequences developed for this study are given in Table 1. Aliquots of cDNA equivalent to 25 ng of total RNA were used for QRT-PCR reactions. Real-time fluorescence was measured for 45 cycles and mRNA levels between treatment and controls were evaluated by comparing cycle threshold (C_T) of the target genes. Quantification of mRNA expression levels were normalized to the median expression of GAPDH and/or β-Actin. Specificity and level of the different mRNA transcripts were assessed by analysis of melting point dissociation curves or by evaluating the bands of PCR products run on agarose gels.

Relative changes in gene expression through quantification of the transcripts from QRT-PCR experiments that involved different treatment groups were performed using the $2^{-(\Delta\Delta C_T)}$ algorithm, also known as the delta-delta-C_T ($\Delta\Delta C_T$) algorithm method (Livak KJ, and Schmittgen TD 2001). The method requires the assignment of one or more housekeeping genes, which are assumed to be uniformly and constantly expressed in all samples and the results are expressed as fold change relative to the untreated control. The $\Delta\Delta C_T$ method in brief is computed as follows: The averaged C_T value for each target mRNA was determined along with the averaged C_T value for a specific house keeping gene. The ΔC_T was calculated as the difference between C_T values of the target and housekeeping gene mRNAs. Subsequently, $\Delta\Delta C_T$ value for each sample was calculated by subtracting the treatment condition $\Delta\Delta C_T$ value from the reference/control ΔC_T and the fold change relative to the reference/control was determined using the formula $2^{-(\Delta\Delta C_T)}$.

Fluorescence-activated cell sorting (FACS):

Cells were grown to 70-80% confluency and exposed to 10 µg/ml of Poly i/c for 24 hrs. Poly i/c treated and untreated control monolayers were dissociated with EDTA (0.5mM). The cells were subsequently washed with FACS solution (1% bovine serum albumin (BSA), 5mM NaN_3 in PBS), and pelleted by centrifugation (1000 x g). Pelleted cells were resuspended in FACS solution and their density adjusted to 10^6/100 µl by hemocytometer counting. Re-suspended cells (100 µl) were then added to a FACS tube and incubated in the dark on ice for 2 hours with 20 µl of phycoerythrin (PE)-labeled mouse anti-human TLR2 antibody (clone TLR2.1; # 12-9922-71; eBioscience, San Diego, CA, USA) or 5 µl of the corresponding PE-labeled mouse immunoglobulin G2a isotype control antibody (clone eBM2a; # 12-4724-81; eBioscience, San Diego, CA, USA) as recommended by the manufacturer. Unlabeled cells served as controls for auto-fluorescence. The labeled and the unlabeled cells were then washed, centrifuged and re-suspended in 500 µl FACS solution and analyzed using a FACS Diva caliber flow cytometer and BD CellQuest software (Becton-Dickinson, San Jose, CA, USA) at Dr. Bruce Walcheck's lab of the Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota . The mean fluorescence intensity (MFI) of cells stained with PE-labeled mouse anti-human TLR2 antibody was reported for 10,000 events for Poly i/c treated and untreated control cells.

Western blotting:

Monolayers treated for 24 hrs with Poly i/c (10 µg/ml), PAM_3CSK_4 (1 µg/ml) and untreated control samples were suspended in lysis buffer to which a cocktail of protease inhibitors was added (Sigma-Aldrich, St. Louis, MO, USA). The protein content of the samples was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Up to 60 µg of protein were loaded onto 10% Tris-glycine gels and run for 150 min at 125 V. The proteins were then transferred onto a polyvinylidene difluoride membrane (PVDF) membrane (Bio-Rad, Hercules, CA) for 2 h at 25 V. Adequate transfer of proteins was confirmed by staining membranes with BLOT-FastStain (Chemicon, Temecula, CA, USA). Subsequently, membranes were blocked in 5% Blotto non-fat dry milk in Tris buffer containing 1% Tween-20 for 1 hour and probed overnight with corresponding primary antibodies (TLR2 (H-175), sc-10739 and TLR2 (C-19), sc-8690) at a dilution of 1:100; TLR5 (H-127) sc-10742 at a dilution of 1:100, CD36 (SMø, sc-7309), at 1:200 dilution and β-Actin (C4) sc-47778) at 1:1000 dilution) all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). To determine the specificity of TLR2 staining, an immunizing peptide blocking experiment was performed. Before proceeding with the Western blot labeling protocol, TLR2 antibody was neutralized by incubation with a five fold excess of peptide (sc-8690P, Santa Cruz Biotechnology Inc.) corresponding to the epitope recognized by TLR2 antibody for two hours at room temperature. The neutralized TLR2 antibody was then used side-by-side with TLR2 antibody alone. After incubating with the respective secondary antibodies (donkey anti-goat IgG-HRP, sc-2020, 1:2000; goat anti-mouse IgM-HRP, sc-2064, 1:2000; and goat anti-mouse IgG-HRP, sc-2005, 1:2000, also from Santa Cruz Biotechnology) for 1 hour, chemiluminescent immunodetection was employed using a kit from Pierce (Rockford, IL, USA). The signal was visualized by exposure of membranes to HyBolt CL autoradiography film from Denville Scientific (Metuchen, NJ, USA). Densitometry was performed using Image J software available from NIH. Densitometry results were expressed as the intensity of TLR2, TLR5 or CD36 relative to β-actin, which served as the loading control.

RNA interference for silencing TLR3 in HBE cells:

The pGIPZ Lentiviral shRNAmir expressions construct with Green Fluorescent Protein (GFP) reporter to which the target TLR3 gene shRNA (clone V2LHS_171349, Accession # NM_003265) was cloned was used to facilitate RNA silencing in immortalized human bronchial epithelial (HBE) cells. Additionally, HBE cells were transfected with pGIPZ lentiviral vector without a shRNAmir insert (empty vector) for generating non-silencing construct containing vector control cells. This lentiviral shRNAmir expression system has the advantage of high transfection efficiency and GFP is used to visualize the expression of target gene short hairpin that can further be utilized for sorting out transfected cells. Incorporating GFP within the vector enabled flow cytometric sorting of cells expressing the TLR3-shRNA instead of relying on more time-consuming antibiotic selection methods. This approach also reduced the problem of selection-based changes in cell phenotype, which can be a problem when comparing cells expressing target and control shRNAs. All flow cytometry sorting was performed in the Flow Cytometry Core Facility at Stem Cell Institute. Cell sorting was done on FACS Vantage Sorters (Becton Dickinson) which are capable of three excitation lines: 488nm, 340-360nm, and 633nm. HBE cells infected with GFP-containing lentivirus were re-suspended at $3x15^6$ cells/ml in HBSS in 1% FBS. Samples were gated on live cells and cells were collected in culture media appropriate to the designated experiment. Collected HBE cells expressing GFP (and thus shRNA) were further propagated and the level of TLR3 knockdown was determined by qRT-PCR and Western blot.

Statistical analysis:

All data are reported as the mean ± SEM. Differences between groups were analyzed using an unpaired, two tailed t-test or an analysis of variance for multiple comparisons and considered to be significant when $p < 0.05$.

2. 4. Results

mRNA expression patterns of TLRs, co-receptor and adaptor molecule in airway epithelial cells:

Calu-3 and NHBE/HBE and A549/NF-κB-luc cells were analyzed for constitutive mRNA expression of TLRs, co-receptors, adaptor proteins and non-TLR PRRs by QRT-PCR. The analysis in Calu-3 cells revealed expression of multiple TLR subtypes including TLRs 1-6 (Fig. 1A) with low expression levels of TLR4 and TLR6 and with TLR3 exhibiting the highest level of mRNA expression followed by TLR5, TLR1 and then TLR2. Of the 10 TLRs tested in NHBE/HBE cells, TLR1-6 (Fig. 1B) were also detected, TLR3 exhibited the highest level of expression, followed by TLR5, TLR2, TLR1, TLR6 and the lowest expression was detected for TLR4 (Fig. 1B). Conversely, in A549/NF-κB-luc cell line all the ten tested TLRs (TLR1-10) were detectable by QRT-PCR and TLR5 was expressed at the highest level, followed by TLR1 (Fig. 1C).

The effects of treatment with various TLR-ligands on the expression of their cognate receptors or other TLR subtypes were also assessed. In Calu-3 cells, the effect of various TLR ligands on the expression of TLRs was relatively minor (Fig. 2A). Only LPS slightly enhanced TLR2 mRNA expression (by about 75%) and despite its low expression, TLR4 was activated by its specific ligand (LPS) and zymosan up-regulated expression of TLR4 mRNA and its own putative receptor (TLR2) by about two fold (Fig. 2A). Twenty-four hour treatment of NHBE cells with different TLR ligands including zymosan, Poly i/c, LPS and flagellin enhanced mRNA expression of TLR2 in all treatment conditions, but Poly i/c produced a remarkable stimulatory effect (Fig. 2B). NHBE cells treated with Poly i/c for 24 hours increased mRNA expression of TLR2 by over sixty-fold; TLR3 by eleven-fold; TLR4 just by two-fold; with no apparent change of TLR1 and TLR6. Conversely, the expression of TLR5 mRNA was significantly suppressed (64.5%) by Poly i/c (Fig. 3A, B, C, D, & E). Overall, Poly i/c activation of TLR3 or RNA helicases in NHBE cells differentially regulated mRNA expression of TLR2, TLR3 and TLR5. However, the modulation of TLR mRNA expression from Calu-3 cells treated with various TLR ligands was not as prominent as in NHBE cells.

Basal and Poly i/c-induced differential expression of TLR proteins in NHBE cells:

To verify that the effect on mRNA expression of TLR2 and TLR5 elicited by Poly i/c treatment translated into changes in protein expression, lysates from NHBE cells grown on membrane filters were analyzed by Western blots using anti-TLR2 and anti-TLR5 antibodies. Two different TLR2 antibodies that recognize epitopes of the extracellular and intracytoplasmic domain were used: H-175, sc-10739, the epitope which corresponds to amino acids 180-354 mapping near the N-terminus of TLR2 of human origin; and C-19, sc-8690, the epitope which corresponds to the C-terminus of TLR2 of human origin. For anti-TLR5 antibody, the epitope corresponding to amino acids 154-280 mapping near the N-terminus of TLR5 of human origin (H-127, sc-10742) was used. Both TLR2 antibodies identified a ~90 kDa protein induced by Poly i/c consistent with the size of intact TLR2 protein, but the complexity of the bands was different. The antibody that recognized the N-terminus, consistently detected multiple bands that fell within the range of the predicted molecular weight of TLR2 (80-100 kDa), seemingly corresponding to the different glycosylation states of the protein (Fig. 4A). The expression level of these multiple bands was enhanced with Poly i/c activation as compared to vehicle control treated NHBE cells. Densitometry analysis of the Western blot gel showed that the highest molecular weight band corresponding probably to the completely glycosylated TLR2 protein (~90 KDa) exhibited an increase in intensity ratios from 0.05 (untreated) to 0.92 after stimulation with Poly i/c (Fig. 4B). Conversely, the antibody that recognized the C-terminal domain revealed a more complex labeling pattern. Multiple bands with sizes ranging from the intact TLR2 protein to several relatively smaller bands were identified. These multiple smaller bands appear to represent various cleavage products of the receptor (Fig. 4C). Moreover, Poly i/c activation enhanced the level of all the TLR2 protein fragments of various sizes that were detected with immunoblotting. The binding specificity of all the bands were assessed by competing with excess epitope peptide (5 fold over the antibody), which neutralized the antibody making it unavailable to bind to protein and indicating that the competed bands were TLR2 specific. Overall, TLR2 labeling was nearly absent from the western blot obtained using the neutralized antibody (Fig. 4C). As displayed by the Western blot in Figure 4D and by the densitometry in Figure 4E, intact TLR2 protein exhibited an 11 fold increase in expression following stimulation by Poly i/c and a 5 fold increase, following stimulation with PAM_3CSK_4 (Fig. 4D and 4E). Down-regulation of TLR5 mRNA expression after

Poly i/c treatment did not result in a reduction in TLR5 protein level as determined by Western blot analysis (Fig. 4F).

Fluorescence-activated cell sorting (FACS) analysis revealed that Poly i/c treatment of NHBE cells for 24 hrs increased plasma membrane expression of TLR2 protein as displayed by the shift to the right of fluorescence histogram (Fig. 5A & B). A significant increase in the level of TLR2 expression was demonstrated by comparing the mean fluorescence intensity (MFI) of untreated NHBE control cells (mean ± SE of MFI= 181.5 ± 24.2, n=4) versus that of cells treated with Poly i/c for 24 hrs (mean ± SE of MFI=420 ± 52.7, n=4 (Fig. 5C).

Basal and Poly i/c-induced expression of adaptor proteins, co-receptors PAR and P2Y receptor subtypes in NHBE cells

NHBE cells also constitutively express MyD88, TIRAP, TRIF, CD14, CD36 and Dectin-1 mRNA, adaptor proteins and major co-receptors that are critical for downstream TLR signaling (Fig. 6A, & B). The mean $\pm$ SE for cycle threshold (C_T) values of these adaptor proteins and co-receptors basally expressed in NHBE cells was MyD88 (22.65 $\pm$ 0.12), CD14 (24.58 $\pm$ 0.70), TIRAP (27.16 $\pm$ 0.07), TRIF (32.01 $\pm$ 0.46), CD36 (28.22 $\pm$ 0.11) and Dectin-1 (31.48 $\pm$ 0.01). In addition, Poly i/c increased mRNA expression of MyD88 (4.5 fold) and TIRAP (2.1 fold), adaptor proteins involved in TLR2 signaling as well as TRIF (5 fold), an adaptor protein involved in TLR3 signaling (Fig. 6C). In contrast, the TLR2 ligand, PAM_3CSK_4, did not alter adaptor protein mRNA expression (Fig. 6C). Treatment with Poly i/c also enhanced mRNA expression of TLR co-receptors CD14 (4.6

fold) and Dectin-1 (10.1 fold), but reduced mRNA expression of CD36 by 44.3 % (Fig. 6D), a co-receptor recently shown to be involved in immune responses to lipothechoic acid (LTA) in macrophages and BEAS-2B epithelial cells (Hoebe et al. 2005). A significant difference in the levels of mRNA expression between Poly i/c treated and untreated control NHBE cells was statistically substantiated for CD36 ($p<0.05$, $n=6$), but not for CD14 and Dectin-1. Western blot and densitometry analysis indicated that the level of CD36 protein expression in Poly i/c treated cells decreased by 1.8-2 fold relative to untreated control and PAM_3CSK_4 treated cells (Fig. 6E, & F).

Besides TLR3, there are other receptors that also mediate the response to Poly i/c, namely; retinoic acid inducible gene 5 (RIG-I) and myeloid differentiation associated gene 5 (MDA5). Basal mRNA expression of these non-TLR dsRNA-sensing cytoplasmic helicase proteins (RIG-I and MDA5) and the associated adaptor protein, IPS-1, was demonstrated in NHBE cells by QRT-PCR. Poly i/c treatment of these cells increased mRNA expression of RIG-I and MDA5, but not IPS-1, a downstream signaling molecule for these helicases (Fig. 7A & B).

Expression of proteinase activated receptor (PAR) and purinergic (P2Y) receptor mRNAs were determined in NHBE cells by QRT-PCR. The mean $\pm$ SE for cycle threshold (C_T) values for basal expression of PARs were: PAR1 (23.62 $\pm$ 0.12), PAR2 (20.18 $\pm$ 0.07), PAR3 (32.03 $\pm$ 0.06) and PAR4 (36.56 $\pm$ 0.73); whereas that of P2Y receptors were: P2Y1 (23.49 $\pm$ 0.06), P2Y2 (21.41 $\pm$ 0.04), P2Y4 (28.80 $\pm$ 0.19), and P2Y6 (24.41 $\pm$ 0.003). Poly i/c treatment of NHBE cells for 24 hrs enhanced mRNA expression of PAR4 as well as of P2Y2 and P2Y6 without significantly changing the other PAR and P2Y receptor subtypes (Fig. 8B, & 9B) Overall, these results show that Poly i/c presumably by activating TLR3 or RIG-I/ MDA5, differentially regulates mRNA expression of specific TLRs as wells as key co-receptors and adaptor molecules.

Toll-like receptor 3 (TLR3) mediates the response of TLR2 to Poly i/c in NHBE cells:

To determine whether Poly i/c-dependent regulation of TLR2 and TLR5 is linked to activation of TLR3, silencing of TLR3 by shRNA was carried out. Short hairpin RNAs (shRNAs) targeting TLR3 (shTLR3) are introduced to immortalized NHBE cells (HBEC) (Fig. 10A) using a lentiviral vector expressing GFP. GFP-expressing cells (Fig. 10B) were then selected using the fluorescence-activated cell sorting to obtain an enriched population of shRNA-expressing cells. As a control, a cell line expressing an empty vector was also prepared. The silencing of TLR3 was determined to be 83.5% as assessed by measuring mRNA levels in shTLR3 and the vector control cells by QRT-PCR (Fig. 10C). The knock-down of TLR3 by shRNA was specific to the cognate receptor and does not appear to affect the expressions of RNA-helicases (RIG-I and MDA5) that are also known to sense Poly i/c (Fig. 11A).

The effects of Poly i/c on TLR2 mRNA and protein expression were also evaluated by comparing TLR3 knockdown cells with the vector control HBE cells after exposing the cells to 10 μg/mL of Poly i/c for 24 hrs. The result shows that in the vector control cells, activation of TLR3 with Poly i/c increased mRNA expression of TLR2 by 38.5 fold (Fig. 11B). But when TLR3 knock-down cells were used, Poly i/c mediated induction of TLR2 mRNA expression was reduced to only 3.2 fold (Fig. 11B), indicating that up-regulation of TLR2 by stimulation of HBE cells with Poly i/c was primarily TLR3 mediated. The effect of Poly i/c on TLR2 protein expression was also assessed by Western blotting and the decrease in TLR2 mRNA induction was verified at the protein level (data not presented). Besides TLR2, other receptors (TLR3, RIG-I, and MDA5) that were shown to be enhanced in their mRNA expression by Poly i/c treatment of HBE cells were also shown to be negatively modulated (by 2.6, 7.6, and 7.0 fold; respectively) following TLR3 silencing (Fig. 11B).

2. 5. Discussion

Exacerbations of inflammatory airway diseases including COPD or asthma have been strongly linked to viral and bacterial infections. These inflammatory conditions are triggered mainly due to biased release of pro-inflammatory mediators by airway epithelial cells and/or other innate immune cells of the lung in response to activation by different microbial molecular patterns that are recognized by Toll-like receptors. To characterize the effects and interactions of various TLR ligands and to study the inflammatory response of the lung epithelium, primary and immortalized human bronchial epithelial (NHBE/HBE) cells, sub-bronchial gland adenocarcinoma cell lines (Calu-3) as well as carcinomic human alveolar basal epithelial cells (A549) were used as models.

In this study, TLR mRNAs that detect viruses (TLR3), bacteria (TLR1, TLR2, TLR4-6) and fungal allergens (TLR1, TLR2, TLR6) were shown to be expressed by Calu-3 and NHBE cells, with the highest expression for TLR3 and the lowest expression for TLR4. On the other hand, TLR1-10 were marginally detectable in A549/NF-κB-luc cell line, in consistent with a previous finding that also showed constitutive expression of TLR1-10 in A549 cells (Hou Y-F et al. 2006). Among the TLR ligands employed to assess the response of the lung epithelium to stimulation with additional TLR ligands during viral and bacterial infections, Poly i/c potently modulated the expression of TLR receptors in NHBE cells. Conversely, the response of Calu-3 cells to TLR ligands, particularly to Poly i/c, was generally weak. This is consistent with the report by Taura and coworkers that demonstrated the activity of TLR3 (and possibly that of other

TLRs as well) to be dependent on p53 status of the cells. Calu-3, lung adenocarcinoma cell line, which harbors mutant p53, may exhibit faulty activation of TLR3 by Poly i/c (Taura M et al. 2008).

In NHBE cells, stimulation with Poly i/c increased TLR2, TLR3 and TLR4, but reduced TLR5 mRNA expression, consistent with earlier studies using primary small airway epithelial cells (Ritter et al. 2005) and airway smooth muscle cells (Sukkar et al.2006). It has also been recently demonstrated that Poly i/c stimulation of esophageal epithelial cells induces the NF-κB-dependent esophageal epithelial expression of TLR2, leading to enhanced epithelial responsiveness of the cells to TLR2 ligand stimulation (Lim DM et al. 2009).

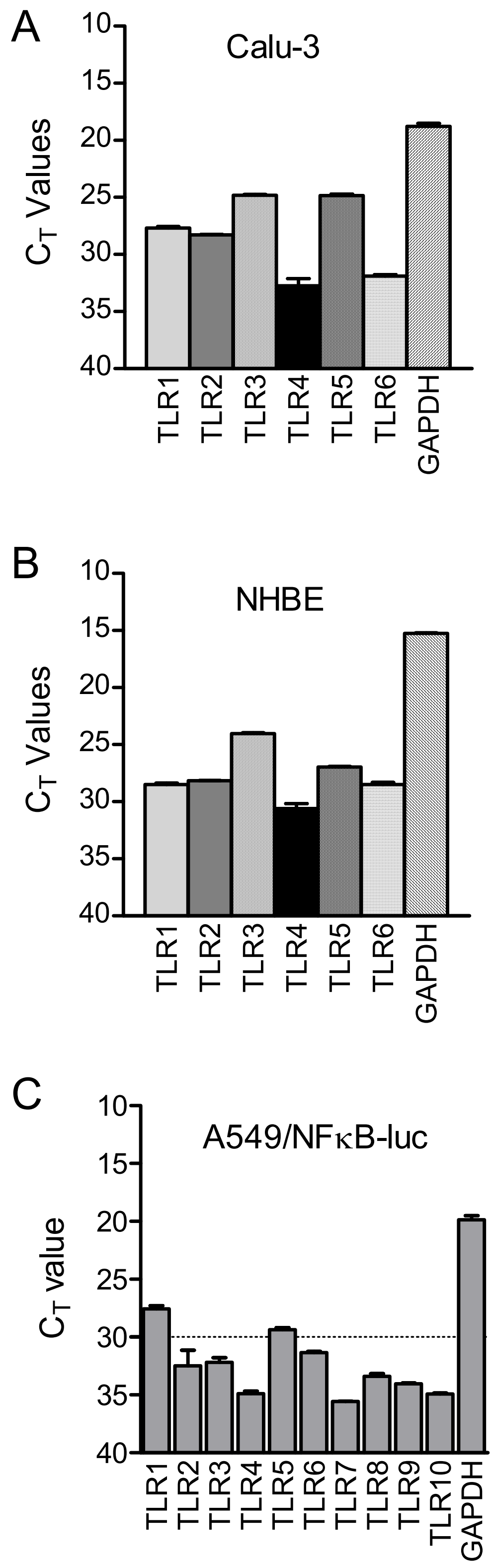
All TLRs are structurally modified by post translational modification processes since they contain *N*-linked glycosylation consensus sites, which presumably influence receptor surface representation, trafficking, and pattern recognition (Weber ANR et al. 2004). Four N-glycosylation sites of TLR2 were reported in the extracellular LRR domain at amino acid positions 114, 199, 414 and 442 (Kataoka H et al. 2006). It has been previously reported that differences in electrophoretic mobility as indicated by the appearance of multiple bands, were attributed to inefficient core glycosylation of TLR2 receptor (Weber ANR et al. 2004). Thus, the complex western blot pattern, showing multiple bands but with closer molecular weight to the size of the intact TLR2 protein, which was detected using the N-terminal antibody, probably reflects glycosylation variants of TLR2 as reported by various investigators under different experimental conditions (Grabiec A et al. 2004, Merx S et al. 2007). On the other hand, the relatively smaller fragments that were identified when the C-terminus antibody was used, appears to represent cleavage products. Supporting this speculation, ectodomain cleavage of endosomal TLR9 and TLR7 has been reported in mouse macrophages and dendritic cells (Ewald SE et al. 2008). Cleavage products of full-length TLR9 were shown to contain approximately half of the ectodomain, the transmembrane domain and the entire cytoplasmic domain (Ewald SE et al. 2008). Likewise, IL-1R1, a receptor that shares a similar intracellular motif (TIR domain) and relies on the recruitment of adapter proteins similar to TLRs to transmit an intracellular signal, exhibited metalloprotease-mediated cleavage within the extracellular domain that liberates soluble IL-1R1 and generates a membrane-bound IL-1R1 C-terminal fragment (Elzinga BM et al. 2009). Additionally, intracellular cleavage of IL-1R1 C-terminal fragment by presenilin-dependent γ-secretase with the release of intracellular soluble fragment, IL-1R1 intracellular domain, has been observed following ectodomain shedding (Elzinga BM et al. 2009). Further evidence for TLR2 protein cleavage has been the well-documented existence of soluble forms of TLR2 (sTLR2) in biological fluids, including human plasma, milk, saliva and amniotic fluid (Dulay AT et al. 2009, Kuroishi T et al. 2006, LeBouder E et al. 2003). Similarly, blood monocytes were also shown to constitutively release sTLR2, the activity of which was increased upon cell activation (LeBouder et al. 2003). Thus, it is likely that the low molecular weight bands detected by Western blotting of cell lysates after Poly i/c activation are cleavage products of TLR2. Furthermore, TLR2 labeling of all the detected bands was eliminated from the Western blot using peptide competition, implying that the lower molecular weight bands contain the TLR2 specific antigen. In summary, the detection of soluble forms of TLR2 in major body fluids and the discovery of ectodomain shedding of TLR7, TLR9 and IL-1R1 strongly suggests that ectodomain shedding of TLR2 may occur. Whether stimulation of NHBE with Poly i/c modulates the expression of either metalloproteinase or γ-secretase enzymes that are involved in intramembrane proteolysis of TLR2 protein leading to TLR2 fragmentation and the physiological significance of this fragmentation warrants further investigation.

Suppression of TLR5 mRNA expression following activation of NHBE cells with 10 μg/ml of Poly i/c can not be corroborated with diminished protein levels of TLR5. The transcriptional suppression of TLR5 gene in response to dsRNA has also been reported in various cell systems that have been subjected to Poly i/c or respiratory syncitial virus (RSV) (Avila PC et al. 2005, Ritter et al. 2005, Sukkar et al. 2006), but has never been substantiated by translational suppression at a protein level. In the current study, though TLR5 mRNA expression was suppressed following Poly i/c treatment of NHBE cells, Western blot analysis indicated that there may be sufficient receptor protein to efficiently carry out the innate immune response of the airway epithelium to TLR5 ligands (recombinant flagellin) and flagellated bacteria that activate this receptor. The results also showed that mRNA expression of adaptor proteins, MyD88 and TIRAP, which are involved in TLR2 signaling, also increased following exposure to Poly i/c. These results and those of a previous study suggested that detection of dsRNA may lead to an overall sensitization of the airway epithelium to TLR2 activating ligands (LeVine et al. 2001). Additionally, RIG-I and MDA5 mRNA expression also increased in response to Poly i/c treatment, suggesting that detection of viral RNA may also involve these RNA helicases, in addition to TLR3.

A similar dramatic increase in mRNA expression of TLR2 in airway epithelial cells and smooth muscle cells when treated with dsRNA has been previously demonstrated; however the receptors mediating the effect were not identified. Pattern recognition receptors for viral double stranded RNA (dsRNA) or its synthetic analogue Poly i/c that simulate aspects of viral infection include TLR3 and cytoplasmic RNA helicases RIG-I and MDA5. This study showed that NHBE cells express mRNA for viral RNA receptors TLR3, RIG-I and MDA5 and all of them have been previously shown to respond to Poly i/c *in vitro* (Yoneyama M et al. 2005). Although further work is required to determine whether RIG-I and MDA5 activation by Poly i/c produce up-regulation of TLR2 and other receptors, the RNA silencing data imply that the phenomenon is predominantly TLR3 mediated.

In summary, this thesis work showed that Poly i/c has a marked effect on the expression of TLRs and molecules involved in TLR signaling in surface airway epithelial (NHBE) cells. Poly i/c induced an elevated expression of TLR2 and TLR3, TLR adaptor proteins and co-receptors including MyD88, TRIF, CD14 and Dectin-1 as well as non-TLR pathogen recognition receptors such as RIG-I, MDA5, PAR4, P2Y2. All or some modulated receptors serve as sensors of molecular patterns of viruses, bacteria, fungal allergens, suggesting the potential influence of viral replicative intermediate (dsRNA) on the immune response of the lung epithelium to viral and bacterial infections and fungal allergens.

Figure 1: Basal mRNA expression profile of Toll-like receptors (TLRs) in different human airway epithelial cell lines: Quantitative RT-PCR was performed using RNA isolated from Calu-3 (A), NHBE (B), and A549-NF-κB-luc (C) cells. The data shows the relative expression of TLRs under basal conditions. Results are expressed as the mean cycle threshold (C_T) of at least three independent experiments and GAPDH was used as an internal control.


A
Calu-3
C_T Values
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TLR2
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TLR6
GAPDH
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C_T Values
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TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
GAPDH
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A549/NFκB-luc
C_T value
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TLR1
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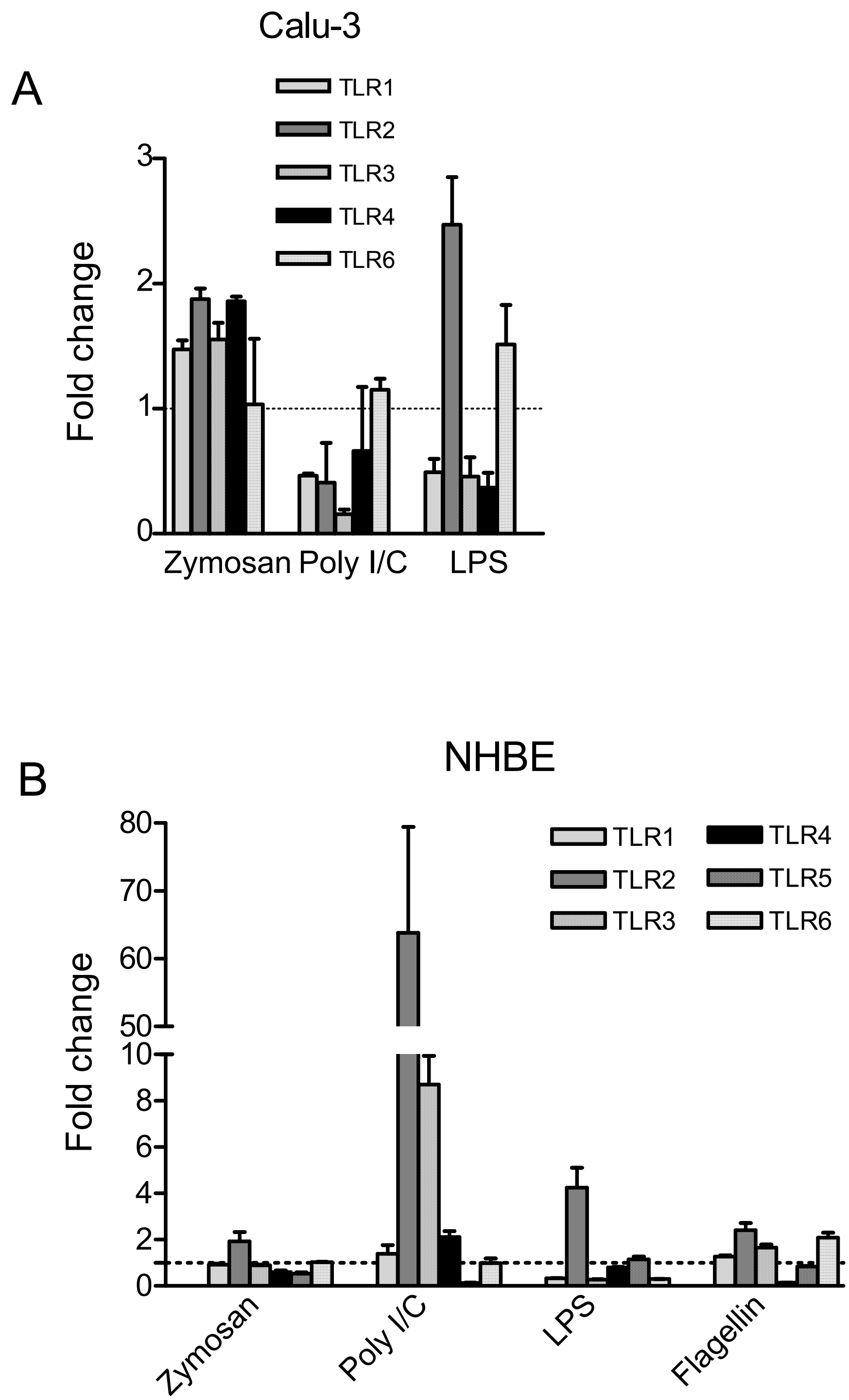
TLR6
TLR7
TLR8
TLR9
TLR10
GAPDH

Figure 2: Effects of treatment with various TLR-ligands of airway epithelial cells on the expression of their cognate receptors or other TLR subtypes: Fold changes in TLR mRNA expression of Calu-3 and NHBE cells treated with Zymosan, Poly i/c, LPS, and/or Flagellin for 24 hrs versus vehicle-treated control cells were compared. Results are expressed as the mean of a minimum of three independent experiments and GAPDH was used as an internal control.


Calu-3
A
TLR1
TLR2
TLR3
TLR4
TLR6
Fold change
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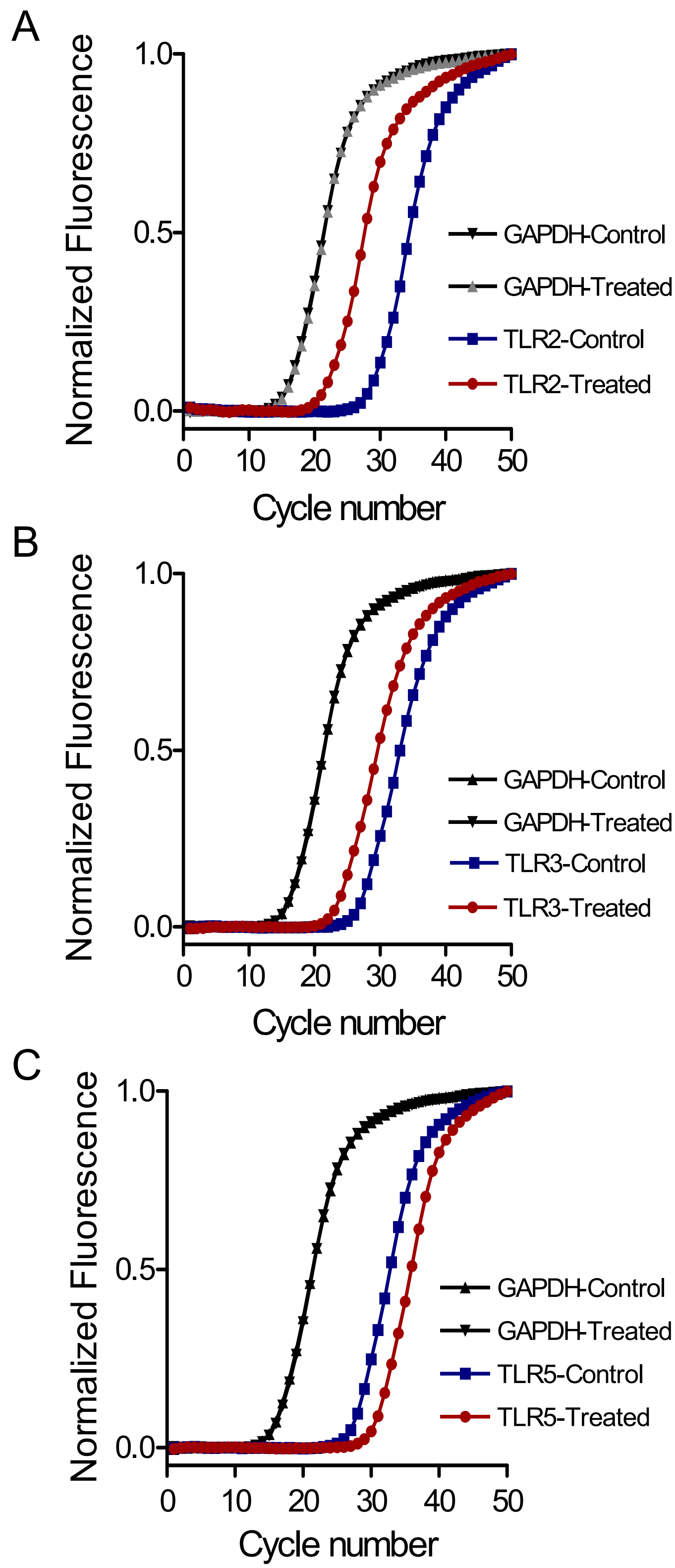
2
1
0
Zymosan
Poly I/C
LPS
NHBE
B
80
70
60
50
10
8
6
4
2
0
Fold change
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
Zymosan
Poly I/C
LPS
Flagellin

Figure 3: Effects of Poly i/c on TLRs mRNA expression: The patterns of TLR2 (A), TLR3 (B) and TLR5 (C) mRNA expression of Poly i/c-treated (T) and vehicle-treated NHBE controls (C) were displayed by comparing normalized fluorescent intensity against cycle threshold run at 50 cycles. Peak differences in fluorescent intensity were captured by running the qRT-PCR reaction at 32 cycles and the amplified products were resolved on 2% agarose gel and stained with Ethidium bromide (D). Analysis of fold change in mRNA expression of Poly i/c treated versus untreated control cells showed that Poly i/c potently induced TLR2, TLR3, but suppressed the expression of TLR5 (E). Results are expressed as the mean of a minimum of three independent experiments and GAPDH was used as an internal control.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Normalized Fluorescence
1.0
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Cycle number
GAPDH-Control
GAPDH-Treated
TLR2-Control
TLR2-Treated
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Normalized Fluorescence
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Cycle number
GAPDH-Control
GAPDH-Treated
TLR3-Control
TLR3-Treated
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Normalized Fluorescence
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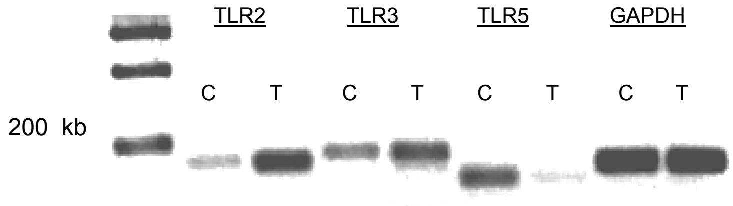

TLR2
TLR3
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GAPDH
C
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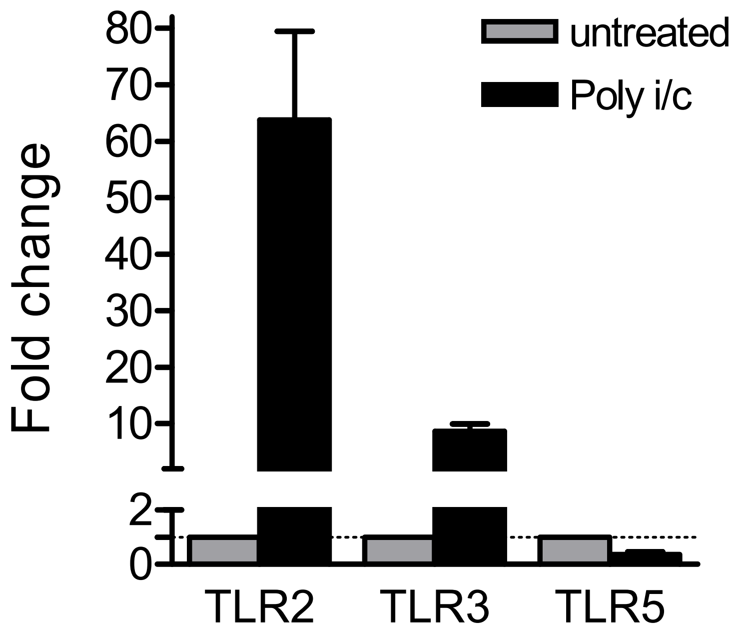
C
T
C
T
C
T
200 kb

E



untreated
Poly i/c
Fold change
80
70
60
50
40
30
20
10
2
0
TLR2
TLR3
TLR5

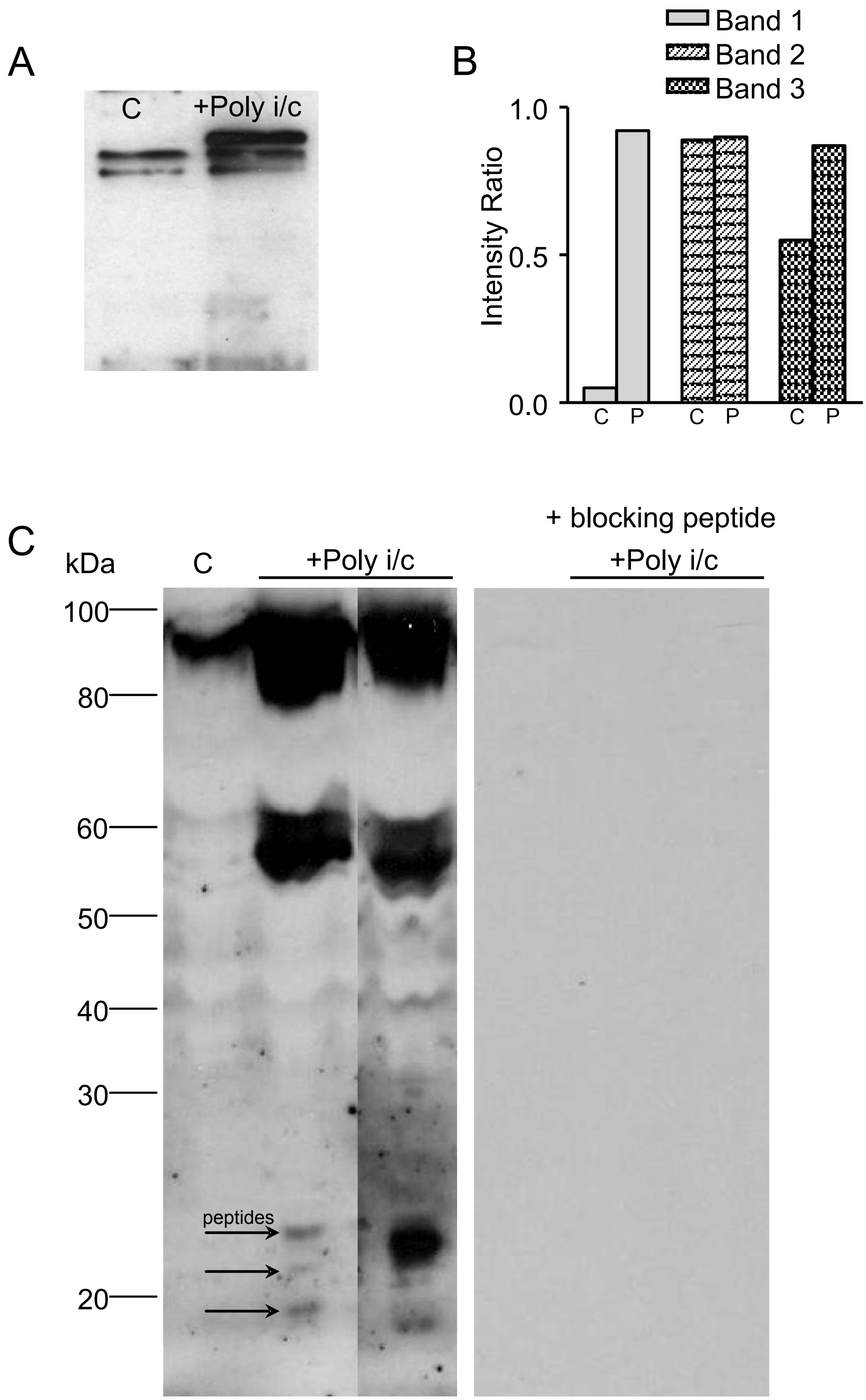
Figure 4: Western blot analysis of basal and induced TLR2 protein level in NHBE cells: (A) Lysates from NHBE cells grown on membrane filters with and without Poly i/c treatment (24 h) were analyzed by Western blots using TLR2 antibody, the epitope of which corresponds to amino acids 180-354 mapping near the N-terminus of TLR2 of human origin (H-175; sc-10739). Multiple bands that fell within the range of the predicted molecular weight of TLR2 (80-100 kDa) were repeatedly detected. The intensities of these bands were enhanced with Poly i/c activation (+Poly i/c) as compared to vehicle-treated control (C) NHBE cells.

(B) Densitometry analysis of the gel showed that the highest molecular weight band corresponding probably to the completely glycosylated TLR2 protein (~90 KDa) exhibited a relative increase in intensity ratios from 0.05 (untreated control; C) to 0.92 (after stimulation with Poly i/c; P). Densitometry results were expressed as (band intensity)/(background intensity) where the intensity range extended from 0-256 shades of gray. (C) Lysates from NHBE cells grown on membrane filters with and without Poly i/c treatment (24 h) were analyzed by Western blots using TLR2 antibody, the epitope maps to the C-terminus of TLR2 of human origin (C-19, sc-8690). Multiple bands with sizes ranging from the intact TLR2 protein to several relatively smaller bands were identified. The intensities of these bands were enhanced with Poly i/c activation (Poly i/c) as compared to vehicle-treated control (C) NHBE cells. To determine the specificity of TLR2 staining, an immunizing peptide blocking experiment was performed. Hence, Panel 1 is in the absence and Panel 2 is in the presence of TLR2 specific blocking peptide (C). Before proceeding with the Western blot labeling protocol, TLR2 antibody was neutralized by incubation with a five fold excess of peptide (sc-8690P) corresponding to the epitope recognized by TLR2 antibody for two hours at room temperature. The neutralized TLR2 antibody was then used side-by-side with TLR2 antibody alone. TLR2 labeling was absent from the Western blot, when the antibody was pre-incubated with the blocking peptide (Fig. 4C, Panel 2)

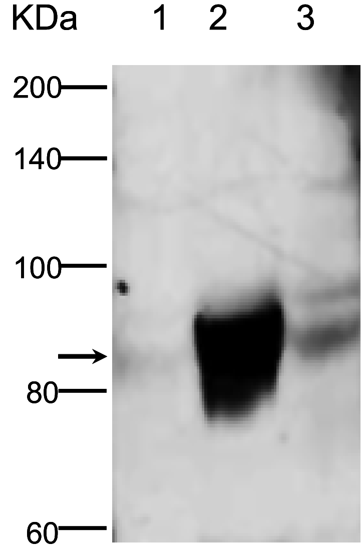
(D) Western blot analysis of TLR2 protein expression in NHBE cell lysates from untreated control monolayers (lane 1) and monolayers treated with 10 μg/ml of Poly i/c for 24 hrs (lane 2) or with 1 μg/ml of PAM_3CSK_4 for 24 hrs (lane 3) are displayed.

(E) Densitometry revealed an 11 fold increase in TLR2 protein expression after Poly i/c exposure compared to untreated controls. Treatment with PAM_3CSK_4, also increased TLR2-protein expression by 5 fold compared to the untreated control cells. Blots were reprobed with an anti–β-actin antibody to ensure equal protein loading. Analyses from three independent experiments were performed.

(F) Analysis of TLR5 protein expression in NHBE cell lysates from untreated control monolayers (lane 1) and monolayers treated with 10 μg/ml of Poly i/c for 24 hrs (lane 2) was performed via immunoblotting using anti-TLR5 antibody, the epitope corresponds to amino acids 154-280 mapping near the N-terminus of TLR5 of human origin (H-127, sc-10742). Blots were reprobed with an anti–β-actin antibody to ensure equal protein loading. Analyses from three independent experiments were performed. No significant change in TLR5 protein expression was observed in monolayers treated with Poly i/c as compared to the control cells.

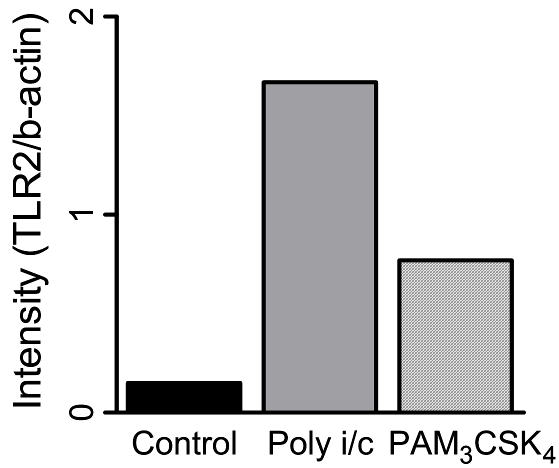

A
C
+Poly i/c
B
Band 1
Band 2
Band 3
Intensity Ratio
1.0
0.5
0.0
C P
C P
C P
+ blocking peptide
C
kDa
C
+Poly i/c
+Poly i/c
100
80
60
50
40
30
20
peptides

D



KDa
1
2
3
200
140
100
80
60

E



Intensity (TLR2/b-actin)
2
1
0
Control
Poly i/c
PAM3CSK4

F

unt
PIC
TLR5
β-Actin

Figure 5: Fluorescence-activated cell sorting (FACS) analysis for surface expression of TLR2 protein in untreated NHBE cells and after Poly i/c treatment for 24 hrs: (A) NHBE cells were incubated only with media and then FACS analysis was performed for unstained cells (gray filled histogram), cells stained with PE-conjugated anti-human TLR2 antibody (open histogram with black solid line) and cells stained with PE-conjugated mouse IgG2a K isotype control antibody (open histogram with gray solid line).

(B) FACS was performed as described above after treatment of NHBE cells with 10 µg/mL of Poly i/c for 24 hours. There is a shift of the fluorescence histogram plot to the right in Poly i/c treated NHBE cells as compared to the untreated controls, corresponding to an increase in the level of Poly i/c-induced surface expression of TLR2.

(C) Mean Fluorescence Intensity (MFI) of FACS-sorted untreated control and 24 hrs Poly i/c-treated (10 µg/mL) NHBE cells was compared after staining with PE-conjugated anti-human TLR2 antibody. The histograms are representatives of 4 independent experiments. Statistical significance was assessed by the unpaired two-tailed student's t-test ("*" represents $p<0.05$, $P=0.034$, $n=4$).

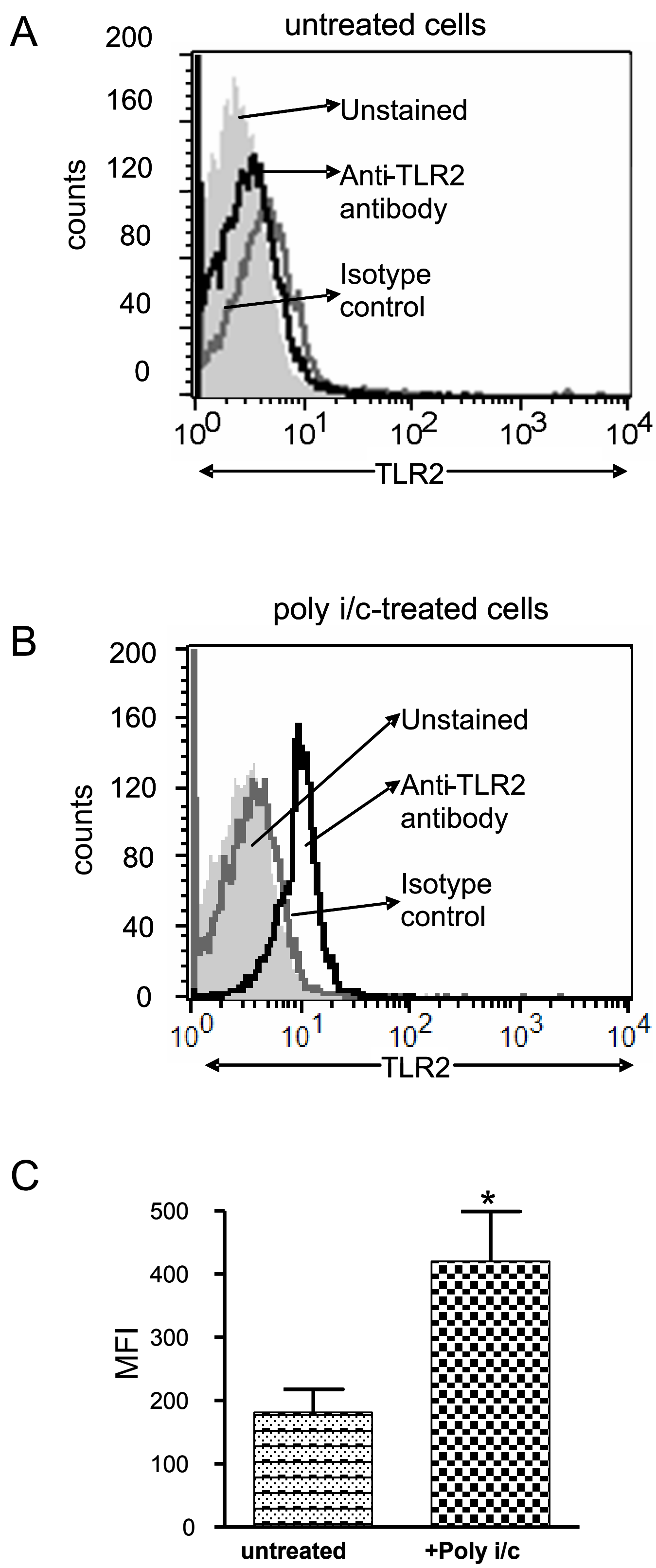

A
untreated cells
200
160
120
80
40
0
counts
Unstained
Anti-TLR2 antibody
Isotype control
10^0
10^1
10^2
10^3
10^4
TLR2
B
poly i/c-treated cells
200
160
120
80
40
0
counts
Unstained
Anti-TLR2 antibody
Isotype control
10^0
10^1
10^2
10^3
10^4
TLR2
C
500
400
300
200
100
0
MFI
*
untreated
+Poly i/c

Figure 6: Basal and Poly i/c-induced mRNA expression of adaptor proteins and co-receptors involved in TLR signaling: (A) Quantitative RT-PCR was performed using RNA isolated from NHBE cells and relative mRNA expression of key adaptor proteins involved in TLR signaling (MyD88, TIRAP, and TRIF) was determined.

(B) Quantitative RT-PCR was performed using RNA isolated from NHBE cells and relative mRNA expression of key co-receptor proteins that function in concert with TLRs (CD14, CD36, and Dectin-1) was determined. All the results on basal mRNA level are expressed in terms of the mean cycle threshold (C_T) of at least three independent experiments and GAPDH was used as an internal control.

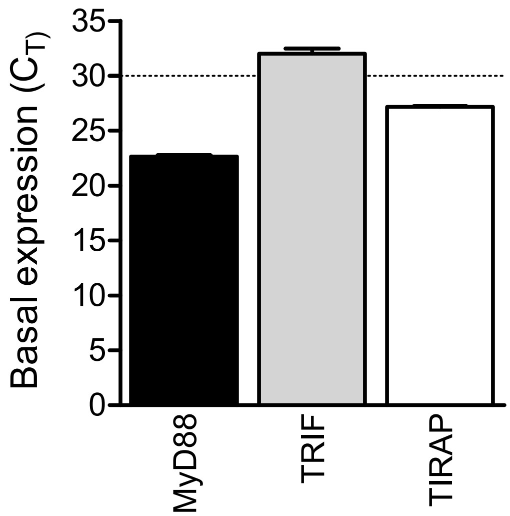
(C) Effect of Poly i/c on mRNA expression of MyD88, TIRAP, and TRIF was carried out after treating monolayers of NHBE with Poly i/c for 24 hrs. Increased mRNA expression of MyD88 and TIRAP was observed; but no effect with PAM_3CSK_4. Poly i/c also increased expression of TRIF, an adaptor protein involved in TLR3 signaling. Results are expressed as the mean of a minimum of three independent experiments and GAPDH was used as an internal control.

(D) Effect of Poly i/c on mRNA expression of CD14, CD36, and Dectin-1 (Co-receptors) was carried out after treating monolayers of NHBE with Poly i/c for 24 hrs. Treatment of NHBE cells with Poly i/c for 24 hrs enhanced mRNA expression of the TLR co-receptors CD14 and Dectin-1 but reduced mRNA expression of the co-receptor CD36.

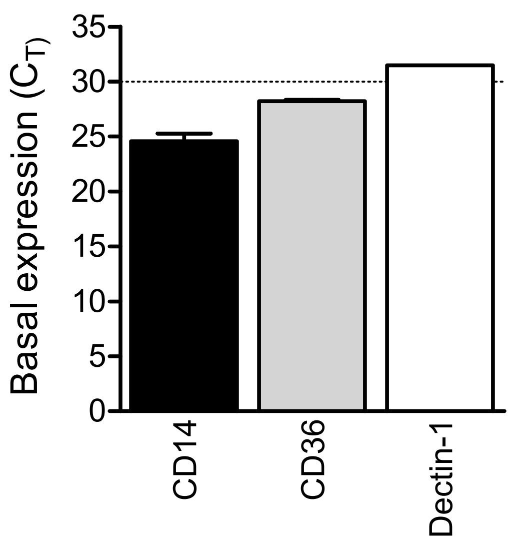
(E) Western blot analysis of CD36 expression in NHBE cell lysates from untreated control monolayers (lane 1) and monolayers treated with 10 µg/ml Poly i/c for 24 hrs (lane 2) or 1 µg/ml PAM_3CSK_4 for 24 hrs (lane 3).

(F) Densitometry indicated that Poly i/c decreased CD36 expression by 1.8-2 fold compared to untreated control monolayers and PAM_3CSK_4 treated monolayers. Blots were reprobed with an anti–β-actin antibody to ensure equal protein loading. Analyses from three independent experiments were performed.

A


Basal expression (C_T)
35
30
25
20
15
10
5
0
MyD88
TRIF
TIRAP

B


Basal expression (C_T)
35
30
25
20
15
10
5
0
CD14
CD36
Dectin-1

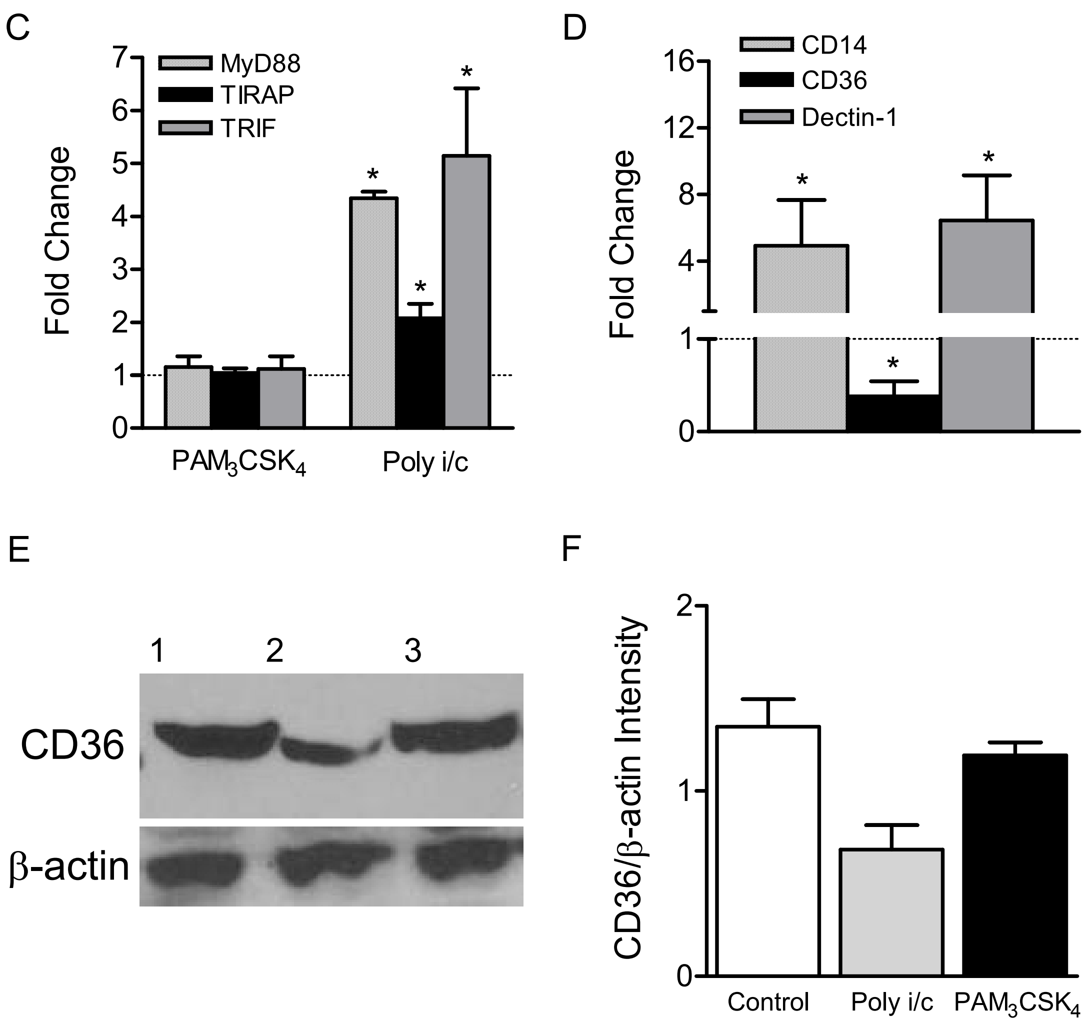

C
Fold Change
MyD88
TIRAP
TRIF
0
1
2
3
4
5
6
7
PAM3CSK4
Poly i/c
D
Fold Change
CD14
CD36
Dectin-1
0
1
4
8
12
16
E
1
2
3
CD36
β-actin
F
CD36/β-actin Intensity
0
1
2
Control
Poly i/c
PAM3CSK4

Figure 7: Basal and Poly i/c-induced mRNA expression of non-TLR dsRNA sensing receptors and their adaptor protein: (A) Basal mRNA expression expressed in terms of the mean cycle threshold (C_T) of non-TLR dsRNA-sensing cytoplasmic helicase proteins (RIG-I and MDA5) and the associated adaptor protein, IPS, was determined in NHBE cells by qRT-PCR. The results are a mean of at least three independent experiments and GAPDH was used as an internal control.
(B) Analysis of the effect of 24 hrs Poly i/c treatment of NHBE on mRNA expression demonstrated enhanced mRNA expression of RIG-I and MDA5 but not IPS, a downstream signaling molecule for these helicases.

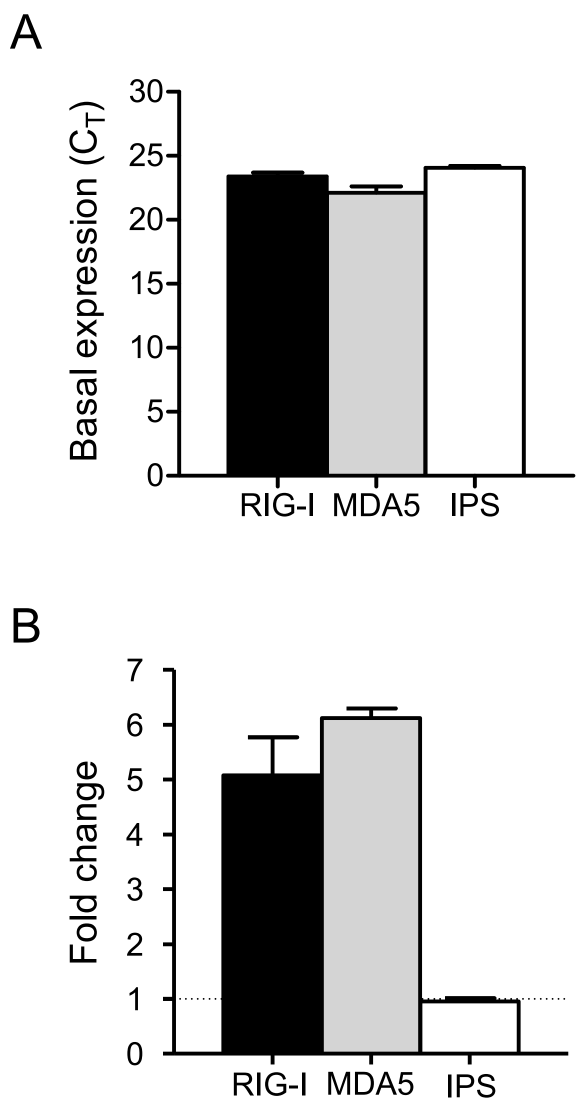

A
Basal expression (C_T)
30
25
20
15
10
5
0
RIG-I
MDA5
IPS
B
Fold change
7
6
5
4
3
2
1
0
RIG-I
MDA5
IPS

Figure 8: Basal and Poly i/c-induced mRNA expression of proteinase activated receptor (PAR): (A) Basal mRNA expression expressed in terms of the mean cycle threshold (C_T) of proteinase activated receptor (PAR) was determined in NHBE cells by qRT-PCR. The results are a mean of at least three independent experiments and GAPDH was used as an internal control. (B) Analysis of the effect of 24 hrs Poly i/c treatment of NHBE on mRNA expression of PARs demonstrated enhanced mRNA expression only of PAR4.

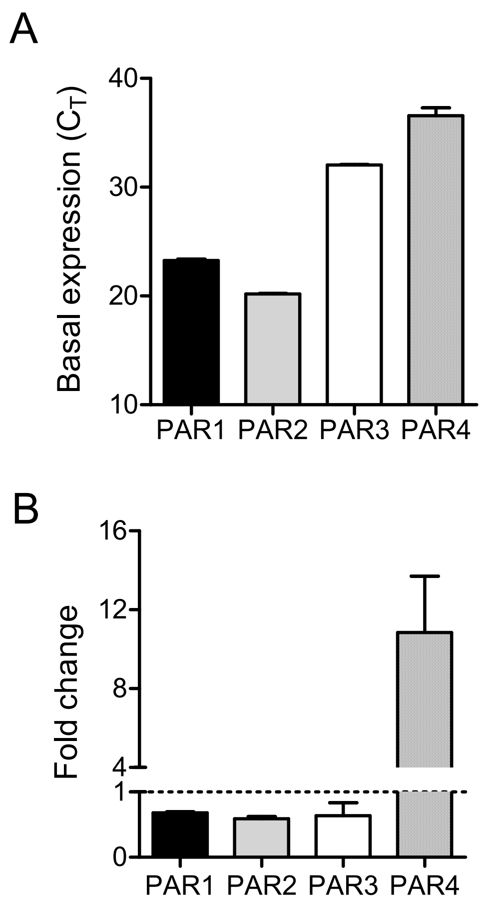
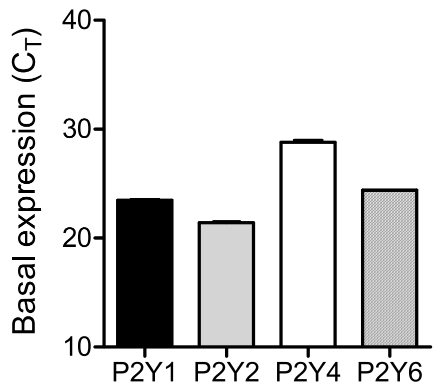

A
40
30
20
10
Basal expression (C_T)
PAR1
PAR2
PAR3
PAR4
B
16
12
8
4
1
0
Fold change
PAR1
PAR2
PAR3
PAR4

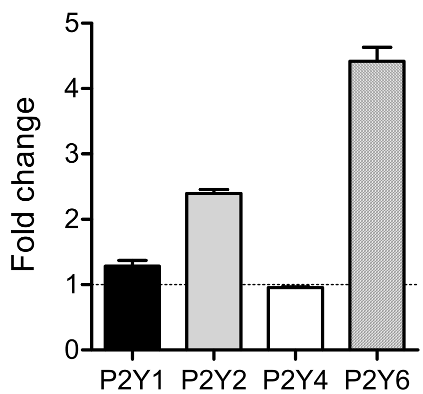
Figure 9: Basal and Poly i/c-induced mRNA expression of purinergic (P2Y) receptor: (A) Basal mRNA expression expressed in terms of the mean cycle threshold (C_T) of non-TLR dsRNA-sensing cytoplasmic helicases proteins (RIG-I and MDA5) and the associated adaptor protein, IPS, was determined in NHBE cells by qRT-PCR. The results are a mean of at least three independent experiments and GAPDH was used as an internal control. (B) Analysis of the effect of 24 hrs Poly i/c treatment of NHBE on mRNA expression of P2Y demonstrated enhanced mRNA expression of P2Y2 and P2Y6 without significantly changing the other PAR and P2Y receptor subtypes.

A



40
30
20
10
Basal expression (C_T)
P2Y1
P2Y2
P2Y4
P2Y6

B



5
4
3
2
1
0
Fold change
P2Y1
P2Y2
P2Y4
P2Y6

Figure 10: Silencing of TLR3: shRNA targeting TLR3 was introduced into immortalized NHBE (HBE) cells using a lentiviral vector expressing green fluorescent protein (GFP). Cells were checked for efficiency of transfection by comparing phase-contrast picture of untransfected control cells (A) with fluorescence detection for GFP positive cells. GFP-expressing cells (B) were then sorted by fluorescence-activated cell sorting (FACS) to obtain an enriched population of shRNA-expressing cells. As a control, shRNA sequence not-targeting TLR3 or no other gene was introduced again via lentiviral vector into HBE cells. The level of TLR3 knock-down was determined by comparing levels of TLR3 expression in shTLR3-cells and the vector control cells by QRT-PCR and expressed in terms of fold change (C). GAPDH was used as an internal control.

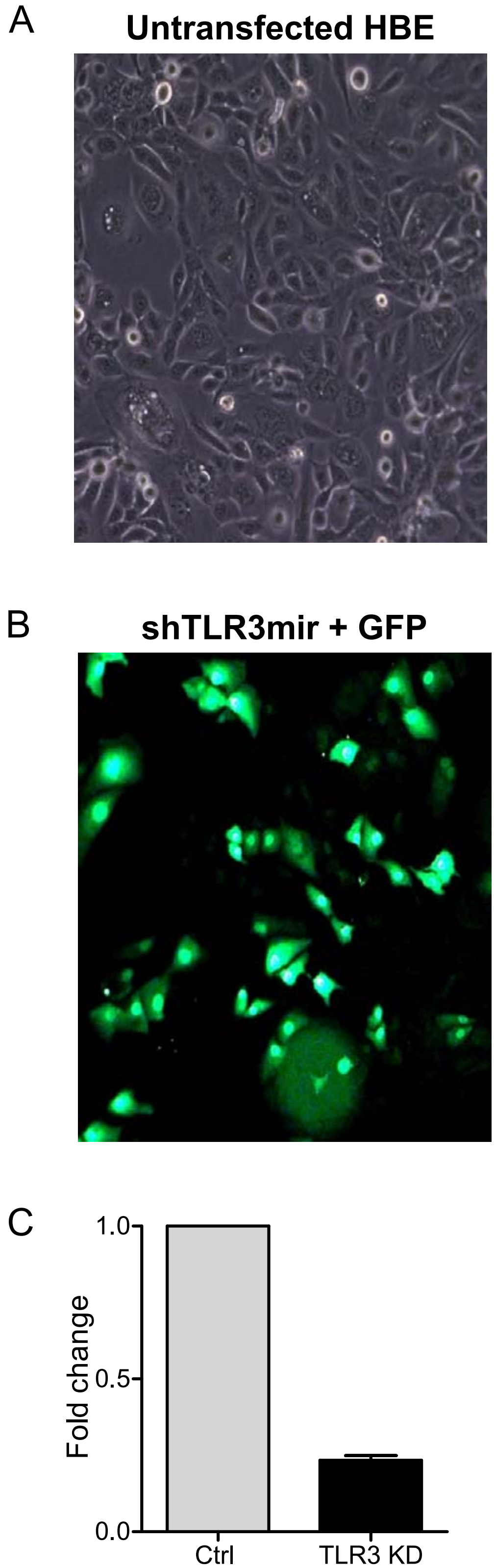

A
Untransfected HBE
B
shTLR3mir + GFP
C
Fold change
1.0
0.5
0.0
Ctrl
TLR3 KD

Figure 11: Effect of TLR3 Silencing on mRNA expression of TLR2 and RNA-helicases: (A) The specificity of silencing TLR3 was determined by assessing the effect that silencing TLR3 has on mRNA expression of the alternative dsRNA-sensing receptors (RIG-I and MDA5) by QRT-PCR. (B) To examine whether Poly i/c-stimulation-dependent induction of TLR2 mRNA and protein expression was TLR3-mediated, TLR2 mRNA levels between TLR3 knockdown and the vector control HBE cells were compared after exposing the cells to 10 μg/mL of Poly i/c for 24 hrs. The result shows that in the vector control cells, activation of TLR3 with Poly i/c increased mRNA expression of TLR2. But when TLR3 knock-down cells were used, Poly i/c mediated induction of TLR2 mRNA expression was reduced by over a dozen fold, indicating that up-regulation of TLR2 by stimulation of HBE cells with Poly i/c primarily TLR3 mediated. The functional consequence of TLR3 silencing on other receptors (TLR3, RIG-I, and MDA5) that were shown to be enhanced in their mRNA expression by Poly i/c treatment of HBE cells were also assessed. Poly i/c dependent induction of mRNA of all the four receptors (TLR2, TLR3, RIG-I, MDA5) were shown to be negatively modulated following TLR3 silencing.

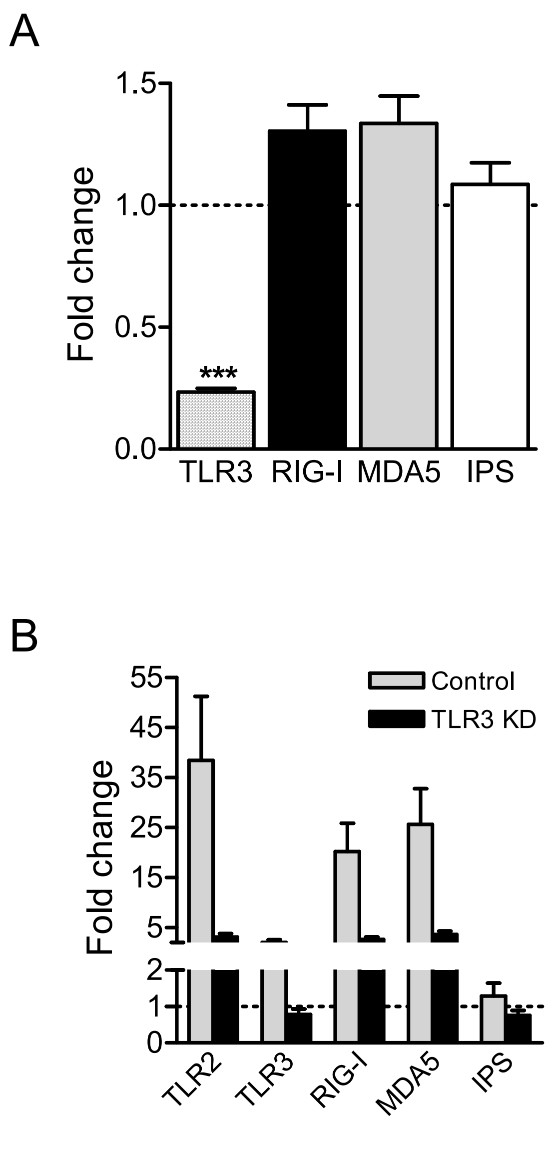

A
1.5
1.0
0.5
0.0
Fold change
***
TLR3
RIG-I
MDA5
IPS
B
55
45
35
25
15
5
2
1
0
Fold change
Control
TLR3 KD
TLR2
TLR3
RIG-I
MDA5
IPS

Table 1: Primer sequences for QRT-PCR for Human Target Genes

Target Gene	Sequences (F and R 5' to 3')	Accession No. for the designed primer or Authors, if taken from a published paper	mRNA Size (bp)	Position	Product Size (bp)	Melting Temp. (Tm) (ºC)
TLR1	ATTCCGCAGTACTCCATTCCT CTTTGCTTGCTCTGTCAGCTT	BC109093	2605	2295-2450	156	55.9 56.0
TLR2	GGGTTGAAGCACTGGACAAT TCCTGTTGTTGGACAGGTCA	NM_003264	3417	202-409	208	55.6 56.2
TLR3	CCGTCTATTTGCCACACACTT TCATCGGGTACCTGAGTCAAC	NM_003265	3057	15-244	230	55.5 56.2
TLR4	TGAGCAGTCGTGCTGGTATC CAGGGCTTTTCTGAGTCGTC	U88880	3811	2443-2609	167	57.0 55.7
TLR5	CCTCATGACCATCCTCACAGT ATTCTGCACCCATGTGAAGTC	BC109118	3000	2297-2465	169	56.4 55.4
TLR6	TGCCCATCTGTAAGGAATTTG TGGGTGAAAAACAAGGTGAAG	NM_006068	2753	473-681	209	53.0 53.5
MyD88	GCACATGGGCACATACAGAC TGGGTCCTTTCCAGAGTTTG	NM_002468	2826	2060-2298	239	56.4 54.7
TIRAP	AGAAGCCTAGAGGCCATTCTG GTCCAAAGGTGAAGATGGTGA	BC032474	2151	1753-1944	192	56.4 54.8
TRIF	CGCCACTCCAACTTTCTGTAG TTCTGTTCCGATGATGATTCC	AB093555	2139	995-1178	184	55.7 52.4
RIG-I	AGGAAAACTGGCCCAAAACT TTTCCCCTTTTGTCCTTGTG	(Le Goffic et al, 2007)				55.0 53.2
MDA5	GTGCATGGAGGAGGAACTGT GTTATTCTCCATGCCCCAGA	(Le Goffic et al, 2007)				57.1 54.4
						55.9

IPS	GCAGCAGAAATGAGGAGACC AAAGGTGCCCTCGGACTTAT	(Le Goffic et al, 2007)				56.1
CD14	CTGCAACTTCTCCGAACCTC CCAGTAGCTGAGCAGGAACC	X13334	1367	224-438	215	55.7 57.6
CD36	TGGCTGTGTTTGGAGGTATTC CTTGAATGTTGCTGCTGTTCA	NM_00100 1548	2338	345-542	198	55.1 54.0
Dectin-1	AGAACCACAGTCAACCCACACA GGAGATTAGAGCCCAGTTGC	AY026769	744	269-467	199	57.4 56.7
IL-6	GCCCAGCTATGAACTCCTTCT TGAAGAGGTGAGTGGCTGTCT	NM_00060 0	1125	55-212	158	56.7 57.9
GM-CSF	CACTGCTGCTGAGATGAATGA GATAATCTGGGTTGCACAGGA	E01817	767	150-360	211	55.1 54.7
RANTES	TTTCTACACCAGCAGCAAGTGC CACACACTTGGCGGTTCCT	(Shi et al, 2003)				58.8 58.1
IFN-β	GATTCATCTAGCACTGGCTGG CTTCAGGTAATGCAGAATCC	(Li et al, 1998)			186	55.4 50.8
GAPDH	ATGACATCAAGAAGGTGGTG CATACCAGGAAATGAGCTTG	(Lee et al, 2007)			177	52.4 51.1

Chapter 3: Functional consequences of altered TLR2 and TLR5 expression induced by Poly i/c on cytokine/chemokines mRNA and protein secretion by airway epithelial cells

3. 1. Introduction

Exacerbation of airway inflammatory disease is likely to involve simultaneous or subsequent activation of TLRs by multiple microbial pathogens (Fransson M et al. 2005, Proud D and Chow CW 2006, Schleimer RP 2004). For instance, sensitization to fungal antigens during asthmatic reactions is exacerbated by viral respiratory infections (Proud D and Chow CW 2006, Beasley R et al. 1988, Gern JE 2004, Groskreutz et al. 2006, Kauffman HF and van der Heide S 2003, Mallia P and Johnston SL 2006, Tan WC 2005). To elucidate the interactions between viruses and allergens and their respective receptors, a synergistic effect between virus and allergen-induced airway inflammation has been proposed (Murray CS et al. 2004), but no definitive molecular mechanism has been described. Previous studies in primary small airway epithelial cells and airway smooth muscle cells showed that activation of TLR3 using a synthetic dsRNA (Poly i/c) increases mRNA expression of TLR2, TLR3 and suppresses mRNA expression of TLR5 (Ritter M et al. 2005, Sukkar MB et al. 2006). In accord with this *in vitro* work, high levels of TLR3 mRNA expression has been reported in *ex vivo* nasal mucosal samples from healthy human subjects and challenge with rhino viruses (RV) increased mRNA expression of TLR2, TLR3, TLR4, TLR7 and TLR8, but reduced mRNA expression of TLR5 (Avila PC et al. 2005). Furthermore, up-regulation of TLR2, 3 and 4 has been reported in the nasal mucosa of patients with allergic rhinitis. TLR2, TLR4 and TLR6 have also been implicated in mediating activation of epithelial cells by fungal allergens (Fransson M et al. 2005, Mallia P and Johnston SL 2006). These *in vitro*, *ex vivo* and *in vivo* findings are suggestive of collaborative interactions among multiple TLRs and various co-receptors to regulate the immune response of the airway epithelium. The results also imply that respiratory viral infections can differentially alter epithelial sensitivity and responses to ligands sensed by multiple TLRs including bacterial agents and aeroallergens.

3. 2. Rationale and objectives for the experiments

Previous studies have shown that individual members of the TLR family and other non-TLR receptors both physically or functionally interact with each other to determine the subsequent nature and outcome of the immune response to a particular pathogen (Murray CS et al. 2004). The least understood phenotype in human asthma, predominantly in children and in adult asthmatics with severe symptoms, is acute exacerbation requiring hospitalization. The consistent observation that at the time of hospitalization most affected patients also carry respiratory viral infections is an important clue to the nature of the underlying trigger. This suggests inflammation arising from host antiviral defense may interact with underlying allergic inflammatory response produces cumulative airway tissue damage above a critical threshold necessary to precipitate severe asthma attacks (Subrata LS et al. 2009).

TLR3, RIG-I and MDA5 recognize viral replicative intermediates, double stranded RNA (dsRNA), or its synthetic analog, Poly i/c (Matsukura S et al. 2007, Sasai M et al. 2006). Although the roles for TLR3, RIG-I and MDA5 in the recognition of several RNA viruses have been investigated, the functional relationships between these dsRNA detectors and other pathogen recognition receptors in modulating airway inflammatory response remain to be determined. On the bases of data presented in Chapter 2 that show Poly i/c stimulation of NHBE cells increases mRNA and protein expression of TLR2 and decreases mRNA expression of TLR5, we hypothesized that following 24 hrs of TLR3 ligand priming, NHBE/HBE cells, would be more responsive to TLR2 ligand stimulation but less responsive to TLR5-dependent stimulation elicited by recombinant flagellin or flagellated bacteria (e.g. *Burkholderia cenocepacia*). Thus the objective of the present study was to investigate the effects of enhanced TLR2 mRNA and protein expression and suppressed TLR5 mRNA expression induced by exposure to Poly i/c on the innate immune response of human airway epithelial cells to TLR2 and TLR5 ligands and microorganisms that activate these receptors.

3. 3. Materials and Methods

Cell culture:

In addition to NHBE/HBE and Calu-3 cells, the human alveolar epithelial carcinoma cell line, A549, originated from type II pneumocytes (Burns JL et al. 1996) were also used in the experiments addressing specific aim 2 of the thesis. This A549 cell line was used by various investigators as established models for studying the role of flagellated bacteria in inflicting human pulmonary diseases, particularly cystic fibrosis. It has also been previously demonstrated that the invasion of A549 cells by different strains of *Burkholderia cenocepacia* was significantly higher (10-20 fold higher) than that of Calu-3 cells and 16HBE14o- cells (Duff C et al. 2006). Furthermore, a strong positive correlation was recognized between *in vitro* invasion of A549 cells and *in vivo* infection of a mouse model by *B. cenocepacia* (Cieri MV et al. 2002). On the contrary, there has been controversy concerning the appropriateness of A549 cells in the study of certain pathogenic phenotypes (such as invasiveness) of flagellated bacteria (*Pseudomona aurogenosa* and *B. cenocepacia*). Since A549 cells do not readily polarize or form tight epithelial monolayers, their suitability has largely been brought to question (Duff C et al. 2006, McClean S and Callaghan M 2009). Despite this controversy, A549/NFκB-luc cells (a modification of the original A549 cell model) are an appealing and powerful tool for experiments involving TLR5 and *B. cenocepacia.* These cells are stably transfected with a nuclear factor kappa B (NF-κB) luciferase reporter plasmid making it a pertinent cell line for assaying NF-κB activation. Hence, we employed the A549/NFκB-luc cells for studying the degree of NF-kB activation and cytokine/chemokine expression during infection of these cells with three strains of *B. cenocepacia.* A549-NF-κB-luc cells constitutively express TLR5; originally purchased from Panomics Inc, (Freemont, CA, USA) were kind gift of Dr. Fekadu Kassie (Masonic Cancer Center, University of Minnesota) and were grown at 37˚C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and were maintained in selection medium containing 100 µg/ml of Hygromycin B.

Cell inoculation and NF-κB reporter activity assay:

A549-NF-κB-luc cells were seeded in 96-well plates at 30,000 cells/well and were then incubated for 24 hrs before they were inoculated with three strains of mid-exponential-phase *Burkholderia (B.) cenocepacia* at a multiplicity of infection (MOI) of 1:100 and incubated at 37°C in the presence of 5% CO_2 for 2, 4, and 6 hours. After incubating the cells with the bacteria for designated time points, bacterial-cell free culture supernatants were collected by centrifugation at 6,000 X *g* for 15 min, filtered through a 0.2 mm pore size filter, and stored at -80 ºC until checked for cytokines/ chemokine levels, where as the cells were then washed five times with phosphate-buffered saline to remove surface bacteria and processed for the luciferase assay. The three strains of *B. cenocepacia* used in this study were: 1) the parental wild type (WT) strain (J2315), a flagellated motile organism isolated from a patient with Cystic Fibrosis; 2) strain CM58, derived from the parental strain J2315, a non-motile bacterium lacking a flagellum, in which the fliI gene was insertionally inactivated using an antibiotic resistance marker (cat-encoding chloramphenicol resistance); and 3) strain CM100, derived from strain CM58 in which a wild-type copy of the fliI gene was introduced, restoring motility to the mutant. All bacterial strains were grown at 37°C in Luria broth (LB). The luciferase activity was measured by luciferase assay system (Promega) using the GloMax luminometer as per the manufacturer's instructions. The fold activation or repression was calculated relative to the control sample and all measurements were done by calculating the average of triplicate samples of at least three independent experiments.

Measurement of cytokine/chemokine secretion:

In NHBE cells treated with Poly i/c for 24 hrs, triplicate samples of basolateral fluid harvested from each treatment condition were screened for several cytokines and chemokines using the Human Cytokine Antibody Array I (RayBiotech, Norcross, GA, USA) following the manufacturer's instructions. Densitometry was performed on the images of the processed cytokine array membrane and the data were expressed as a percentage of the positive control spots present on the membrane. The effect of Poly i/c treatment on the expression of each of the proteins was determined by computing its percentage expression relative to the untreated controls. Cytokines and chemokines for which absolute quantitation was desired in the conditioned media were examined by specific ELISAs. Duoset kits for IL-6 (samples diluted at 1:20) and Quantikine kit for GM-CSF (samples diluted at 1:10) (R & D systems, Minneapolis, MN, USA) were used for the ELISA assays.

Statistical analysis:

All data are reported as the mean ± SEM. Differences between groups were analyzed using an unpaired, two tailed t-test or an analysis of variance for multiple comparisons and considered to be significant when $p < 0.05$.

3. 4. Results

Poly i/c differentially regulates mRNA expression of TLR2 and TLR5 and adaptor proteins

Using quantitative RT-PCR, NHBE cells were shown to express TLR1 through TLR6 mRNA under basal conditions. Stimulation of NHBE cells with Poly i/c (10 µg/mL) for 24 hours profoundly increased TLR2 but suppressed TLR5 mRNA expression as described in Chapter 2 (Fig. 1A). Poly i/c increased TLR2 mRNA expression by approximately 47-fold but pretreatment with Poly i/c for 24 hrs and then with a TLR2 ligand (PAM_3CSK_4 ; 1 µg/mL) for another 24 hrs had a marked synergistic effect on TLR2 mRNA expression (53-fold) but treatment with PAM_3CSK_4 alone produced only a modest effect on TLR2 mRNA expression (Fig. 1B).

Poly i/c had also affected mRNA expression of adaptor proteins (MyD88, TIRAP and TRIF) involved in TLR signaling. Poly i/c induced a 4-6 fold up-regulation of MyD88, and TRIF mRNA expression either alone or after treatment with PAM_3CSK_4 (1 µg/mL) for another 24 hrs compared to the untreated control cells. However, the increase in mRNA expression of TIRAP was barely 2-fold in cells treated with Poly i/c alone or in cells pretreated with Poly i/c for 24 hrs and then with PAM_3CSK_4 for another 24 hrs compared to the untreated control cells. PAM_3CSK_4 alone does not appear to alter mRNA expression of these adaptor proteins (Fig. 1C). Unlike the robust induction of TLR2 mRNA expression in NHBE cells, Poly i/c stimulated very little change in Calu-3 and no change in A549-NF-κB-luc cells. Furthermore, initial screening of anti-inflammatory and immune responses of these two human lung epithelial cell lines to various TLR ligands was not as pronounced as the response exhibited by NHBE cells; therefore detailed cytokine/chemokine profiling studies were performed only on NHBE/HBE cells.

Poly i/c stimulation of NHBE/HBE enhanced mRNA expression of TLR2 that augment epithelial responsiveness to TLR2 ligand

Since the airway epithelium is a major source of inflammatory mediators in various viral and bacterial infections (Diamond G et al. 2000, Bals R et al. 2004), we determined which cytokines/chemokines are released from NHBE cells in response to activation by different TLR ligands. Among the tested TLR-ligands (zymosan, poly i/c, and LPS), Poly i/c prompted the strongest proinflammatory response; including increased secretion of IL-6, GM-CSF, MCP-1, GRO-α, RANTES, TNF-α, IL-10, MCP-2 and G-CSF (Fig. 2A-L). Secretion of IL-6, GM-CSF, MCP-1, GRO-α and RANTES was stimulated to the greatest degree (Fig. 3). In contrast to poly i/c, ligands for TLR2 and TLR4 were less effective in stimulating cytokine or chemokine release by NHBE cells (Fig. 2A-L).

To examine whether the Poly i/c-induced increase in TLR2 expression was associated with enhanced TLR2 function, NHBE/HBE cells were stimulated with various combinations of Poly i/c and then TLR2 ligand, PAM_3CSK_4. Then expression levels of transcripts of selected cytokines in these sequentially stimulated cells were compared with that of cells that were stimulated individually with either Poly i/c or PAM_3CSK_4 for 24 hrs. A comparison of mRNA expression for IL-6, GM-CSF, RANTES and IFN-β, a cytokine typically associated with viral infection, after Poly i/c treatment for 24 hrs, followed by PAM_3CSK_4 stimulation for an additional 24 hrs was carried out by QRT-PCR and the result is shown in Figure 5. Stimulation with PAM_3CSK_4 for 24 hours produced only a modest increase in mRNA expression of IL-6 and GM-CSF but did not affect RANTES or IFN-β. However, expression of IL-6 mRNA was augmented by forty- fold in response to PAM_3CSK_4 when cells were pretreated for 24 hrs with Poly i/c, which was shown to up-regulate mRNA expression of TLR2 (Fig. 5A). To determine the kinetics of IL-6 and GM-CSF mRNA expression, NHBE cells were exposed to Poly i/c at different time points and induction of IL-6 and GM-CSF mRNA expression following stimulation with Poly i/c was found to be time-dependent (Fig. 4). Moreover, IL-6 mRNA levels were profoundly enhanced within 3 hours following stimulation and reached a plateau that was ~50 fold higher than baseline after 24 hours (Fig. 4). On the other hand, induction of GM-CSF mRNA synthesis was more gradual, achieving a similar maximum fold increase as IL-6 after 24 hours (Fig. 4).

Apical* Poly i/c *exposure enhances secretion of IL-6 and GM-CSF across the apical membrane

Unlike PAM_3CSK_4 and flagellin stimulation alone, apical exposure of NHBE monolayers to poly i/c alone for 24 hrs or following sequentially stimulation for another 24 hrs with PAM_3CSK_4 or flagellin after initially priming with Poly i/c for 24 hrs induced robust, polarized secretion of IL-6 mainly across the apical membrane (Fig. 7A, & B). The effect of Poly i/c on GM-CSF secretion was lower than that observed for IL-6, although a greater amount of secretion was directed into the apical solution similar to IL-6 (Fig. 7C, & D). Stimulation of monolayers with PAM_3CSK_4 or flagellin, following 24 hour pretreatment with Poly i/c resulted in significant enhancement of IL-6 secretion into both the apical and basolateral solutions (Fig. 7A, & B). Similarly, an increase in apical and basolateral secretion of GM-CSF was also measured in response to PAM_3CSK_4 or flagellin following apical exposure to Poly i/c (Fig. 7C, & D). These results demonstrate significant directional secretion of IL-6 secretion across the apical membrane of NHBE monolayers following apical Poly i/c exposure that is significantly enhanced by PAM_3CSK_4 or flagellin.

PAM_3CSK_4 mediated increase in IL-6 secretion was dependent on TLR2 activation

The effect of known TLR2 ligands (PAM_3CSK_4 and Alternaria alternata extract, zymosan, and LTA) on apical IL-6 secretion was measured after pretreatment of NHBE cells with Poly i/c for 24 hours. Both PAM_3CSK_4 and Alternaria extract produced a significant increase in secretion compared to their effects in the absence of Poly i/c (Fig. 8A). In contrast, zymosan and LTA did not produce significant changes in IL-6 secretion alone and no significant increase was detected when monolayers were pretreated with Poly i/c (Fig. 8A).

To verify that the increase in IL-6 secretion evoked by PAM_3CSK_4 was dependent on binding to TLR2, monolayers were pretreated with TLR2 blocking antibody during the time period they were exposed to PAM_3CSK_4. The results (Fig. 8B) show that monolayers simultaneously treated with TLR2 blocking antibody failed to exhibit a significant increase in IL-6 secretion in response to PAM_3CSK_4, demonstrating that the TLR2 blocking antibody was effective at inhibiting the PAM_3CSK_4 response after Poly i/c treatment. In contrast, pretreatment with TLR2 blocking antibody failed to block the increase in IL-6 secretion evoked by Alternaria extract following Poly i/c exposure, suggesting that TLR2 was not responsible for the increase in responsiveness to *Alternaria alternata* extract.

Silencing TLR3 reduces the inflammatory response to* Poly i/c *stimulation

Given the existence of multiple, redundant dsRNA signaling pathways (TLR3, RIG-I, MDA5, PKR), we determined the relative contribution of TLR3 signaling to the Poly i/c-induced inflammatory response in immortalized normal human bronchial epithelial cells. We quantified changes in TLR2 mRNA expression and secretion of selected cytokines in HBE cells stimulated with Poly i/c following stable knockdown of TLR3 with lentiviral mediated introduction of shRNA against TLR3. Furthermore, compared with control empty vector-transfected cells, TLR3 shRNA-transfected cells demonstrated abrogated inflammatory responses to Poly i/c stimulation with 83% reduction in IL-6 level (Fig. 9A). No significant difference in Poly i/c-dependent IL-8 secretion was observed between controls versus TLR3 silenced HBE cells with and without without subsequent exposure to PAM_3CSK_4 or *Alternaria extrac*t. Relatively higher levels of IL-8 secretion were induced by stimulating both cell types with *Alternaria extrac*t (Fig. 9B). Together, these results suggested that, despite the existence of redundant dsRNA signaling pathways, TLR3 contributes significantly to the Poly i/c-induced inflammatory response in the airway epithelium.

Poly i/c-mediated reduction of TLR5 mRNA expression in NHBE cells failed to exhibit a corresponding decline on cytokine/chemokine levels

As shown in Figure 5E to 5H, Poly i/c mediated down-regulation of TLR5 mRNA did not exhibit a corresponding reduction in IL-6, GM-CSF, RANTES or IFN-β mRNA expression with flagellin treatment of NHBE cells for 24 hrs, following 24 hrs of TLR3 ligand priming. Furthermore, IL-6 and GM-CSF protein secretion from NHBE cells stimulated with flagellin following Poly i/c priming did not match Poly i/c-dependent abrogation of TLR5 (Fig. 7B, & D). Conversely, apical exposure of NHBE cells to Poly i/c for 24 hrs and subsequent treatment with flagellin for another 24 hrs resulted in significant augmentation of IL-6 and GM-CSF secretion into both the apical and basolateral solutions (Fig. 7B, & D).

Flagella are required for NF-kB activation and cytokine/chemokine mRNA and protein expression in A549 cells infected with B. cenocepacia

A549/NFκB-luc cells (a modification of the original A549 cell model) constitutively expressing TLR5 (Fig. 10A) were employed for studying the degree of NF-kB activation and cytokine/chemokine expression during infection with three strains of *B. cenocepacia* that vary in their degree of motility as a result of molecular manipulation of their flagella (Fig. 10B). To determine whether impaired motility of *B. cenocepacia* affects cytokine induction, A549/NFκB-luc cells were exposed to stain J2315, CM58 and CM100 of *B. cenocepacia* and IL-8 mRNA expression and protein secretion were compared at different time points. Challenging A549/NFκB-luc cells with all the three strains of *B. cenocepacia* for 6 hrs enormously stimulated IL-8 mRNA expression by over 50 to 200 fold. The level of induction of the IL-8 transcript elicited with wild type *B. cenocepacia* (strain J2315; intact flagella) was comparable to the level induced by the flagella-complemented *B. cenocepacia* (strain CM100; flagella-complemented). However, fliI mutant *B. cenocepacia* (strain CM58; flagella-deprived) suppressed IL-8 mRNA expression by about 5 fold relative to the levels induced by both flagellated and flagella-complemented strains (Fig. 11A). Incubation of A549/NFκB-luc cells with both the wild type and flagella complemented strains induced statistically significant IL-8 protein secretion than cells exposed to the flagella mutant strain (Fig. 11B). However, the same level of induction as the wild type strain (1.5 fold more) was not achieved by the complemented strain (Fig. 11B). Interestingly, stimulation with flagella-deprived strain resulted in IL-8 secretion that paralleled the level detected from unstimulated control cells. Moreover, no significant synergistic or additive effect on IL-8 secretion was observed in cells incubated for 6 hrs with all the three strains of *B. cenocepacia* subsequent to exposure to poly i/c for 24 hrs (Fig. 12B).

Since flagella-mediated induction of cytokines/chemokines involve the NF-kB pathway triggered by TLR5 activation, we tested the effect of intact or mutant bacterial flagella on activation of the NF-kB pathway by incubating A549/NFκB-luc cells with the three strains of *B. cenocepacia* for different time points (2, 4, and 6 hrs) and measured the activity of this transcription factor by luciferase assay. With exposure to all the three strains of *B. cenocepacia,* A549/NFκB-luc cells exhibited relatively higher level of NF-kB activation at 6 hrs. At 4 and 6 hrs of incubation, NF-kB activation elicited by flagella-complemented strain of *B. cenocepacia* was as high as the activation elicited by the wild type strain with intact flagella. However, the level of NF-kB activation induced by the flagella mutant (motility impaired) strain was significantly lower than the level induced by both the flagella complemented and the wild type strains. Incubation of cells with the bacteria only for two-hours was not sufficient to distinguish the intensity of NF-kB activation elicited by the three strains of bacteria (Fig. 12A).

3. 5. Discussion

In this study, we demonstrate that TLR3 is the most abundant and functionally active TLR expressed largely in primary and immortalized surface bronchial epithelial cells (NHBE/HBE) compared to glandular (Calu-3) or alveolar (A549) epithelial cells. This difference may point to potential differences in innate immune signaling between primary and transformed airway epithelial cell lines as reported for primary and transformed esophageal epithelial cell lines (Lim DM et al. 2009) The data also revealed that primary and immortalized human airway epithelial cells can function autonomously as innate immune effector cells in response to TLR ligand stimulation. Interestingly, TLR3 stimulation of NHBE/HBE by Poly i/c leads not only to robust induction of cytokine/chemokine secretion by airway epithelial cells but also differentially regulates the expression of Toll-like receptors, notably of TLR2 and TLR5. The ability of TLR3 agonist stimulation of NHBE cells to modulate the expression of other TLRs implies the potential of viral infection in regulating the host response to pathogens sensed by these altered pathogen recognition receptors.

The profile of cytokines/chemokines induced by Poly i/c stimulation of NHBE cells is consistent with what others have previously reported in other human airway epithelial cell lines; in small airway epithelial cells (SAEC) (Ritter M et al. 2005), and in human bronchial epithelial cells, transformed by SV40 T-antigen (BEAS-2B cells) (Guillot L et al. 2004). In our study, NHBE cell stimulation with Poly i/c resulted in increased secretion of several pro-inflammatory cytokines including IL-6, GM-CSF, G-CSF and TNF-α, an anti-inflammatory cytokine, IL-10 and inflammatory chemokines including RANTES, GM-CSF, MCP-1, MCP-2 and GRO-α. These signaling molecules have been previously shown to be involved in airway inflammation by enhancing immune cell activation and recruitment (Bishop B and Lloyd CM 2003, Gern JE et al. 2003, Gonzalo J-A et al. 1998, Lukacs NW et al. 1996, Yamashita K et al. 2005). These sets of Poly i/c- induced cytokines/chemokines are part of a pronounced Th1 response leading to the recruitment and activation of neutrophils, macrophages and Th1 cells, which may contribute to viral exacerbations found in type-1 pulmonary diseases like COPD (Ritter M 2005). In addition, chemokines such as RANTES and GM-CSF have been shown to be involved in the recruitment and survival of eosinophils (Lampinen M et al. 2004) that contribute to Th2-mediated pulmonary immune responses like allergic asthma. NHBE cells also exhibited a significant increase in mRNA expression of IFN-β in response to Poly i/c stimulation, which is a well established antiviral response that occurs following activation of TLR3 (Meylan E and Tschopp J 2006, Hewson CA et al. 2005, Kumar A et al. 2006, Matsukura S et al. 2006). Overall, cytokines and chemokines important in the recruitment of various leukocytes and dendritic cells and which have relevance in allergic airway inflammatory diseases were shown to be released from the airway epithelium following stimulation with poly i/c.

The effect of Poly i/c on IL-6 and GM-CSF secretion was investigated further using polarized monolayers under apical air interface conditions instead of cells grown on standard tissue culture plates. Apical stimulation with Poly i/c resulted in preferential secretion across the apical membrane. Polarized secretion of cytokines and chemokines has been reported for several epithelial cell types but directionality of secretion varies depending on the stimulus or surface of exposure. Earlier studies with mouse and human endometrial epithelial cells showed that IL-6 and GM-CSF were secreted into the apical fluid by as much as 4-5 fold over basolateral secretion (Fahey JV et al. 2005, Jacobs AL et al. 1992). Similarly, experiments with primary human nasal epithelial cells under air interface conditions and alveolar epithelial cell monolayers have shown that exposure of the apical surface to particulates, in the case of nasal epithelial cells or to *Francisella tularensis*, in the case of alveolar epithelial cells, evokes IL-6 and GM-CSF secretion across the apical membrane (Auger F et al. 2006, Gentry M et al. 2007). In contrast, experiments using rat intestinal epithelial cell line, IEC-6, have demonstrated basolateral secretion of IL-6 in response to IL-1β stimulation, and nearly equal amounts of apical and basolateral secretion following stimulation with TNF-α (Mascarenhas JO et al. 1996). Results of the present study suggest that apically directed IL-6 and GM-CSF secretion would most likely activate and recruit immune cells already present in the airway lumen to the site of viral infection or epithelial damage. Given the large surface area of the epithelium and a relatively dispersed population of immune cells, greater amounts of IL-6 and GM-CSF secretion may partially compensate for the limitations of diffusion.

In order to investigate the functional consequences of TLR2 and associated co-receptor up-regulation on the epithelial immune response to various TLR2 ligands, monolayers were first pretreated with Poly i/c for a period of 24 hours to enhance (TLR2, Dectin-1 and CD14) or reduce (TLR5, CD36) receptor expression and subsequently stimulated with TLR2 and TLR5 ligands for another 24 hrs. Subsequent stimulation with a TLR2 specific ligand, PAM_3CSK_4, produced an increase in apical IL-6 secretion compared to the level evoked from monolayers that were not treated with Poly i/c. Enhanced IL-6 secretion was inhibited in monolayers that were simultaneously treated with anti-TLR2 blocking antibody, indicating that the effect of PAM_3CSK_4 was dependent on binding to TLR2. Previous studies have shown that triacyl lipopeptides such as PAM_3CSK_4 stimulate TLR1-TLR2 heterodimers, ultimately activating nuclear factor-kappa B (NF-κB)-dependent cytokine/chemokine expression and secretion (Takeuchi O et al. 2002, Triantafilou M et al. 2006). The increase in both IL-6 mRNA and protein secretion observed in Poly i/c stimulated NHBE cells was consistent with this interpretation. Moreover, increasing the level of TLR2 expression appears to be necessary for enhancing the responsiveness of airway epithelial cells to triacyl lipopeptides.

Although apical IL-6 secretion evoked by Alternaria extract was also enhanced by pretreatment with Poly i/c, it was not inhibited by simultaneous treatment with TLR2 blocking antibody. *Alternaria alternata* is a common saprophyte that is found in the soil and often on decaying vegetation in many locations worldwide (Fung F et al. 2000). Inhaled Alternaria spores can cause respiratory distress and airway hyper-responsiveness, particularly in asthma patients and individuals sensitized to Alternaria are three times more likely to develop asthma (Kauffman HF and van der Heide S 2003). *Alternaria alternata* was recently shown to constitutively express heat shock protein 60 (Hsp60) (Buzina W et al. 2008). Hsp60 possesses intrinsic immunostimulatory activity mediated by TLR2 and TLR4 signaling pathways leading to the activation of NF-κB and mitogen-activated protein kinases (Vabulas RM et al. 2001), implicating the potential importance of this airborne mold in airway inflammation. The results of the present study would suggest the possibility that exposure to dsRNA associated with viral infection may predispose some individuals to sensitization to Alternaria, but upregulation of TLR2 expression does not appear to be responsible for the effect. Therefore, Poly i/c may regulate the expression of another unidentified receptor or signaling pathway that mediates the increase in responsiveness to ligands associated with *Alternaria*.

Stimulation of monolayers with zymosan, a cell wall preparation extracted from yeast (*S. cerevisiae*), did not stimulate IL-6 secretion in either control or Poly i/c treated monolayers. Previous studies with macrophages and dendritic cells showed that TLR2 and Dectin-1 function collaboratively in the detection and response to fungal cell wall components, including zymosan (Gantner BM et al. 2003, Underhill DM 2003). Dectin-1 is known to activate Src and Syk kinases, triggering phagocytosis and production of reactive oxygen species (ROS). In addition, Dectin-1 is known to enhance cytokine secretion synergistically with TLR2 by activating NF-κB through stimulation of CARD9, although it is not clear whether Dectin-1 activation in the absence of TLR stimulation is sufficient to induce NF-κB-mediated transcription (Gross O et al. 2006). Our results are consistent with a previous study using human bronchial epithelial cells (BEAS-2B cells) which also failed to show activation in response to zymosan (Mayer AK et al. 2007). BEAS-2B cells do not appear to express Dectin-1 receptors, which would explain the lack of activation. Although Poly i/c exposure increased Dectin-1 mRNA expression, levels were much lower compared to CD14 (basal mRNA expression of Dectin-1 (C_T = 31.7) was approximately 1000 fold lower than the expression of CD14 (C_T = 21.8). It is worth noting however that failure to detect stimulation of cytokine secretion does not necessarily mean that NHBE cells do not respond to zymosan. Unlike macrophages, Dectin-1 signaling through CARD9 may not occur in airway epithelial cells, but activation of Src/Syc kinase-dependent signaling, perhaps leading to ROS production or phagocytosis, may have taken place but was not measured in the present study.

Experiments with LTA also failed to show stimulation of IL-6 secretion in either control or Poly i/c treated monolayers. This result was again consistent with data from BEAS-2B cells (Mayer AK et al. 2007). However, when BEAS-2B cells were transfected with TLR2 and CD36 as a means to increase protein expression for both receptors, cytokine secretion occurred in response to LTA and the Gram-positive bacterium, *Staphylococcus aureus*. It was concluded from the results with BEAS-2B cells that hypo-responsiveness to Gram-positive bacteria observed in airway epithelial cells was due to low expression of TLR2 and CD36 and that expression of both receptors was necessary to observe responses from the complete set of TLR2 ligands. Results from the present study support the idea that both TLR2 and CD36 up-regulation are necessary for airway epithelial cells to respond to LTA since Poly i/c was shown to decrease

CD36 mRNA expression by 62% and protein expression by approximately 1.8-2 fold. Based on these findings it appears that reduced expression of CD36 in monolayers exposed to Poly i/c may have contributed to the lack of enhanced responsiveness to LTA, in spite of elevated TLR2 expression.

Enhanced TLR2 expression and signaling in response to Poly i/c activation of the airway both in vitro and in vivo has been previously observed (Ritter M et al. 2005, Sukkar MB et al. 2006, Jenssen R et al. 2007). Expanding on the earlier findings, in the current work poly i/c treatment of NHBE/HBE cells resulted in enhanced mRNA expression of RIG-I and MDA5, suggesting that detection of viral RNA may also involve these RNA helicases in addition to TLR3. Silencing TLR3 reduced Poly i/c–mediated induction of TLR2 mRNA expression by 83.6%, indicating that Poly i/c induced enhancement of TLR2 mRNA and protein expression is TLR3-mediated. However, the role played by the RNA helicases in regulating TLR2 expression needs to be examined using a molecular approach involving RLR silencing.

The insight that TLR3 activation modulates the expression and function TLR2 and other PRRs, thereby regulating the host response to potential pathogens, has been recognized in the past (Ritter M et al. 2005, Sukkar MB et al. 2006, Jenssen R et al. 2007, Sato S et al. 2000, Bagchi A et al. 2007, Lim DM et al. 2009). However, no molecular mechanism has been described in cells or tissues of the airway to explain the mechanism. Based on the premises that several likely binding sites for NF-κB were recognized in the human TLR2 promoter (Haehnel V et al. 2002, Furuta T et al. 2008), the potential involvement of NF-κB in the transcriptional regulation of the human TLR2 gene has been speculated. The findings as of now are controversial. A few studies have demonstrated that NF-κB binding is required for the inducible regulation of TLR2 expression in human endothelial cells (Satta N et al. 2002), in murine hepatocytes (Matsumura T 2003), and in human esophageal epithelial cells (Lim DM et al. 2009). Conversely, others have demonstrated that no direct role was played by NF-κB in the activation of the TLR2 promoter in human monocytes and macrophages (Haehnel V et al. 2002). Since these opposing findings underline that NF-κB-mediated regulation of TLR2 induction may occur in a tissue- and cell-specific mode, thus the mechanism by which Poly i/c enhances TLR2 gene transcription and translation in NHBE cells remains to be determined.

The major pulmonary cells that support the survival of *B. cenocepacia* are primary type II pneumocytes, and the transformed lung epithelial cell line A549 is an accepted surrogate for these cells (Keig PM et al. 2001, Reddi K et al. 2003). Hence, in this study, we used A549 cells to evaluate the role that *B. cenocepacia* flagella has in activating NF-kB and in inducing IL-8 transcription and protein secretion. The induction of IL-8 by *B. cenocepacia* strain J2315 (flagellated strain) in A549 cells was consistent with the findings reported by previous investigators (Palfreyman RW et al. 1997**,** Tomochi et al. 2002). Production of the IL-8, a major neutrophil chemotactic cytokine, is a hallmark of the pathology of cystic fibrosis (CF) (Reddi K et al. 2003). Consequently, neutrophils, accounting for up to 90% of inflammatory cells in CF lung, release enzymes that contribute to the tissue damage and chronic lung inflammation (Stone P et al. 1995, Brennan S et al. 2001). Of the two bacterial agents known to cause chronic inflammation of the lung in CF patients, *B. cenocepacia* causes the most severe form paralleled with more IL-8 induction and greater neutrophilic recruitment than that caused by *Pseudomonas aeruginosa* (Reddi K et al. 2003). The difference in the degree of IL-8 secretion and neutrophil recruitment elicited by the two bacterial types may have been attributed to the potency of their molecular patterns in activating their matching recognition receptors. However, data supporting this claim is controversial. Our results show that the level of secreted IL-8 and the degree of NF-kB activation exhibited by A549/NFκB-luc cells treated with *fliI* mutant *B. cenocepacia* was comparable to untreated A549/NFκB-luc cells, suggesting that the IL-8-inducing and NF-kB activating component of *B. cenocepacia* is mainly the flagella. A TLR5 agonist, flagellin, extracted from bacterial flagellae, has been shown to be strong mediator for pulmonary inflammation (Liaudet L et al. 2003). This finding is in agreement with a study by Palfreyman and coworkers that implicated a non-LPS extracellular factor from *B. cepacia* to be responsible for the induction of IL-8 secretion in alveolar epithelial cells (Palfreyman RW et al. 1997). In contrast, other investigators have reported that the LPS from isolates of *B. cepacia* induced TNF-α in human monocytes (Zughaier SM et al. 1999) and IL-8 in A549 cells (Reddi K et al. 2003) in a CD14-dependent manner. These differences in the relative role of various components of microbes to elicit inflammatory responses may be due to differences in cell types, bacterial strains used and the duration of exposure of the cells to the agonists.

Poly i/c-induced differential regulation of TLR2 and TLR5 expression observed in NHBE/HBE cells has not been noticed in A549-NF-κB-luc cells, explaining why Poly i/c priming of A549-NF-κB-luc cells with flagellated *B. cenocepacia* did not produce synergistic or an additive outcome as compared to cells that were not Poly i/c primed. Since there might be differences in response to Poly i/c among the different cell types derived from different anatomical sites of the pulmonary system, the down-regulation of TLR5 by Poly i/c and the consequence for pulmonary infections with flagellated bacteria in other cells of upper airway origin, including NHBE/HBE requires further investigation.

In summary, experiments with NHBE cells demonstrated that exposure to synthetic dsRNA as a means to activate TLR3 resulted in up-regulation of TLR2 and certain co-receptors that conferred increased responsiveness to triacyl lipopeptides and to constituents present in extracts from Alternaria alternata. Failure to detect LTA was correlated with decreased mRNA expression of CD36, providing support for the contention that up-regulation of both TLR2 and CD36 receptor expression are necessary for increasing responsiveness of the airway epithelium to LTA and Gram-positive bacteria. The experiments with A549-NF-κB-luc cells indicated that flagella of *B. cenocepacia* play a major role in triggering NF-kB activation and IL-8 mRNA expression and protein secretion.

Figure 1: Effects Poly i/c on the expression of TLR2, TLR5 and respective adaptor proteins: (A) Comparison of Poly i/c-treated versus untreated cells showing that Poly i/c differentially regulates TLR2 and TLR5 mRNA expression. Both PAM_3CSK_4 (A) and flagellin (B) did not affect the expression of their cognate receptors. Poly i/c increased mRNA expression of MyD88 and TIRAP (C, D), adaptor proteins involved in downstream signaling of TLR2, unlike the TLR2 ligand PAM_3CSK_4 and TLR5 ligand flagellin. Poly i/c also had an effect on TRIF (C, D), an adaptor protein involved in TLR3 signaling (n=3). GAPDH was used as an internal control to normalize measurements between control and Poly i/c treated conditions.

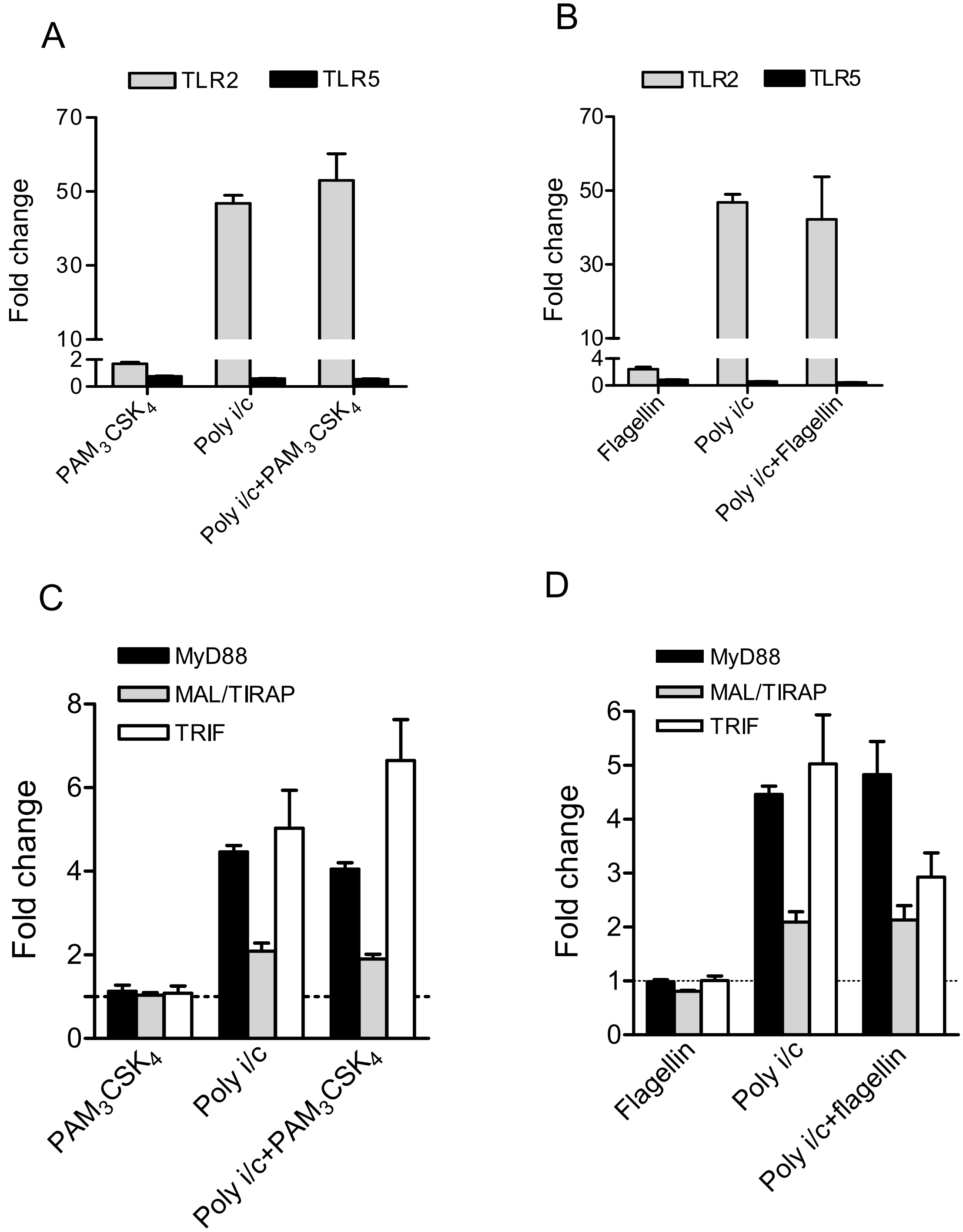
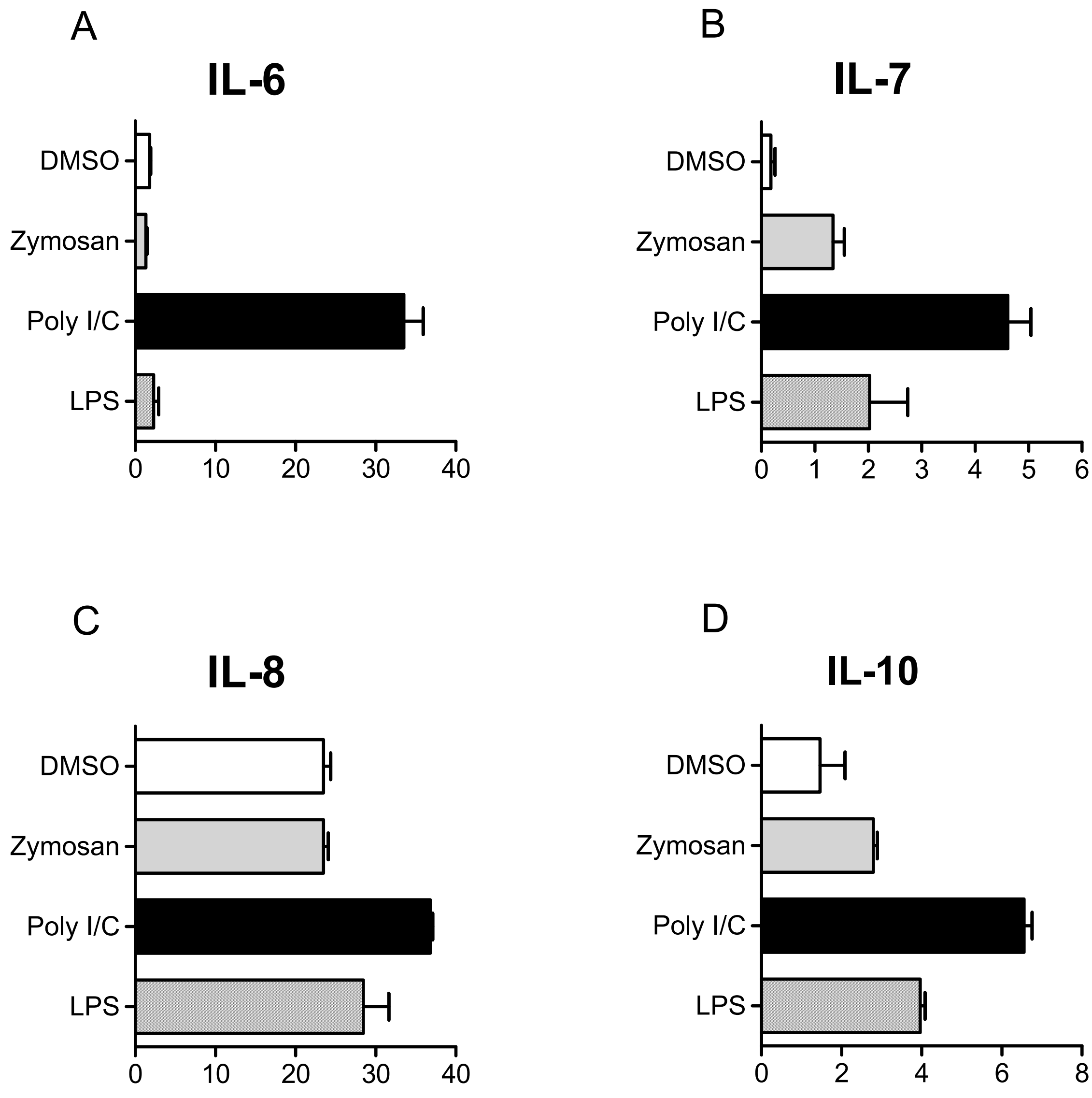
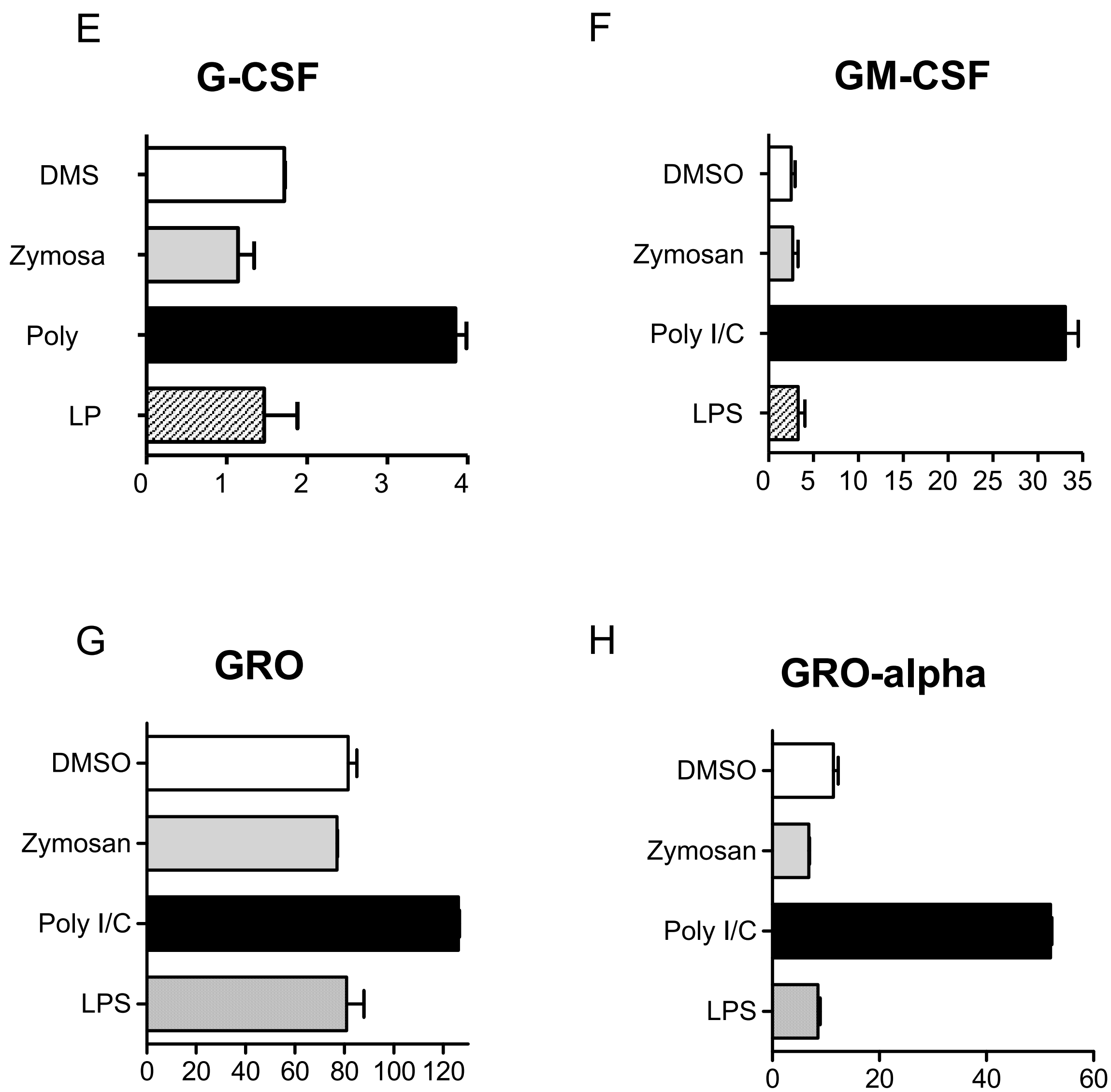

A
TLR2
TLR5
Fold change
70
50
30
10
2
0
PAM_3CSK_4
Poly i/c
Poly i/c+PAM_3CSK_4
B
TLR2
TLR5
Fold change
70
50
30
10
4
0
Flagellin
Poly i/c
Poly i/c+Flagellin
C
MyD88
MAL/TIRAP
TRIF
Fold change
8
6
4
2
0
PAM_3CSK_4
Poly i/c
Poly i/c+PAM_3CSK_4
D
MyD88
MAL/TIRAP
TRIF
Fold change
6
5
4
3
2
1
0
Flagellin
Poly i/c
Poly i/c+flagellin

Figure 2: Effects of Poly i/c treatment on NHBE cell cytokine and chemokine secretion: (A-L) Cytokines and chemokines released from NHBE cells into basolateral fluid in response to activation by different TLR ligands for 24 hrs (10 μg/mL of zymosan, 25 μg/mL of Poly i/c, and 10 μg/mL of LPS) were collected. The harvested fluid from each treatment condition was screened for 23 different cytokines and chemokines using the Human Cytokine Antibody Array I (RayBiotech, Norcross, GA, USA) as described in the Methods. Densitometry was performed on the images of the processed cytokine array membranes and the data expressed as a percentage of the positive controls present on the membrane (n=4).


A
IL-6
DMSO
Zymosan
Poly I/C
LPS
0
10
20
30
40
B
IL-7
DMSO
Zymosan
Poly I/C
LPS
0
1
2
3
4
5
6
C
IL-8
DMSO
Zymosan
Poly I/C
LPS
0
10
20
30
40
D
IL-10
DMSO
Zymosan
Poly I/C
LPS
0
2
4
6
8


E
G-CSF
DMS
Zymosa
Poly
LP
0
1
2
3
4
F
GM-CSF
DMSO
Zymosan
Poly I/C
LPS
0
5
10
15
20
25
30
35
G
GRO
DMSO
Zymosan
Poly I/C
LPS
0
20
40
60
80
100
120
H
GRO-alpha
DMSO
Zymosan
Poly I/C
LPS
0
20
40
60

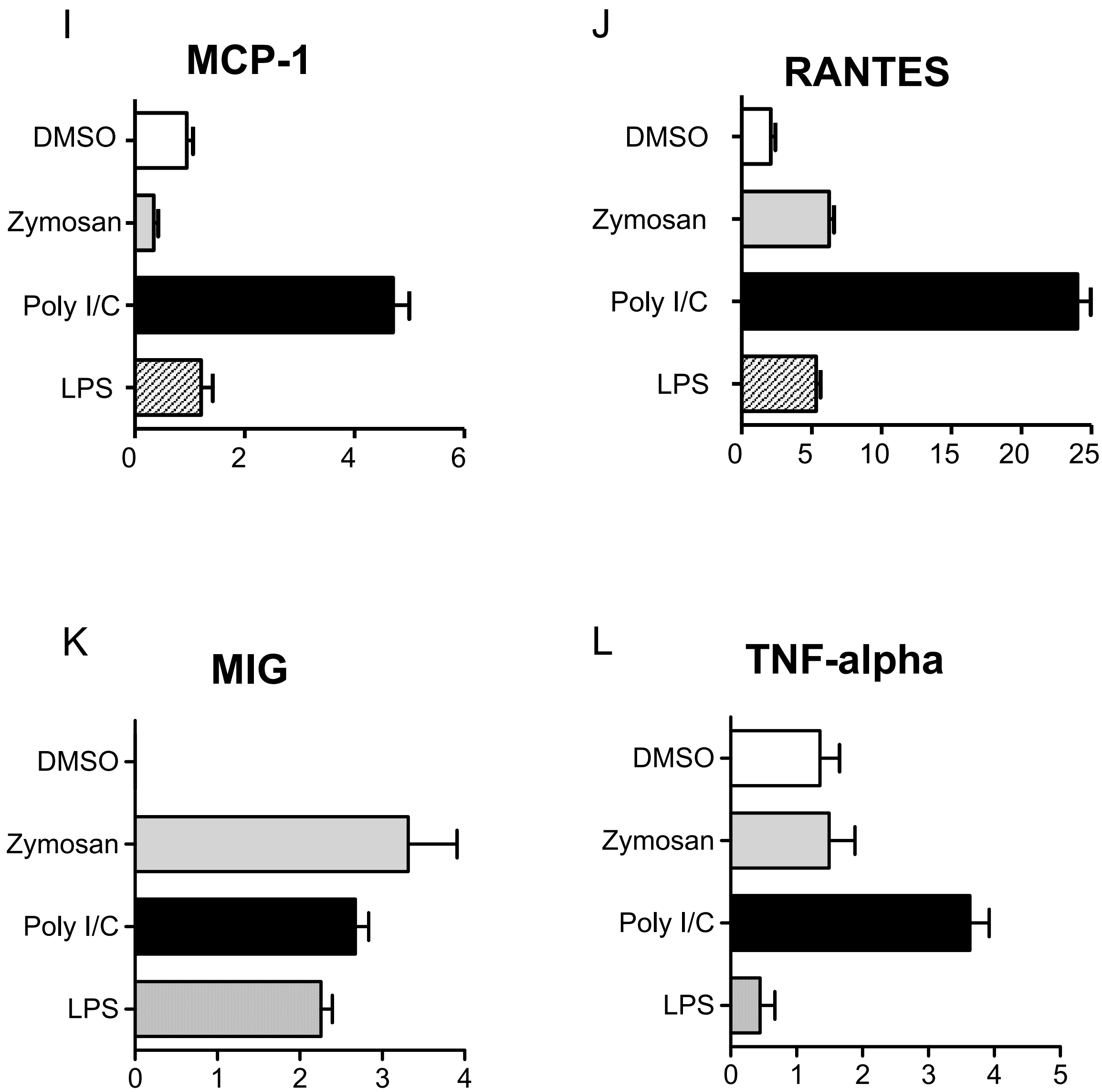

I
MCP-1
DMSO
Zymosan
Poly I/C
LPS
0
2
4
6
J
RANTES
DMSO
Zymosan
Poly I/C
LPS
0
5
10
15
20
25
K
MIG
DMSO
Zymosan
Poly I/C
LPS
0
1
2
3
4
L
TNF-alpha
DMSO
Zymosan
Poly I/C
LPS
0
1
2
3
4
5

Figure 3: Profiling of select cytokines and chemokines in response to Poly i/c stimulation in NHBE cells: Of the 23 detected cytokines and chemokines, analysis of protein levels of a select group of 9 exhibited ≥ 200% induction over untreated controls (n=4).

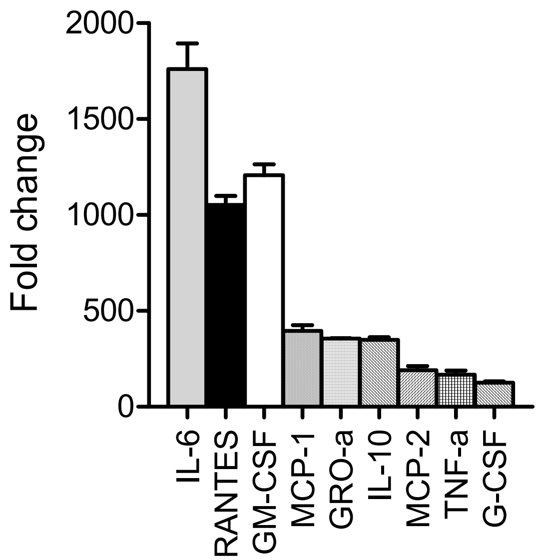

2000
1500
1000
500
0
Fold change
IL-6
RANTES
GM-CSF
MCP-1
GRO-a
IL-10
MCP-2
TNF-a
G-CSF

Figure 4: Time course showing mRNA expression of IL-6 and GM-CSF following exposure to 10 µg/mL Poly i/c: NHBE cells were exposed to Poly i/c at different time points, and induction of IL-6 and GM-CSF mRNA expression following stimulation with Poly i/c was found to be time-dependent (n=3).

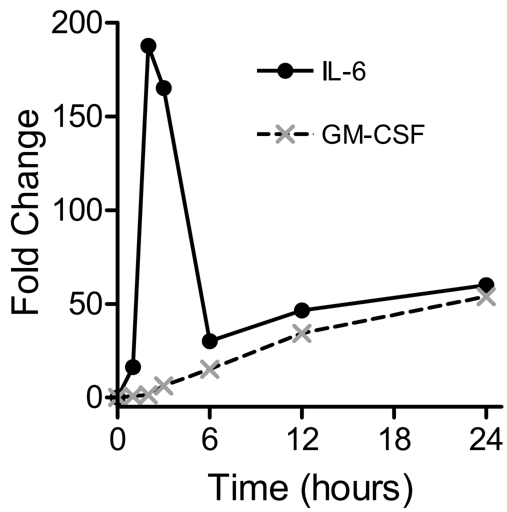
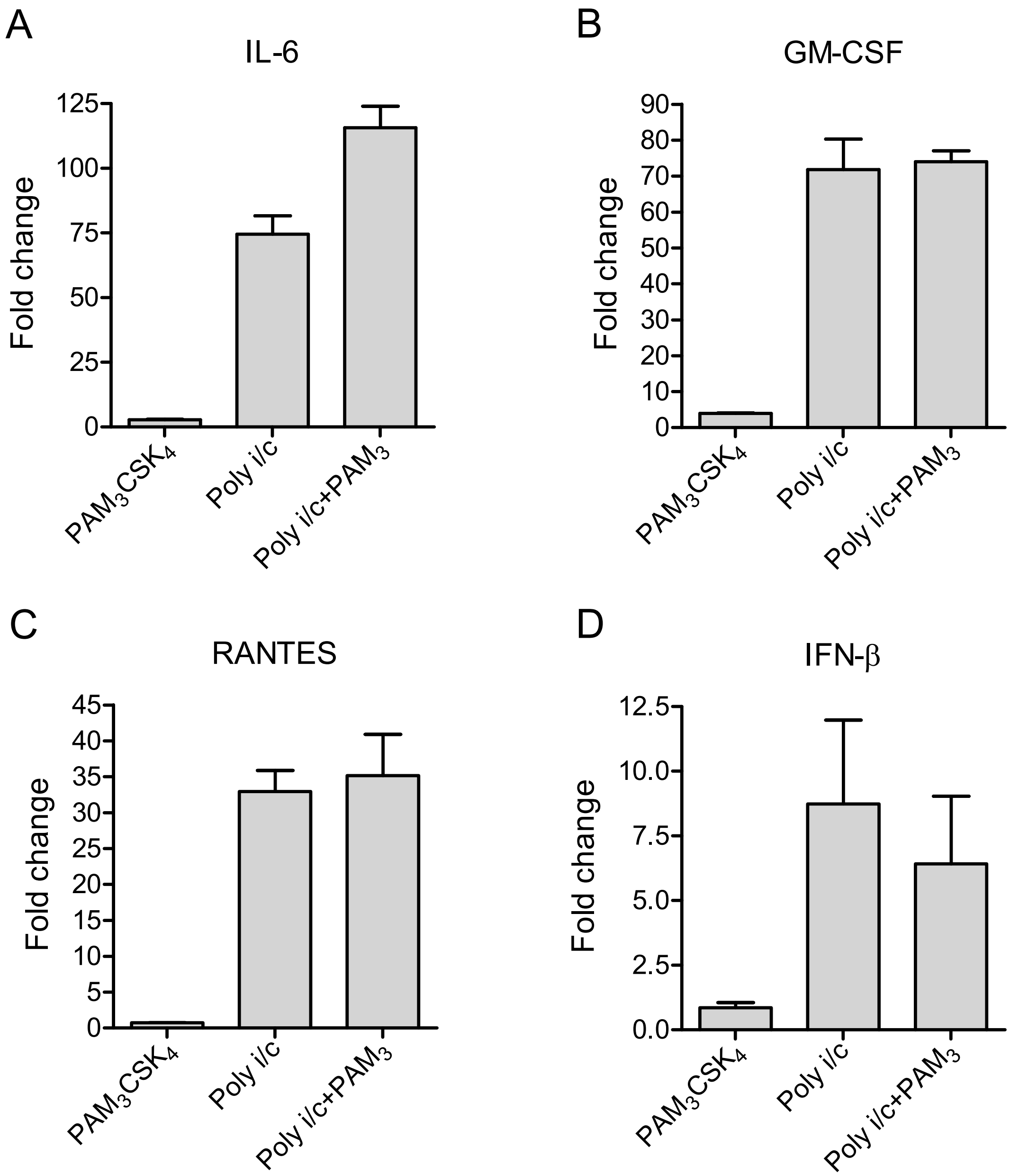

200
150
100
50
0
Fold Change
IL-6
GM-CSF
0
6
12
18
24
Time (hours)

Figure 5: Effects of TLR2 and TLR5 activation on mRNA expression of selected cytokines/chemokines after pretreatment of NHBE cells with Poly i/c: NHBE cells were stimulated with various combinations of Poly i/c and then TLR2 ligand, PAM_3CSK_4, (A-D) as well as TLR5 ligand, flagellin (E-H). Expression levels of transcripts of selected cytokines in these sequentially stimulated cells were then compared with cells that were stimulated individually with either Poly i/c or PAM_3CSK_4/flagellin for 24 hrs. Enhanced mRNA expression of pro-inflammatory cytokines (IL-6) and chemotactic chemokines (GM-CSF, RANTES) and the antiviral cytokine IFN-β was observed in cells treated with 10 μg/mL Poly i/c for 24 hours in contrast to 1 μg/mL of PAM_3CSK_4 which slightly increased mRNA expression of IL-6 and GM-CSF but did not affect RANTES or IFN-β (A-D). No reduction in cytokine mRNA levels was observed in Poly i/c-primed and flagellin-treated cells (E-H) (n=3).


A
IL-6
Fold change
0
25
50
75
100
125
PAM3CSK4
Poly i/c
Poly i/c+PAM3
B
GM-CSF
Fold change
0
10
20
30
40
50
60
70
80
90
PAM3CSK4
Poly i/c
Poly i/c+PAM3
C
RANTES
Fold change
0
5
10
15
20
25
30
35
40
45
PAM3CSK4
Poly i/c
Poly i/c+PAM3
D
IFN-β
Fold change
0.0
2.5
5.0
7.5
10.0
12.5
PAM3CSK4
Poly i/c
Poly i/c+PAM3

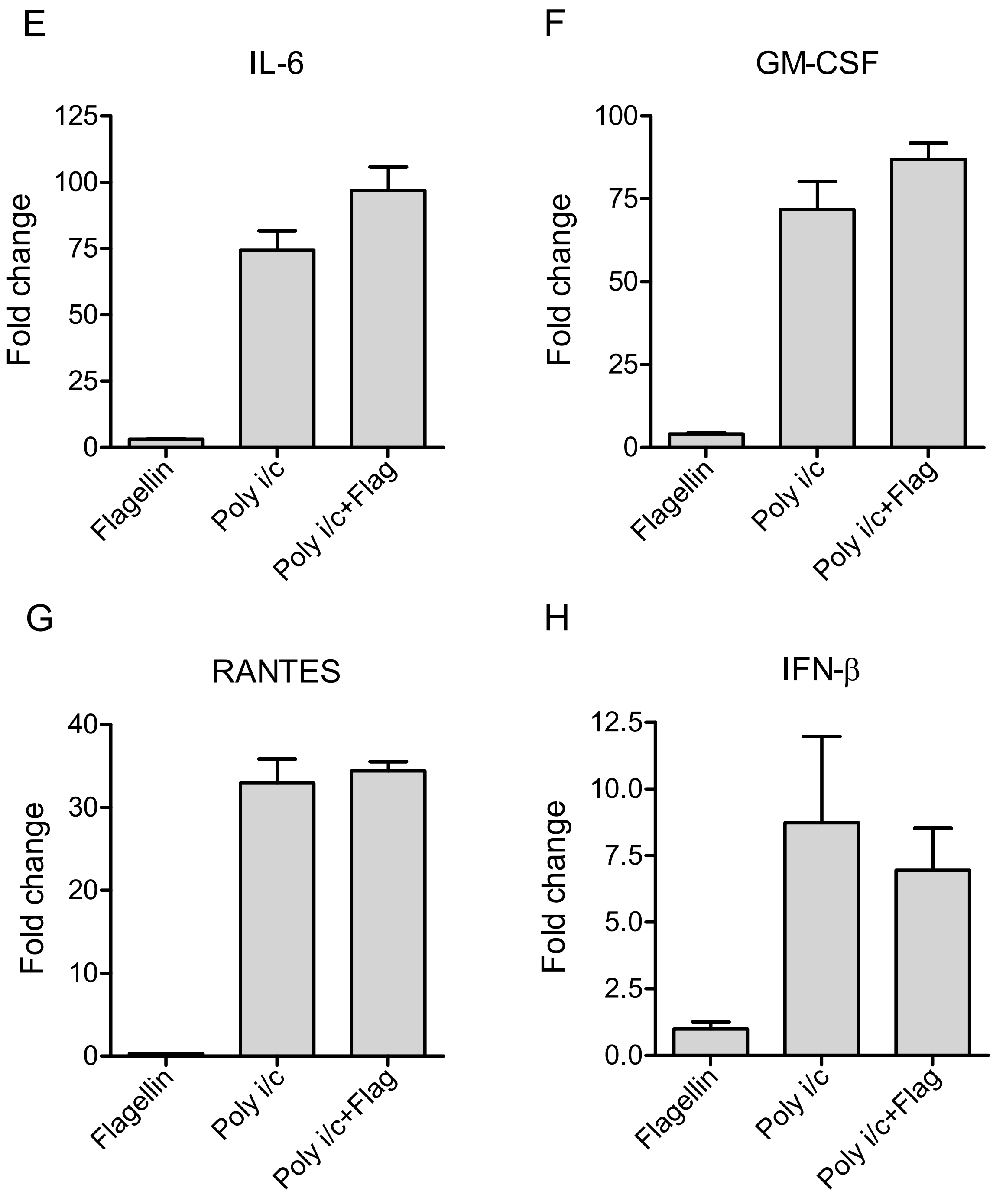

E
IL-6
125
100
75
50
25
0
Fold change
Flagellin
Poly i/c
Poly i/c+Flag
F
GM-CSF
100
75
50
25
0
Fold change
Flagellin
Poly i/c
Poly i/c+Flag
G
RANTES
40
30
20
10
0
Fold change
Flagellin
Poly i/c
Poly i/c+Flag
H
IFN-β
12.5
10.0
7.5
5.0
2.5
0.0
Fold change
Flagellin
Poly i/c
Poly i/c+Flag

Figure 6: Polarity of cytokine and chemokine secretion in relation to the side of exposure to the ligand: NHBE cells grown on transwell filters were exposed to Poly i/c on both apical and basolateral sides for 24 hrs. The fluid was collected from both sides, and levels of GM-CSF (A) and IL-6 (B) were determined using ELISA kit. The side to which cytokines and chemokines are preferentially secreted depended upon the side of exposure of NHBE cells to the activating ligand Poly i/c. Apical exposure tended to direct the secretion towards apical fluid, while basolateral exposure preferentially leads the release into the basolateral fluid.

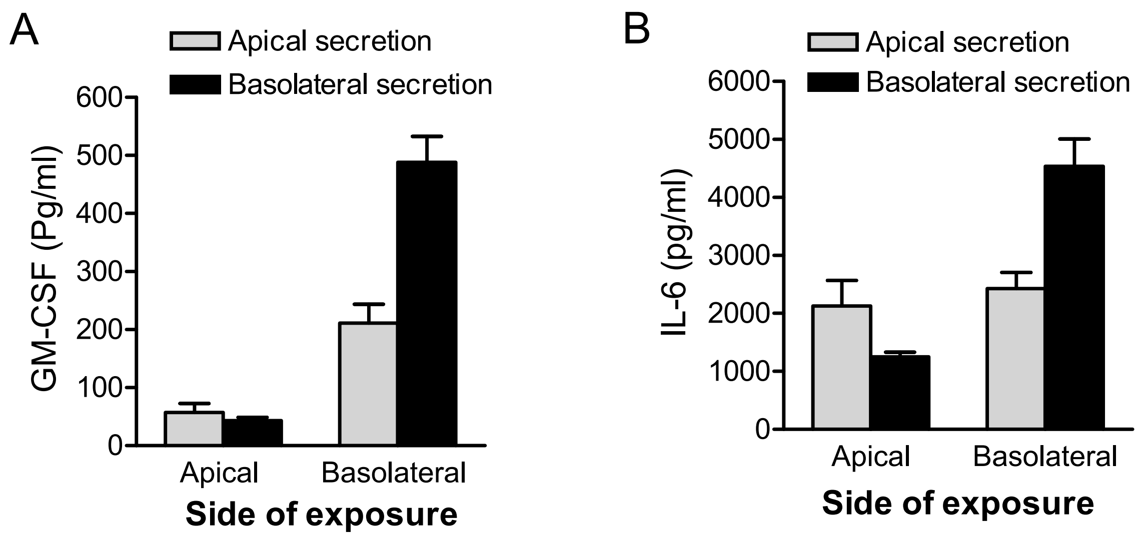

A
Apical secretion
Basolateral secretion
600
500
400
300
200
100
0
GM-CSF (Pg/ml)
Apical
Basolateral
Side of exposure
B
Apical secretion
Basolateral secretion
6000
5000
4000
3000
2000
1000
0
IL-6 (pg/ml)
Apical
Basolateral
Side of exposure

Figure 7: Effect of Poly i/c pretreatment of NHBE cells on sensitivity to a TLR2 ligand (PAM_3CSK_4) and TLR5 ligand (flagellin): Apical exposure of NHBE cells to Poly i/c (10µg/mL) induced robust release of IL-6 into the apical and basolateral fluid that was greater than that produced by PAM_3CSK_4 (A) or flagellin (B) alone**.** A significantly greater amount of IL-6 was secreted into the apical solution compared to the basolateral solution (n=6) (A, B).

Apical Poly i/c treatment also increased GM-CSF secretion into the apical and basolateral fluid relative to that produced by PAM_3CSK_4 (C) and flagellin (D) (n=3). Again, a significantly greater amount of secretion into the apical solution was observed relative to the basolateral solution (C, D).

The secretion of both IL-6 and GM-CSF into the apical and basolateral fluids was enhanced by pretreatment of cells with 10 µg/mL of Poly i/c for 24 hrs before stimulation with 1 µg/mL of PAM_3CSK_4 (or 0.5 µg/mL of flagellin) for another 24 hrs.

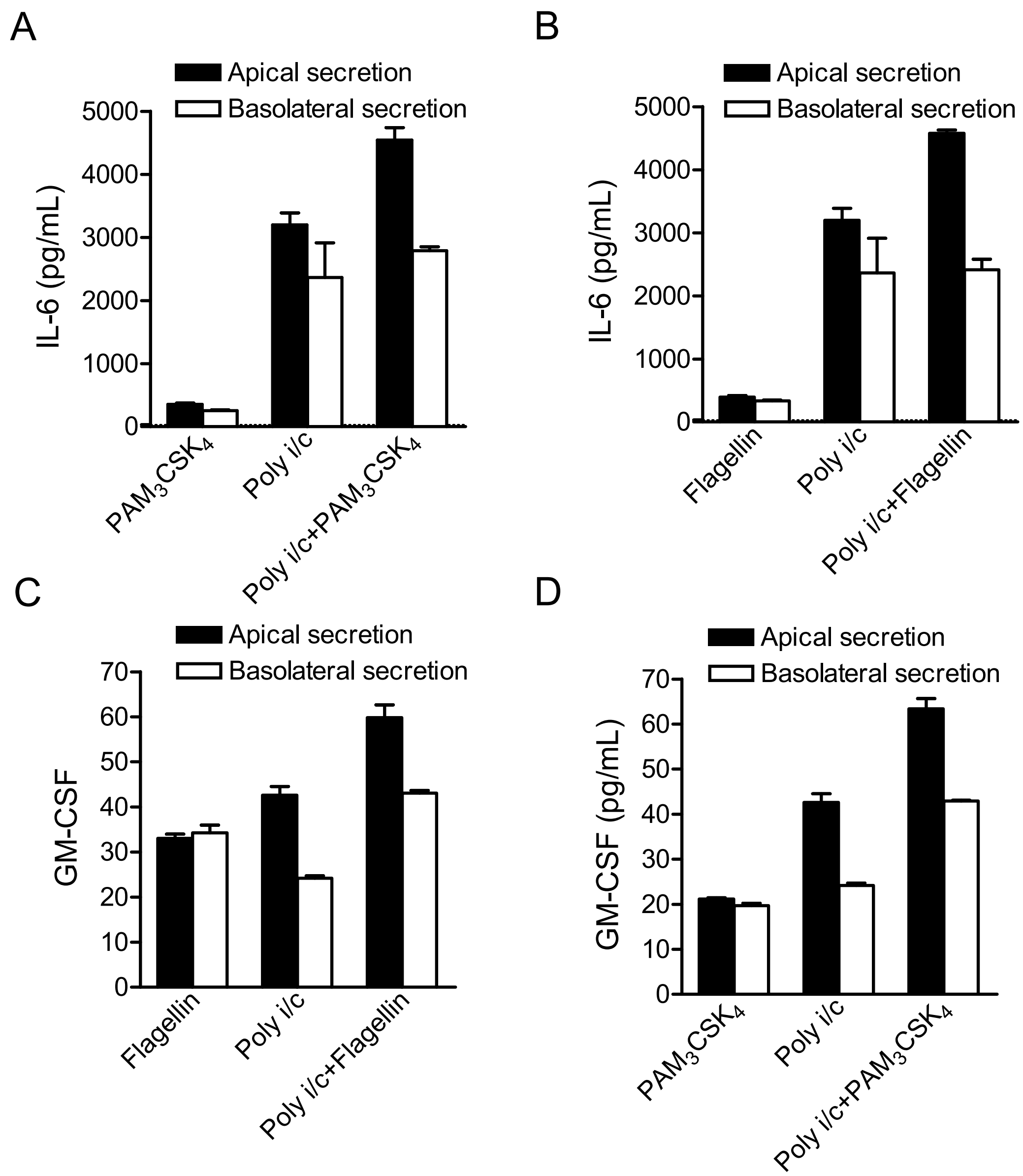
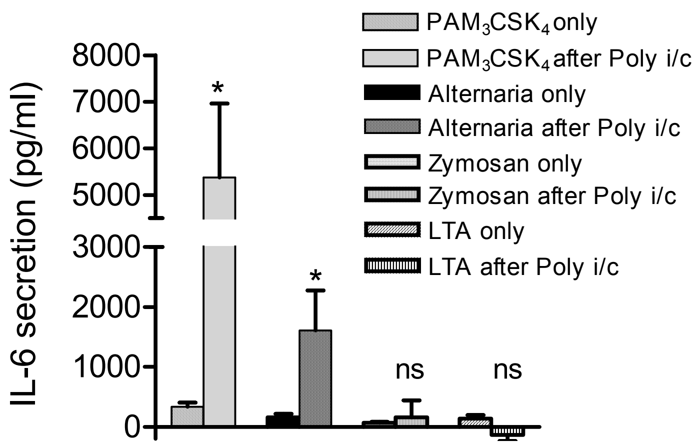

A
Apical secretion
Basolateral secretion
IL-6 (pg/mL)
5000
4000
3000
2000
1000
0
PAM3CSK4
Poly i/c
Poly i/c+PAM3CSK4
B
Apical secretion
Basolateral secretion
IL-6 (pg/mL)
5000
4000
3000
2000
1000
0
Flagellin
Poly i/c
Poly i/c+Flagellin
C
Apical secretion
Basolateral secretion
GM-CSF
70
60
50
40
30
20
10
0
Flagellin
Poly i/c
Poly i/c+Flagellin
D
Apical secretion
Basolateral secretion
GM-CSF (pg/mL)
70
60
50
40
30
20
10
0
PAM3CSK4
Poly i/c
Poly i/c+PAM3CSK4

Figure 8: Apical IL-6 secretion in response to specific TLR2 ligands following pretreatment with Poly i/c: (A) Secretion of IL-6 into the apical fluid was compared in monolayers treated with PAM_3CSK_4 (1 μg/mL), *Alternaria alternata extract* (100 μg/mL), Zymosan (10 μg/mL) and LTA (10 μg/mL) in the absence and presence of Poly i/c (10 μg/mL). Polarized monolayers were incubated with apical Poly i/c for 24 hours followed by the specified ligand for another 24 hrs. Basal secretion was determined by subtracting the level of IL-6 measured in untreated monolayers from the amount measured in TLR2 ligand treated monolayers. Secretion induced by each TLR2 ligand after Poly i/c treatment was determined by subtracting the level of IL-6 secretion measured in Poly i/c treated monolayers from the amount measured in Poly i/c + TLR2 ligand exposed monolayers. Significant increases in IL-6 secretion were observed in monolayers pretreated with Poly i/c and then treated with PAM_3CSK_4 and Alternaria extract but not with Zymosan or LTA.
(B) The increase in apical IL-6 secretion induced by PAM_3CSK_4 after Poly i/c pretreatment was inhibited by TLR2 blocking antibody. Monolayers of NHBE were apically treated with Poly i/c for 24 hrs then treated with anti-TLR2 antibody (Functional Grade purified TL2.1 monoclonal, at 20 μg/mL, for 2 hours before and during incubation with PAM_3CSK_4.

A


PAM3CSK4 only
PAM3CSK4 after Poly i/c
Alternaria only
Alternaria after Poly i/c
Zymosan only
Zymosan after Poly i/c
LTA only
LTA after Poly i/c
IL-6 secretion (pg/ml)
8000
7000
6000
5000
3000
2000
1000
0
*
*
ns
ns

B

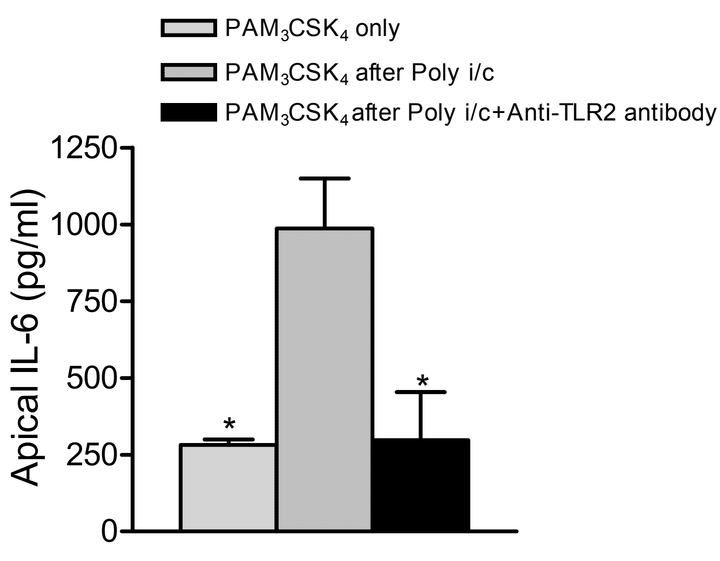

PAM3CSK4 only
PAM3CSK4 after Poly i/c
PAM3CSK4 after Poly i/c+Anti-TLR2 antibody
Apical IL-6 (pg/ml)
1250
1000
750
500
250
0
*
*

Figure 9: Effect of Silencing TLR3 in HBE cells on the response to Poly i/c: The relative contribution of TLR3 to the Poly i/c-induced cytokine response was assessed by quantifying IL-6 (A) and IL-8 (B) secretion in TLR3 knockdown ($TLR3^{-/-}$) and vector control HBE cells. Monolayers of both cell types were stimulated with PAM_3CSK_4 (1 µg/mL) and *Alternaria alternata extract* (100 µg/mL) in the absence and presence of Poly i/c (10 µg/mL). No basal secretion of IL-6 was detected in untreated cells. No detectable level of IL-6 secretion was observed in both cell types exposed to PAM_3CSK_4 or *Alternaria extrac*t. Poly i/c stimulation with or without subsequent exposure to PAM_3CSK_4 or *Alternaria extrac*t resulted in a marked abrogation of IL-6 secretion in HBE cells that exhibit silenced TLR3 as compared to the control cells (n=6). (B) No significant difference in Poly i/c-dependent IL-8 secretion was observed between controls versus TLR3 silenced HBE cells with and without without subsequent exposure to PAM_3CSK_4 or *Alternaria extrac*t. Relatively higher level of IL-8 secretion was induced by stimulating both cell types with *Alternaria extrac*t.

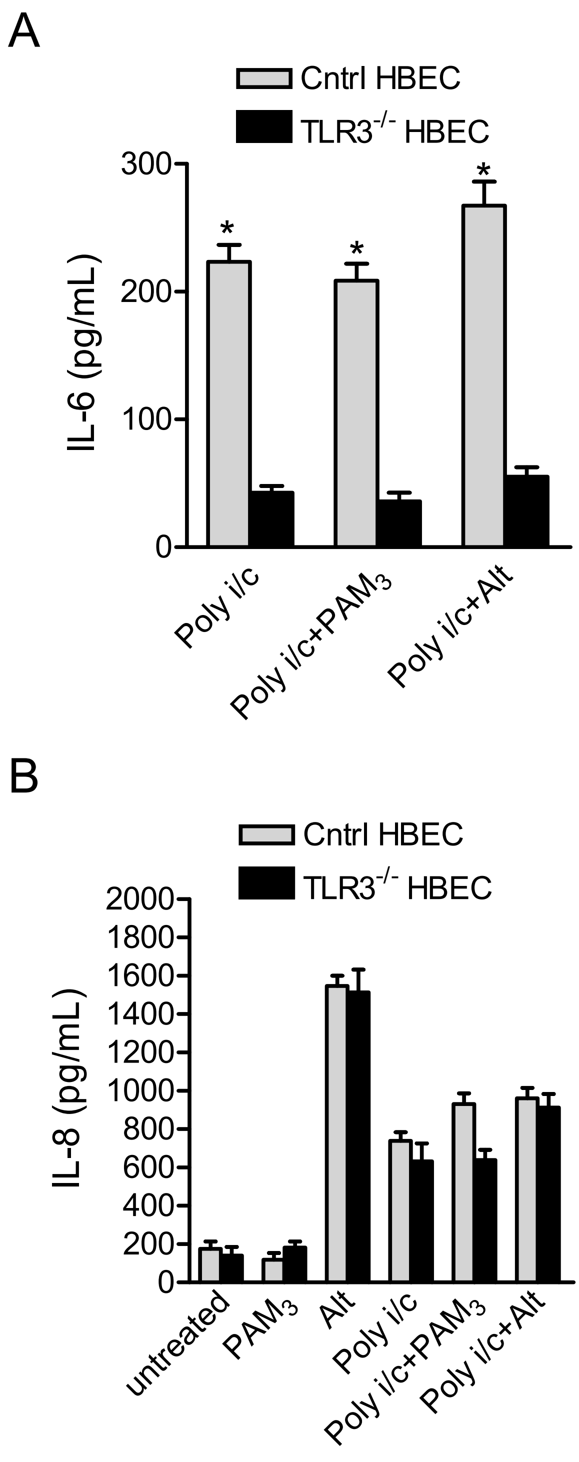
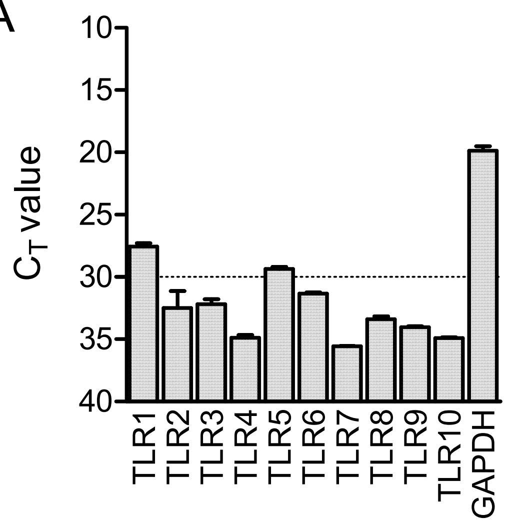

A
Cntrl HBEC
TLR3-/- HBEC
IL-6 (pg/mL)
300
200
100
0
*
*
*
Poly i/c
Poly i/c+PAM3
Poly i/c+Alt
B
Cntrl HBEC
TLR3-/- HBEC
IL-8 (pg/mL)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
untreated
PAM3
Alt
Poly i/c
Poly i/c+PAM3
Poly i/c+Alt

Figure 10: TLR profiling of A549/NFκB-luc cells and phenotypes of *Burkholderia cenocepacia*: (A) Basal mRNA expression of A549/NFκB-luc cells was determined by QRT-PCR. The results show TLR5, which detects flagellated bacteria and flagella, is highly expressed in this cell line.

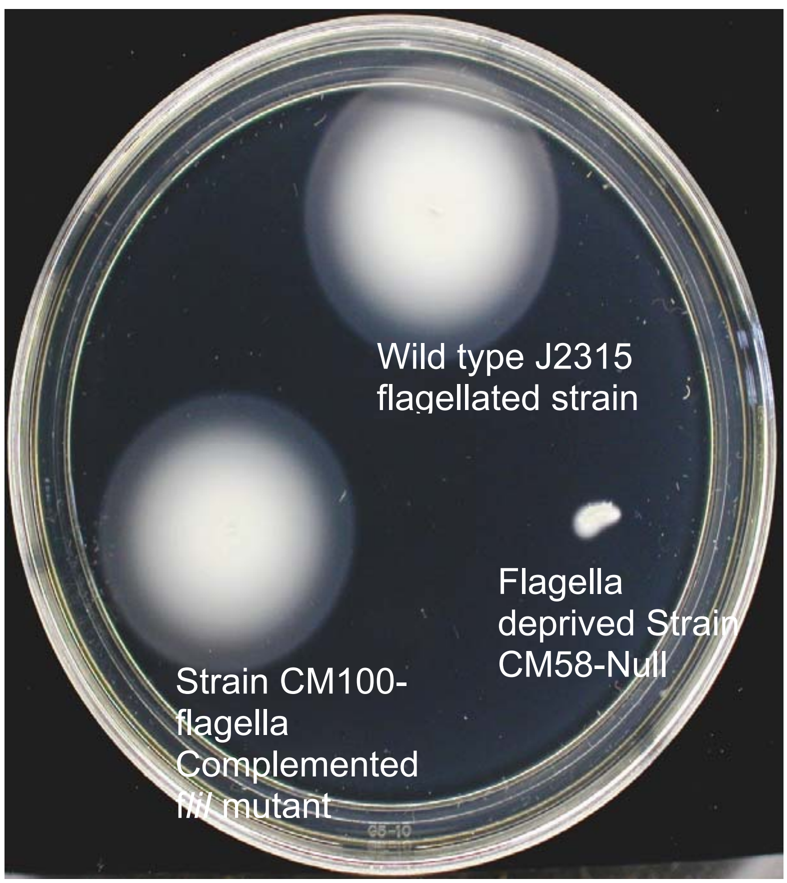
(B) Phenotypes of *Burkholderia cenocepacia* wild type strain and mutant strains with impaired and restored motility. Strains were stabbed into semisolid LB medium plates (0.25% agar) and incubated at 37˚C for 24 hrs. Flagellated wild type strain 2315, flagella-deprived mutant strain CM58 (*fli*I Mutant), and flagella-deprived strain CM58 complemented with *fli*I (strain CM100) are depicted in the figure.

A



10
15
20
25
30
35
40
C_T value
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
TLR10
GAPDH

B



Wild type J2315
flagellated strain
Flagella
deprived Strain
CM58-Null
Strain CM100-
flagella
Complemented
fliI mutant

Figure 11: Change in IL-8 mRNA expression and protein secretion after treatment of A549/NFκB-luc cells with *Burkholderia cenocepacia* strains: A549/NFκB-luc cells were exposed to all three strains of *B. cenocepacia*—J2315 (Wt), CM58 ($Fl^{-/-}$) and CM100 (Comp)—for 6 hrs. Total RNA was isolated from the exposed and control cells (Unt) and used for determination of IL-8 mRNA expression (A). Media from exposed cells was collected, and bacterial cells were sedimented by centrifugation. The supernatant fluid was collected in order to quantify IL-8 protein secretion using ELISA kit (B).

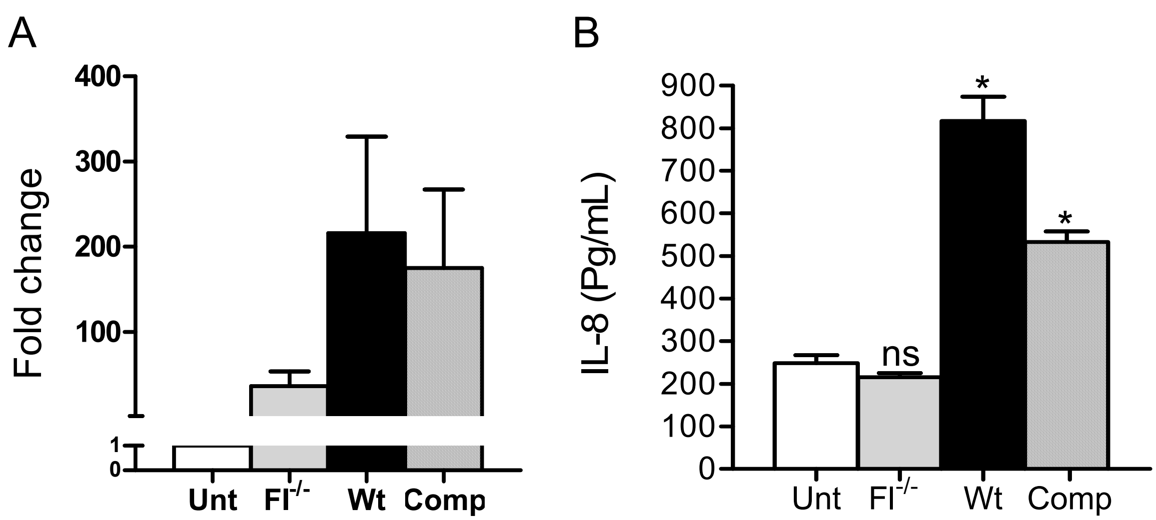
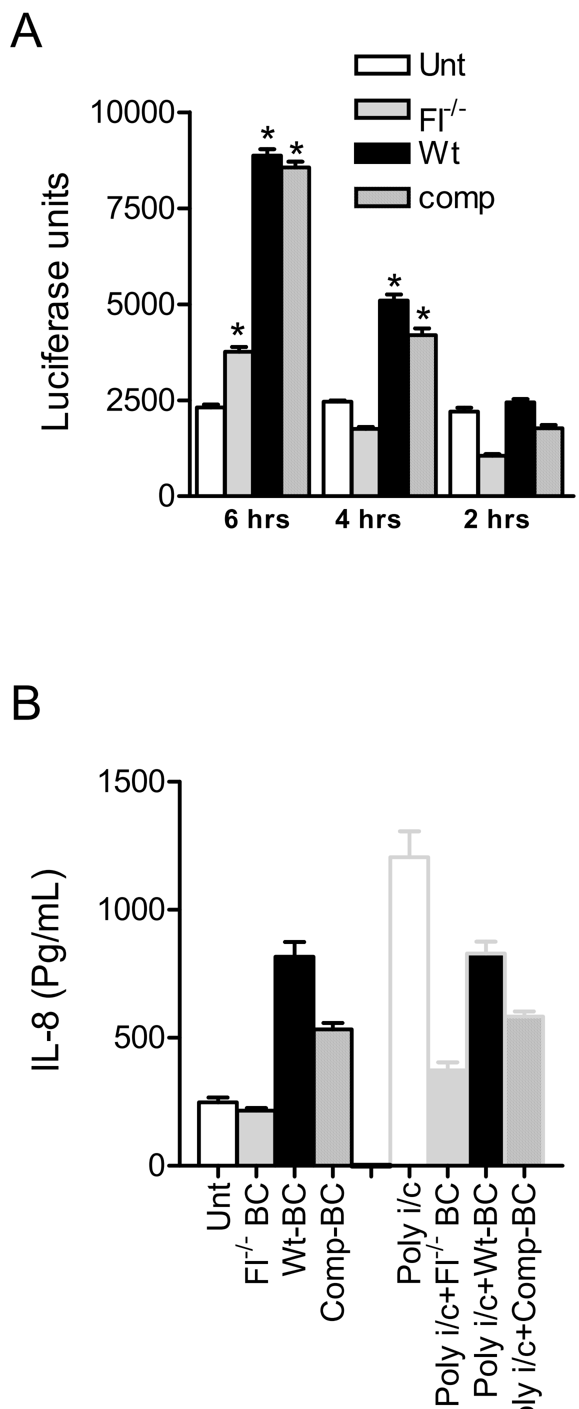

A
B
Fold change
400
300
200
100
1
0
Unt
Fl-/-
Wt
Comp
IL-8 (Pg/mL)
900
800
700
600
500
400
300
200
100
0
ns
*
*

Figure 12: Activation of NF-κB and induction of IL-8 secretion with the three strains of *Burkholderia cenocepacia* in A549/NFκB-luc cells: (A) The degree of NF-kB activation was determined by a Luciferase-based assay following exposure of A549/NFκB-luc cells to the three strains of *B. cenocepacia* (Wt, $Fl^{-/-}$, and Comp). Luciferase activity measured at different time points (2, 4, and 6 hrs) showed that the same level of NF-κB activation can be achieved in cells treated by the wild type bacteria (flagellated) and flagella-complemented strains, but activation was impaired when cells are treated by the mutant strain.
(B) This same pattern was detected with regards to IL-8 protein secretion; both wild type bacteria (flagellated) and flagella-complemented strains could induce IL-8 secretion.

A



A
Unt
Fl-/-
Wt
comp
Luciferase units
10000
7500
5000
2500
0
6 hrs
4 hrs
2 hrs
B
IL-8 (Pg/mL)
1500
1000
500
0
Unt
Fl-/- BC
Wt-BC
Comp-BC
Poly i/c
Poly i/c+Fl-/- BC
Poly i/c+Wt-BC
Poly i/c+Comp-BC

Chapter 4: The in vivo effects of enhanced TLR2 expression following TLR3 activation by dsRNA (Poly i/c) on airway inflammatory responses to TLR2 selective ligand (PAM_3CSK_4) and *Alternaria alternata*

4. 1. Introduction

Virus-mediated acute exacerbations account for a significant amount of the morbidity and mortality in chronic inflammatory airway diseases (Johnston SL et al. 1995, Kang M-J et al. 2005, Sapey E and Stockley RA 2006). Thus, mediators of the exacerbations are now considered to be reasonable targets for therapy against these diseases. In view of that, a thorough understanding of the underlying pathophysiology is crucial in identifying effective therapeutic strategies for asthma exacerbations. Viral exacerbations of chronic pulmonary diseases are believed to be due to inflammatory responses that overwhelm protective anti-inflammatory defenses (Sapey E and Stockley RA 2006, Papi A et al. 2006). Neutrophilic and lymphocytic airway influxes are recognized as dominant cellular components of the exaggerated inflammatory response during these exacerbations (Jarjour NN et al. 2000, Fraenkel DJ et al. 1995). Eosinophilic inflammation is also noticed during viral infection-induced asthmatic exacerbations, but not as robust as in atopic inflammation that involves no concomitant viral infection (Sigurs NR et al. 2000, Park SW et al. 2003). However, the mechanisms of recruitment of these cells and how resident and recruited cells in the lungs sense viruses and mount immune and inflammatory responses are poorly defined.

Stimulation of lung epithelial cells *in vivo* with Poly i/c elicited the secretion of multiple cytokines, chemokines, the induction of transcription factors and increased expression of TLRs (Sha Q et al. 2004, Ritter M et al. 2005, Melkamu T et al. 2009). The key mediator of these responses was suggested to be TLR3. Moreover, impaired responses to viral dsRNA and to Poly i/c have been documented in human cells and in mice deficient in TLR3 (Alexopoulou L et al. 2001, Rudd DB et al. 2005, Stowell NC et al. 2009, Lim DM et al. 2009). Yet, other nucleic acid-recognition proteins, including RIG-I and MDA5, have been implicated as viral sensors. Using actual virus (influenza A virus; IAV) and various molecular tools, an earlier *in vitro* study explored the respective role of TLR3 versus RIG-I/MDA5 signaling in human airway epithelial cells (Le Goffic R et al. 2007). Their data demonstrated that recognition of IAV by TLR3 primarily regulates a proinflammatory response, whereas RIG-I (but not MDA5) mediates both a type I interferon (IFN)-dependent antiviral signaling and a proinflammatory response. The relative importance of these proinflammatory cytokine and IFN responses in aggravation of asthma have been investigated by various investigators. A few studies involving *in vitro* human airway epithelial cells as well as analysis of bronchoalveolar lavage and sputum of patients revealed a positive correlation between the induction of proinflammatory (IL-1β, IL-6) and chemotactic (IL-8 and RANTES) cytokine and severity of disease (Grunberg K et al. 1997, Norzila MZ et al. 2000, de Kluijver J et al. 2003). Conversely, other studies failed to find differences in IL-6, IL-8, IL-11, GM-CSF and RANTES levels in nasal lavage or sputum samples from patients with virus-associated asthma exacerbations compared to virus-infected healthy subjects (Fleming HE et al. 1999, Grissell TV et al. 2005). These conflicting results highlight the need for further studies evaluating the inflammatory profile in asthma exacerbation models *in vitro* in various pulmonary cells, *in vivo* in animal models, and eventually in human subjects. Moreover, an impaired innate antiviral immune response, including impaired production of the IFN-β and reduced apoptosis have been documented in epithelial cells derived from asthmatic patients as compared to control cells (Wark PA et al. 2005). The consequence of these functional impairments is enhanced viral replication that eventually leads to cytotoxic cell death with the release of alarmins, inflammatory mediators and large numbers of intact viral particles, exacerbating the disease. The administration of IFN-β restores the virus protection observed in epithelial cells from normal airways. Whether these *in vitro* observations translate into new therapies aimed at augmenting or replacing deficient IFN-β production in asthma models warrants investigation.

In this study, we investigated the *in vivo* role of viral replicative intermediate, dsRNA, sensing receptors (TLR3/RIG-I/MDA5) in modulating the expression and functional consequence of TLR2. In our study design, we primed mice with Poly i/c or PBS for 12 hours, and then challenged with, a TLR2 specific agonist, a bacterial lipoprotein, PAM_3CSK_4, or with a fungal extract of *Alternaria alternata*. Exposure to Alternaria is implicated in the development and exacerbation of asthma and its effect is speculated to be TLR-mediated. Using heterologous (ovalbumin and *Alternaria alternata*) airway sensitization model of mice, Kobayashi and coworkers have demonstrated an enhanced sensitization to ovalbumin when supernatants of *Alternaria alternata* is co-administered as measured by airway eosinophlic inflammation, Th2 cytokine production , and serum IgE levels. The sensitization was significantly minimal in the absence of *Alternaria alternata* culture supernatant, implicating the significance of this fungal allergen in exacerbating allergic airway diseases (Kobayashi T et al. 2009). Employing knockout mice, the same researchers found out those TLR4-deficient mice exhibited the same Th2-type immune responses to OVA similar to wild-type mice. On the contrary, TLR2 knockout mice developed more robust Th2-type responses to ovalbumin and *Alternaria alternata* extract compared to wild type mice and underscore the possible involvement of TLR2 in sensitization with *Alternaria alternata* (Kobayashi T et al. 2009). In line with our experiments, a most recent *in vivo* study demonstrated that Poly i/c administration into mouse lungs induced a TLR3-dependent production of pro-inflammatory mediators that recruited inflammatory cells into the airways (Stowell NC et al. 2009). Furthermore, the study showed TLR3-mediated impairment of lung function by Poly i/c, implying the potential role of TLR3 activation during viral infections in contributing towards exacerbation of inflammatory airway diseases (Stowell NC et al. 2009).

4. 2. Rationale and objectives for the experiments

We and others have shown that *in vitro* stimulation of human airway epithelial and smooth muscle cells with Poly i/c augments levels of TLR2 mRNA and protein (Sha Q et al. 2004, Ritter M et al. 2005, Sukkar MB et al. 2006, Melkamu T et al. 2009). The functional consequence of this effect has also been determined by demonstrating that IL-6 secretion elicited by the TLR2 ligand, PAM_3SK_4, is potentiated when NHBE cells have been previously exposed to Poly i/c (Melkamu T et al. 2009). In line with these *in vitro* studies, increased mRNA expression of multiple TLRs including TLR2 and TLR3 as well as reduced mRNA expression of TLR5 were previously reported in *ex vivo* human nasal epithelial cells challenged with rhinovirus (Avila PC et al. 2005). In addition, microarray data from lung and bronchial lymph nodes of mice infected with respiratory syncitial virus also revealed that TLR2 and TLR3 are among the up-regulated genes (Jenssen R et al. 2007). Furthermore, ovalbumin-sensitized mice stimulated with Poly i/c exhibited an enhanced allergic inflammatory response, suggesting a role for dsRNA-sensing receptors (TLR3, RIG-I, MDA5 and PKR) in exacerbation of allergic airway inflammation during viral infection (Nagase H et al. 2006). Based on the results of these *in vitro* studies and the existing ex vivo human and *in vivo* mouse data, we hypothesize that *in vivo* activation of dsRNA-sensing receptors with Poly i/c or virus may alter the innate immune response to TLR2 activators and potentially increase the sensitivity of the airway epithelium to fungal allergens, but at this time there have been no reported studies that have addressed this possibility. Therefore, the objective of the studies presented in this chapter is to elucidate the functional consequence of dsRNA (Poly i/c) on the inflammatory response of mouse airways to a selective TLR2 ligand (PAM_3CSK_4) and to a common fungal allergen (*Alternaria alternata)* known to induce inflammation, in part through activation of TLR2.

4. 3. Materials and Methods

Mouse types:

For initial characterization of the effect of Poly i/c on the expression of TLR2 mRNA, protein and the functional consequences of this modulation, female C57BL/6 wild type mice 8 weeks of age were used. The mice were purchased from Jackson laboratory (Bar Harbor, ME). Prior to challenging with various TLR-ligands, mice were allowed to acclimatize by housing them for one week at Mayo clinic animal facility. The mice were then randomly assigned into six groups as follows:

1. Mice challenged with PBS for 24 hrs and then PBS for another 12 hrs.
2. Mice challenged with Poly i/c for 24 hrs and then PBS for another 12 hrs.
3. Mice challenged with Poly i/c for 24 hrs and then with PAM_3CSK_4 for another 12 hrs.
4. Mice challenged with PBS for 24 hrs and then with PAM_3CSK_4 for another 12 hrs.
5. Mice challenged with Poly i/c for 24 hrs and then with Alternaria for another 12 hrs.

6. Mice challenged with PBS for 24 hrs and then with Alternaria for another 12 hrs.

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC), at Mayo Clinic, Rochester, Minnesota.

Administration of TLR-agonists into the lungs of mice:

Mice were anesthetized with intraperitoneal injection of 200-400 µl of Avertin/mouse (100% Avertin: 10 g of 2,2,2-tribromoethyl alcohol and 10 ml of tert-amyl alcohol, Sigma). An additional 100 to 200 µl of Avertin was given when the mouse was not fully anesthetized within 5 minutes. The mice remained anesthetized for approximately 15-20 minutes and recovered within 30-60 minutes. Mice were kept warm during recovery. Each mouse was then intranasally (I.N.) instilled with Poly i/c 50 µg in 50 µl sterile PBS, or with 50 µl sterile PBS alone. Twenty-four hours following Poly i/c (or PBS) administration, the mice were re-anesthetized with Avertin and then I.N. challenged with PAM_3CSK_4 (10 µg in 50 µl sterile PBS/mouse) or with Alternaria extract (50 µg in 50 µl sterile PBS/mouse). Twelve hours after administering PBS, PAM_3CSK_4 or Alternaria extract, mice were euthanized with overdose of Avertin and then the trachea was cannulated with a 19 gauge cannula and bronchoalveolar lavage (BAL) was performed by injecting 1 mL of Hank's balanced saline solution (HBSS) into the lungs and the effluent fluid was collected by gentle aspiration.

Collection of lung tissue for histopathological and molecular assessment:

Following collection of BAL samples, the left lobes of the lung were removed and placed in 10% neutral buffered formalin for histopathological analysis. The leftover lung tissue with the airways was split in two; one-half was snap frozen in liquid N_2 for protein determination and Western blot analysis. The other half of lung tissue was placed in 1 ml of RNA-stabilizing reagent (RNAlater) (Ambion Inc., Austin, TX) for RNA extraction and quantitative RT-PCR. Samples for protein and RNA determination were stored at -80 ºC until processing.

Processing of the bronchoalveolar lavage samples:

Bronchoalveolar lavage was performed by intratracheal instillation of 1.0 ml of Hank's balanced saline solution (HBSS). The BAL fluids were fractionated by centrifugation (1200 rpm, 10 minutes) and the cell-free supernatants were collected and stored at -80°C until analyzed. The cell pellet was resuspended in 200 µl of 1ml PBS and total leukocyte counts were determined with a hemocytometer after Randolph's staining. For differential cell counts, cytospin preparations from BAL fluids were stained with Wright-Giemsa stain. Total and differential cell counting was performed using standard morphological criteria and was enumerated as the multiple of the total cell count and differential cell type percentage after counting at least 500 cells/slide under oil immersion at x1,000 magnification (Axolab, Zeiss). The cell-free supernatants were assayed for IL-1β, IL-5, IL-6, and IFN-β using duosets ELISA kits following the manufacturer's protocol (R & D systems, MPLS, MN). Limits of detection for the analytes ranged from 3 – 20 pg/ml.

Tissue sample processing for mRNA and protein determination:

Lung tissue was snap frozen at collection and stored at -80 ˚C until processing. Aliquots of frozen normal lungs, lung tissue from different treatment groups were then dispersed and ground into powder with a mortar and a pestle in liquid nitrogen. For RNA isolation, the powdered whole lung tissue was homogenized by adding Trizol reagent to it. RNA was isolated using manufacturer's instructions (Life Technologies, Gaithersburg, MD). Quality and quantity of the RNAs were assessed by A260/A280 nm reading and by running on a gel and checking for its integrity. Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) assay was performed as it was described in the methods section of Chapter 2 of this thesis. Primer sets for the target genes were developed using Primer 3 software or taken from published work and all sequences used for real time PCR analysis of mouse lung tissue are given in Table 2.

For Western blot analysis, the powdered tissue was transferred into an eppendorf tube and homogenized by adding lysis buffer (RIPA) and incubated with agitation for 1 hour at 4 ºC. The homogenate was then clarified by centrifugation at 13000 RPM for 10 minutes, the supernatant was transferred into a new tube and the protein concentration determined by BCA and the samples were aliquoted and stored at -80°C. For Western immunoblotting, 50 μg of protein per sample were loaded onto a 4-12% Novex Tris-glycine gel (Invitrogen, Carlsbad, CA) and electrophoresis performed for 60 min at 200 V. The proteins were then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) for 2 h at 30 V. Subsequently, membranes were blocked in 5% Blotto non-fat dry milk in Tris buffer containing 1% Tween-20 for 1 h and probed overnight with anti-TLR2 primary antibody (1:200), sc-12504, from Santa Cruz Biotechnology.

After incubating the membranes with a secondary antibody (donkey anti-goat IgG, 1:5,000, sc-2020, Santa Cruz Biotechnology) for 1 h, chemiluminescent immunodetection was employed. Signal was visualized by exposing membranes to HyBolt CL autoradiography film. To check for differences in the amount of protein loaded in each lane, membranes were stripped and probed with anti-β-tubulin, at 1:200, sc-9104 also from Santa Cruz Biotechnology.

Histological Analysis:

The left lobes of the lung placed in 10% neutral buffered formalin were submitted for histopathological analysis. Tissue was processed by routine methods of sectioning and hematoxylin and eosin staining. Histopathological analysis of lung sections were performed by a board certified veterinary pathologist.

4. 4. Results

Basal and Poly i/c-induced changes in TLR mRNA levels in whole lung of C57BL/6 wild type mice:

Basal mRNA expression of TLR1–9 in was measured by QRT-PCR in whole lung tissue of PBS treated control mice. All of the TLRs were expressed: TLR8, TLR3, and TLR7 were expressed at relatively higher levels (mean C_T values 27.4, 27.4, and 28.6 respectively); TLR5, TLR6, TLR2, and TLR1 at intermediate levels (mean C_T values 30.5, 31, 31.1, and 33.9 respectively), and TLR4 at the lowest level (mean C_T value 38.4) (Fig. 1A). In an effort to understand how intranasal instillation of Poly i/c to mice modulates mRNA expression of TLRs of the lung, QRT-PCR analysis was performed. Several TLRs exhibited more than a 2-fold increase following Poly i/c instillation, including TLR2 (10.9), TLR1 (4.9), TLR9 (3.6), TLR8 (2.8), TLR3 (2.4) and TLR6 (2.2) whereas TLR4 and TLR5 exhibited down-regulation and TLR7 did not show any significant change (Fig. 1B). Moreover, TLR2 mRNA expression, which exhibited the highest augmentation in the lung following Poly i/c challenge, also exhibited enhanced protein expression as revealed by western blot analysis (Fig. 2).

Quantitative and qualitative changes in cellularity of bronchoalveolar lavage (BAL) fluid following Poly i/c exposure and TLR2 activation:

Total and differential cell counts in BAL fluid were performed to determine changes in immune cell infiltration of the airways following challenge with Poly i/c alone or subsequently with selected TLR2 ligands. BAL fluid from mice challenged only with Poly i/c did not exhibit a significant difference in total cell count from lavage fluid of vehicle-treated control mice. However, there was a remarkable difference in the composition of cells; in BAL of vehicle-treated mice 92.5% of cells were of monocytes/macrophage lineage, while in BAL of Poly i/c-treated mice over half (52%) were neutrophils (Fig. 3B). Unlike mice that were challenged only with Poly i/c for 24 hrs, mice that were either primed with Poly i/c or treated with vehicle (PBS) for 24 hrs and then sequentially treated with PAM_3CSK_4 for 12 hrs exhibited a dramatic increase in cellularity of BAL as compared to the BAL from control mice (Fig. 3A). The remarkable boost in total cellularity of lavage fluid in PAM_3CSK_4 treated mice following Poly i/c-priming or PBS-treatment was largely due to a significant increase of neutrophils (89.6% and 75.2%, respectively) (Fig. 3B). Mice challenged with Alternaria extract with and without Poly i/c-priming also exhibited a phenotypic change in BAL cells as compared to BAL fluid from vehicle-treated control mice, showing similar amplification in the proportion of neutrophils (75.4% and 53.4% in Poly i/c primed and unprimed mice, respectively). Furthermore, the fraction of lymphocytes was found to be relatively larger whenever the mice were primed with Poly i/c.

Changes in BAL protein levels and in lung tissue mRNA level of selected cytokine/chemokines following Poly i/c priming and TLR2 activation:

To elucidate the mechanism by which intranasal instillation of Poly i/c exacerbates the allergen-induced airway sensitization; we analyzed secretion profiles in BAL fluids and mRNA expression patterns in lung tissue of selected cytokines. We assessed levels of IL-1β, IL-6, (T helper 1 (Th1) cytokines), IL-5 (Th2 cytokine) and IFN- β (antiviral-response) in BAL fluid. We also analyzed lung tissue mRNA expression of signature cytokines for Th1 (IL-1β, IL-6), Th2 (IL-5, IL-13), and anti-viral dsRNA (IFN-γ, IFN-β) responses. No measurable amount of the tested Th1 or Th2 cytokines were detected in the lavage fluid of vehicle-treated control mice (Fig. 4A-D). In contrast to the non-detectable cytokine level in the lavage fluids from vehicle-treated control mice, stimulation with Poly i/c, PAM_3CSK_4 and Alternaria extract generated a significantly higher level of the Th1 cytokines (IL-1β, IL-6 and IFN-β) in BAL fluid (Fig. 4A, & 4B). Priming mice with intranasal instillation of Poly i/c for 24 hrs potentiated the secretion of Th1 cytokines by 3 to 5 fold (IL-1β (3 fold), IL-6 (5 fold), and IFN-β (5 fold) when mice were subsequently challenged for 12 hrs with TLR2 specific ligand (PAM_3CSK_4) than those challenged with the vehicle (PBS) (Fig. 4C). Twelve-hours after intranasal instillation of mice with Alternaria extract, slightly enhanced secretion of Th1 cytokines (1.6 fold for IL-1β, 1.9 fold IL-6, and 1.7 fold for IFN-β) was detected in the BAL fluid of mice pretreated with Poly i/c compared to PBS pretreated mice (Fig. 4C, & 5A). Interestingly, mice challenged with Alternaria extract alone (in 4 mice out of 6) produced measurable amounts of a Th2 cytokine (IL-5) in BAL fluid. Notably, IL-5 secretion into the BAL fluid elicited by intranasal instillation with Alternaria extract was abrogated when the mice were primed with Poly i/c 24 hrs before Alternaria challenge (Fig. 4D, & 5B).

As an indicator of a Th1 immune response, IL-6 mRNA expression was remarkably upregulated in the lungs of mice treated with Poly i/c (Fig. 6A, C, &

D). However, enhanced (>10 fold) mRNA expression of IL-1β and IFN-γ were observed in lung tissue collected from samples under all treatment conditions, as compared to the lung tissue collected from PBS-treated control mice (Fig. 6B, & 6C). As expected, it was only the lung tissue of mice treated with Poly i/c that exhibited increased mRNA levels of IFN-β (Fig. 6D). The mRNA expression of signature cytokines for allergic immune response, IL-5 and IL-13, were found to be augmented in the lung tissue of mice treated with Alternaria extract, both with and without Poly i/c pretreatment (Fig. 6E, & 6F). TLR2 ligation with PAM_3CSK_4 also resulted in mildly enhanced mRNA expression of these Th2 cytokines (Fig. 6E, & 6F).

Histopathological analysis of lung tissue of mice primed with Poly i/c and challenged with PAM_3CSK_4, or with Alternaria alternata extract

Histopathological analysis of unprimed (vehicle-treated) lungs challenged with PAM_3CSK_4, a specific TLR2 agonist, revealed patchy, low grade minimal to occasionally mild, perivascular and peribronchial inflammation characterized by low numbers of neutrophils around the medium to small airways and blood vessels, and occasionally within the lumen of small airways and alveoli (Fig. 7B). Slight perivascular edema was sometimes present (Fig. 7B). Interpretation of findings was complicated by the presence of some foci of inflammation around foreign body material that was presumably inhaled. Inflammation was not observed in the lung of one animal primed with Poly i/c and challenged with PAM_3CSK_4.

Unprimed (vehicle-treated) lungs challenged with *Alternaria alternata* also had similar minimal to mild inflammation, and a few eosinophils were present along with neutrophils in one animal. Poly i/c-primed lungs that were challenged with *Alternaria alternata* had similar minimal to mild inflammation. In addition, apoptosis was frequently observed in the lining epithelium of small airways of mice primed with Poly i/c (Fig. 7C). Priming with Poly i/c did not appear to augment the subsequent *Alternaria alternata*-induced inflammation.

4. 5. Discussion

Various studies involving human subjects and supportive data from animal experiments have shown the existence of molecular interactions between respiratory allergies and viral infections. Early childhood viral infection, for instance with RSV, was shown to enhance development of airway allergen sensitization (Siegel JS et al. 2010). Studies in mice showed that ovalbumin-sensitized mice stimulated with Poly i/c exhibited an enhanced allergic inflammatory response, suggesting a role for dsRNA-sensing receptors (mainly TLR3) in exacerbation of allergic airway inflammation during viral infection (Nagase H et al. 2006). Although the roles for TLR3, RIG-I and MDA5 in recognition of several RNA viruses have been investigated, the functional relationships between these dsRNA detectors and other pathogen recognition receptors remain to be determined.

We and others have shown that TLR2 expression and function are augmented by stimulation of airway epithelial cells with Poly i/c (Ritter M et al. 2005, Melkamu T et al. 2009). Concomitant to this, an *in vivo* microarray analysis of lung and bronchial lymph nodes of mice infected with respiratory syncitial virus (RSV) revealed enhanced gene expression of TLR2 (Jenssen R et al. 2007). The results of these *in vitro* and *in vivo* studies suggest that *in vivo* activation with Poly i/c or virus may alter the innate immune response to TLR2 activators and potentially increase the sensitivity of the airway epithelium to TLR2-specific agonist or to fungal allergens sensed by TLR2. Therefore, this chapter of the thesis presents an *in vivo* study that addresses the functional consequence of dsRNA-priming of mice on the inflammatory response of the airways to TLR2 ligands (PAM_3CSK_4 and *Alternaria alternata)*. In this mice model, we elicited Poly i/c-dependent stimulation of dsRNA sensing receptors (TLR3, RIG-I, MDA5 and PKR) to mimic the physiologic disease state associated with virally-induced disease exacerbations.

Results of the present study show that transcripts of TLR1-9 are detectable in lung tissue of female C57BL/6 mouse strains and that TLR2 and TLR3 receptors are functionally active. Poly i/c administration for 24 hrs increased not only its cognate receptor, TLR3, but also mRNA expression of TLR2, TLR1, TLR9, TLR8 and TLR6 contrary to TLR4 and TLR5 that showed down-regulation and TLR7 that did not show significant change. Moreover, TLR2 mRNA expression that exhibited the highest augmentation in the lung following Poly i/c challenge of mice was also shown to be enhanced at a protein level. This is partly in agreement with the findings of Stowell and coworkers who recently reported up-regulation of TLR3, TLR2, TLR7, and TLR9 gene expression following repeated (3-times every 24 hrs) intra-tracheal challenge of mice with poly i/c (Stowell NC et al. 2009). The slight divergence in the findings (the relatively higher TLR9 and TLR7 expression than our findings) may be attributed to the sample size and method of collection (we analyzed individual animals of sample size ranging from 3-6 while theirs are pooled tissue from 6-8 mice) and the duration of exposure to Poly i/c (one time exposure in the present study compared to 3 exposure periods in 24 hrs).

Since whole lung was used for mRNA and protein determination, the potential source of augmented TLR mRNA expression could be either through up-regulation in resident lung cells (alveolar macrophages) or as a result of enhanced expression in infiltrating inflammatory cells (neutrophils and other polymorphonuclear cells, lymphocytes and other mononuclear cells) or through increased expression on structural cells (epithelial and smooth muscle cells). The initial possibility is supported by the fact that amplification of lung inflammatory response to PAMPs in LPS- or virus-infected mice has been ascribed to airway macrophages, which can include the resident population or recruited monocytes (Didierlaurent A et al. 2004, Maus UA et al. 2006). Indeed, viral infection dependent modulation of PRRs was reported in epithelial cells and tissue monocytes from asthmatic patients (Norzila MZ et al. 2000, de Kluijver J et al. 2003, Welliver RV Sr 2007). As determined by cytological analysis of the BAL fluid and histopathology of the lung, our findings showed that the major cellular components of the inflammatory milieu are neutrophils. This neutrophilic infiltration into the airways of mice evoked by Poly i/c is in agreement with previous findings reported by other investigators (Nagase H et al. 2007, Shiraishi Y et al. 2008). However, the notion that neutrophils play a role in eliciting Poly i/c-enhanced expression of TLRs is not likely, since neutrophils are known to poorly express TLR3 (Hayashi F et al. 2003). With regard to structural cells, however, there are several compelling *in vitro* studies in airway epithelial and smooth muscle cells that indicate modulation of TLR expression by various ligands, including Poly i/c (Sha Q et al. 2004, Ritter M et al. 2005, Sukkar MB et al. 2006, Melkamu T et al. 2009). In an *in vivo model,* direct evidence for a critical role of airway epithelial cells in innate immune response, particularly in controlling viral replication has been recently published using the Stat1$^{-/-}$ mouse infection model with paramyxovirus (Shornick LP et al. 2008).

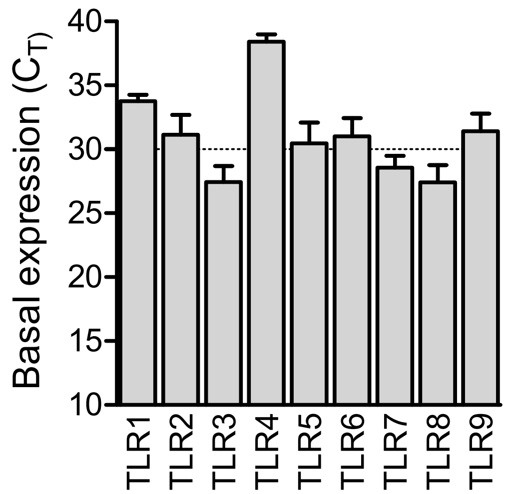
The differential modulatory effect of Poly i/c on the expression of TLRs in mice lung may indicate how concomitant viral infections can potentially lead to hypo- or hyper-responsiveness to PAMPs of subsequent microbial or allergen challenge resulting in either a truncated or augmented innate immune response of the airway to the ensuing insults. In accord with this hypothesis, infection of airway epithelial cells with *Hemophilus influenza* was shown to up-regulate TLR3 expression, and increased the responsiveness to secondary challenge with *Rhinovirus*. Through siRNA based silencing, these researchers verified that this enhanced sensitivity to *Rhinovirus* was TLR3 dependent (Sajjan et al. 2006). Furthermore, though no molecular mechanism has been worked out, enhanced inflammatory responses and increased bacterial burden following *Streptococcus pneumoniae* exposure have been observed in mice exposed to influenza virus or to RSV compared to control mice (Levine M et al. 2001).

In agreement with preceding studies, the results of this study showed that Poly i/c-dependent stimulation of dsRNA-sensing PRRs culminates in the synthesis of antiviral cytokines, such as type I IFNs, IL-1β, and IL-6, which may directly suppress viral replication (Thompson JM and Iwasaki A 2007, Stowell NC et al. 2009). Based on the analysis of the intensity and types cytokines detected in BAL fluid, our data also revealed that priming of either one or more dsRNA-sensing receptors by intranasal instillation of Poly i/c stimulates expression of TLR2 and enhances the sensitivity and magnitude of the airway inflammatory response to a TLR2-selective ligand PAM_3CSK_4. Interestingly, challenging mice with Alternaria extract alone, produced measurable amounts of a Th2 cytokine (IL-5) in BAL fluid. Hence, prior sensitization is not required for eliciting atopic inflammation following exposure to *Alternaria*. IL-5 induces proliferation and differentiation of eosinophils, and acts with IL-4 to enhance the production of IgE antibodies, the entire hallmark features for allergic inflammation. Notably, this IL-5 secretion into the BAL fluid was abrogated when the mice were primed with Poly i/c 24 hrs before *Alternaria* challenge. In agreement with the BAL cytokine profile (IL-5), histopathological analysis of the lung revealed mild eosinophilic infiltration into the lung. Conversely, eosinophils were not detected in the lavage fluid probably as the recruitment may have not been reached a threshold level to detect them in BAL fluid. Prior instillation of Poly i/c with ovalbumin has been previously reported to significantly suppress BAL eosinophilia in sensitized mice (Shiraishi Y et al. 2008). Interestingly, the event was accompanied with increased levels of interferon-gamma and decreased levels of IL-4 and IL-13. On the other hand, instillation of Poly i/c 8-16 hours after ovalbumin sensitization, i.e. after substantial number of eosinophils had already infiltrated into the lungs, significantly enhanced BAL eosinophilia (Shiraishi Y et al. 2008). Similarly, the present study suggests that Poly i/c priming and sensitization with *Alternaria* may have a potential role in modulating eosinophilic airway inflammation and Th2 cytokine profile differently depending on the airway microenvironment. Hence, understanding the mechanism by which Poly i/c interferes with the *Alternaria*-evoked Th2 response needs further investigation. The extent to which this *in vivo* effect is dependent on TLR3 or cytoplasmic dsRNA sensing receptors remains to be explored by employing knockout mice. Histopathologically, one noteworthy finding in the inflamed lung was the presence of bronchial epithelial cell hypertrophy and apoptosis in mice primed with Poly i/c. This was expected since Poly i/c is known to cause apoptosis and is now being explored as therapeutic agent for cancer (Salaun B et al. 2006, Shir A et al. 2009, O'Neill LA et al. 2009).

The current *in vivo* mice study showed that innate immune stimulation of the airway by Poly i/c amplified the level and activity of TLR2. It also demonstrated that viral dsRNA can promote a subsequent allergen sensitization, particularly if the allergen is recognized by TLR2. These data, taken along with the *in vitro* findings suggest that Poly i/c-dependent activation of dsRNA-sensing receptors, particularly TLR3, may play an essential role in respiratory disease pathogenesis. Furthermore, the data imply that viral infections can modulate the local inflammatory response of the lung and determine the severity and the course of chronic pulmonary diseases. Hence, unraveling the pathways and the outcomes involved during activation viral dsRNA-sensing receptors provides additional insight into the mechanisms underlying viral-induced exacerbations of chronic airway diseases. The findings may provide a potential to identify suitable targets for therapeutic intervention for respiratory disease exacerbations.

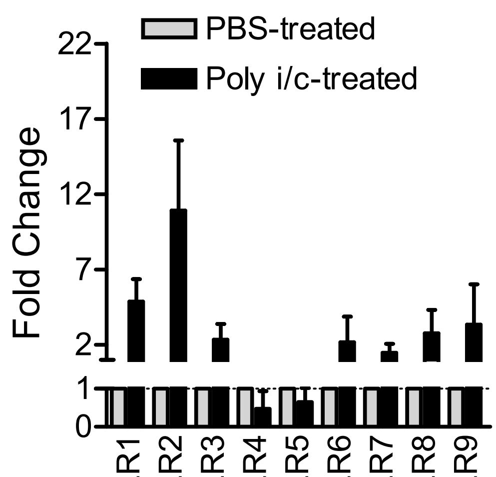
Figure 1: Profiling of TLR mRNA expression in mouse lung: (A) Basal expression of TLR mRNAs in the whole lung of C57BL/6 mice was quantified using QRT-PCRs. Results are provided as relative cycle threshold numbers (C_T). (B) Modulation of TLR mRNA expression in the lungs of C57BL/6 mice challenged with Poly i/c was determined by comparative C_T value analysis. Data are expressed as fold-expression over controls exposed to vehicle control (PBS). β-Actin was used as a normalizer. TLR2 mRNA expression exhibited the highest augmentation in the lung following Poly i/c challenge.

A



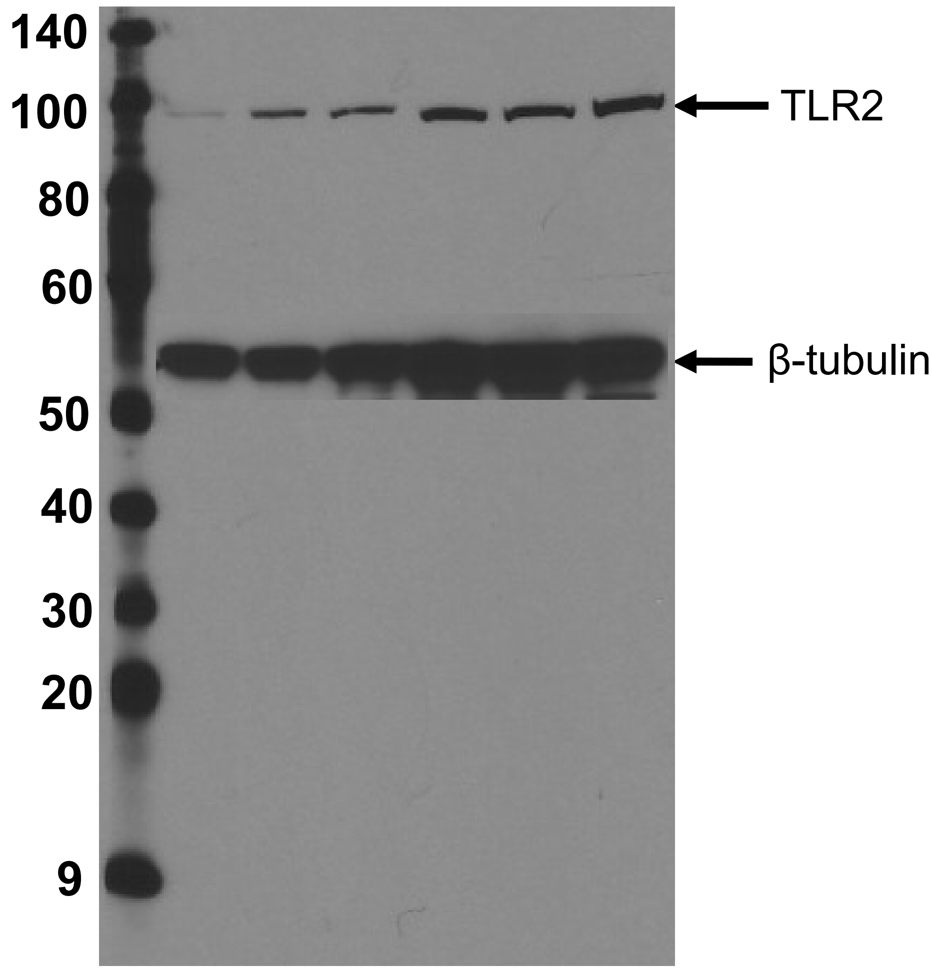
Basal expression (C_T)
40
35
30
25
20
15
10
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9

B



PBS-treated
Poly i/c-treated
Fold Change
22
17
12
7
2
1
0
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9

Figure 2: TLR2 protein determination in mouse lung after ligand stimulation: Western blot analysis of whole mouse lung allowed determination of TLR2 protein levels. Mice were exposed to TLR2 ligands, both with and without Poly i/c pretreatment. Priming with Poly i/c generally augmented TLR2 protein expression, as compared to the vehicle treated control mice. TLR2 ligands PAM_3CSK_4 and *Alternaria* extract also induced TLR2 protein expression, but not to the extent of Poly i/c stimulation.



Lane 1= 24 hrs PBS +12 hrs PBS
Lane 2= 24 hrs Poly i/c +12 hrs PBS

Lane 3= 24 hrs PBS +12 hrs PAM_3CSK_4
Lane 4= 24 hrs Poly i/c + 12 hrs PAM_3CSK_4

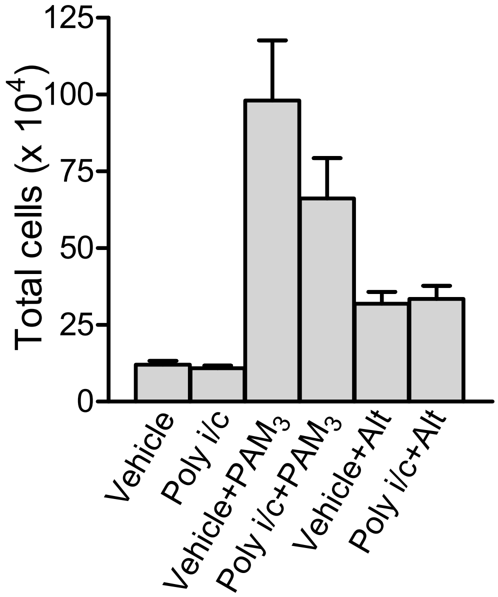
Lane 5= 24 hrs PBS +12 hrs Alternaria
Lane 6= 24 hrs Poly i/c +12 hrs Alternaria

Figure 3: Quantitative and qualitative changes in cellularity of bronchoalveolar lavage (BAL) fluid following Poly i/c exposure and TLR2 activation: Total and differential cell counts in BAL fluid were performed following challenge with Poly i/c alone, or subsequently with selected TLR2 ligands.

(A) BAL fluid from mice challenged only with Poly i/c did not exhibit a significant difference in total cell count from lavage fluid of vehicle-treated control mice; however, a clear increase in total cell count is apparent in all conditions consisting of a combination of challenges.

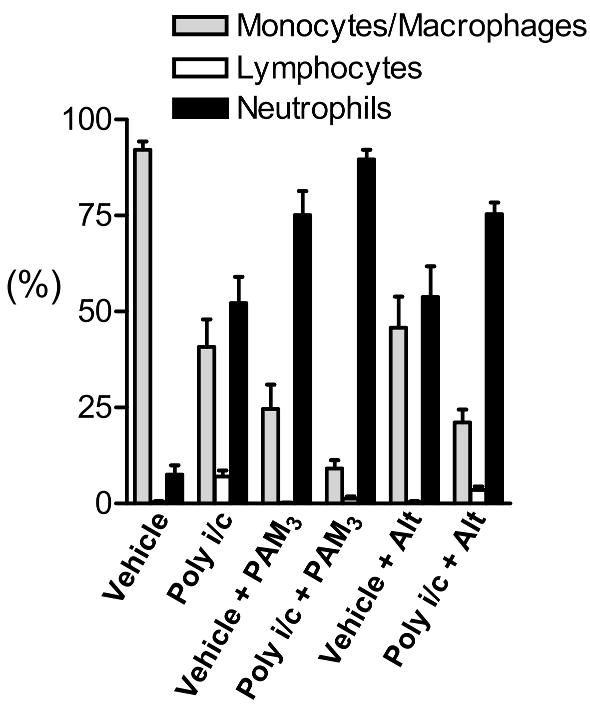
(B) BAL fluid from mice that are either primed with Poly i/c or treated with vehicle control (PBS) for 24 hrs and then sequentially treated with PAM_3CSK_4 for 12 hrs exhibited a significantly large increase in cellularity, as compared to that of control mice. The jump in total cellularity was largely due to a significant increase of neutrophils.

A



Total cells (x 10^4)
125
100
75
50
25
0
Vehicle
Poly i/c
Vehicle+PAM_3
Poly i/c+PAM_3
Vehicle+Alt
Poly i/c+Alt

B



Monocytes/Macrophages
Lymphocytes
Neutrophils
(%)
100
75
50
25
0
Vehicle
Poly i/c
Vehicle + PAM_3
Poly i/c + PAM_3
Vehicle + Alt
Poly i/c + Alt

Figure 4: T helper 1 (Th1) and Th2 cytokine secretion into bronchoalveolar lavage (BAL) fluid after Poly i/c challenge with and without other TLR2 ligands: Cytokine secretion profiles in BAL fluid were determined following challenge with Poly i/c alone, or subsequently with selected TLR2 ligands. Stimulation with Poly i/c and PAM_3CSK_4 generated an elevated level of Th1 cytokines, IL-1β and IL-6 (A, B). Poly i/c triggered secretion of IFN-β, characteristic of a typical antiviral response (C). Th2 cytokine response (IL-5) was detected in the BAL fluid of mice challenged only by *Alternaria* (D).

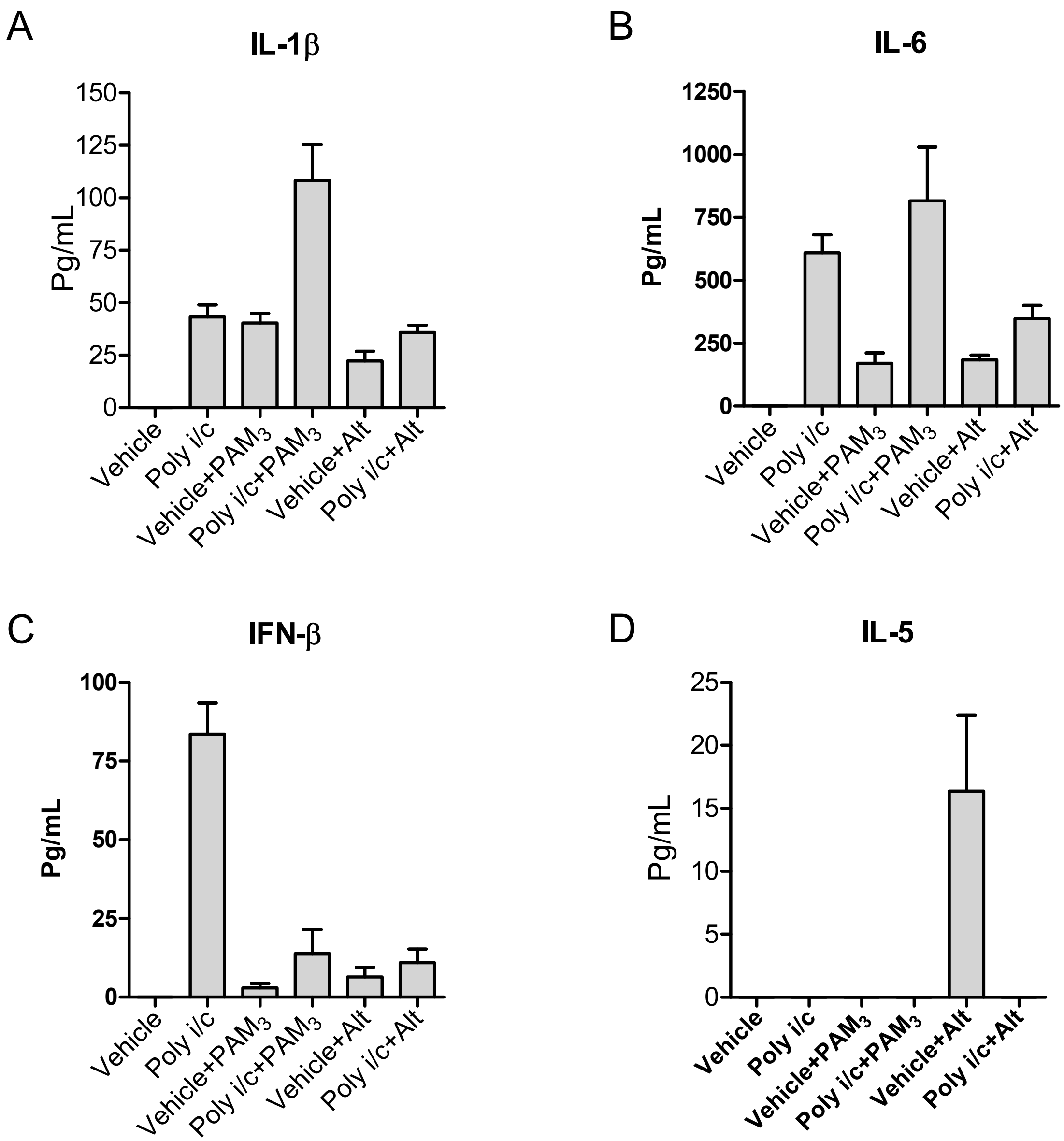

A
IL-1β
Pg/mL
150
125
100
75
50
25
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt
B
IL-6
Pg/mL
1250
1000
750
500
250
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt
C
IFN-β
Pg/mL
100
75
50
25
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt
D
IL-5
Pg/mL
25
20
15
10
5
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt

Figure 5: The effect of priming with Poly i/c on bronchoalveolar lavage (BAL) fluid cytokine level following challenge with TLR2 ligands: (A) Pretreatment of mice with Poly i/c appears to potentiate the IL-6 and IFN-β response, especially when subsequently challenged with TLR2 ligand, PAM_3CSK_4.
(B) Conversely, Poly i/c pretreatment causes complete truncation of Th2 cytokine response (IL-5) elicited by *Atlernaria*.

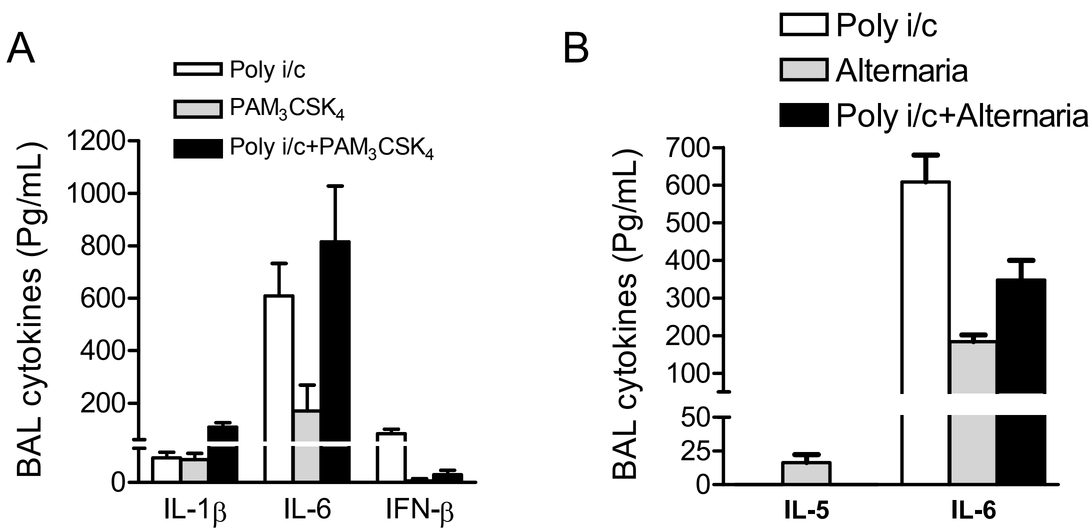
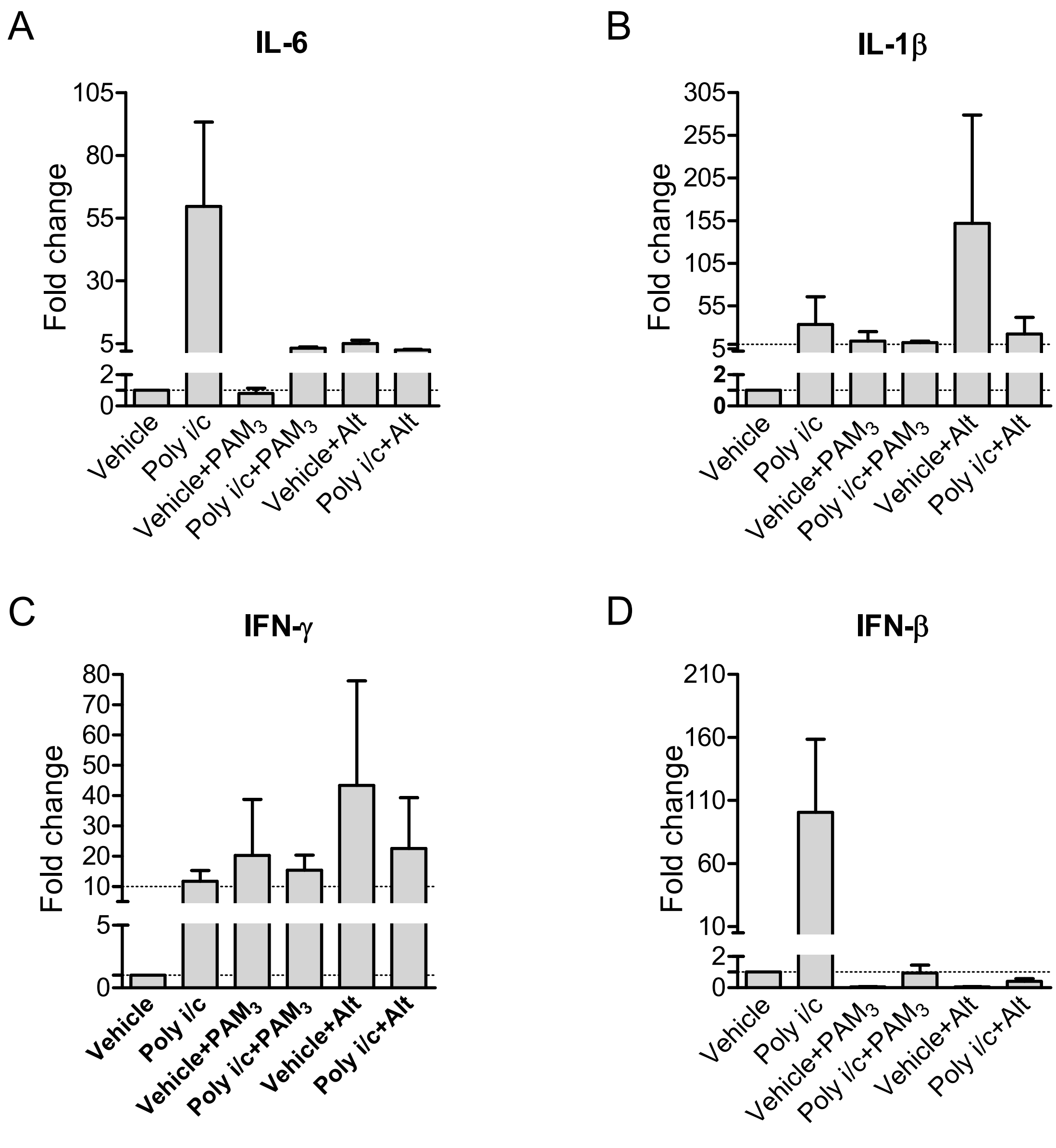

A
Poly i/c
PAM3CSK4
Poly i/c+PAM3CSK4
BAL cytokines (Pg/mL)
1200
1000
800
600
400
200
0
IL-1β
IL-6
IFN-β
B
Poly i/c
Alternaria
Poly i/c+Alternaria
BAL cytokines (Pg/mL)
700
600
500
400
300
200
100
50
25
0
IL-5
IL-6

Figure 6: Assessment of T helper 1 (Th1) and Th2 cytokine mRNA expression in mouse lung tissue after Poly i/c challenge with and without other TLR2 ligands: Th1 type cytokines are robustly induced by Poly i/c (A, C, and D). All treatment conditions enhanced IL-1β mRNA expression, but induction by *Alternaria* alone was significantly greater (B), as is also the case for IFN-γ (C). The presence of *Alternaria* triggered a significant amount of Th2 cytokine (IL-5 and IL-13) mRNA expression (E, F).


A
IL-6
Fold change
105
80
55
30
5
2
1
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt
B
IL-1β
Fold change
305
255
205
155
105
55
5
2
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt
C
IFN-γ
Fold change
80
70
60
50
40
30
20
10
5
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt
D
IFN-β
Fold change
210
160
110
60
10
2
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt

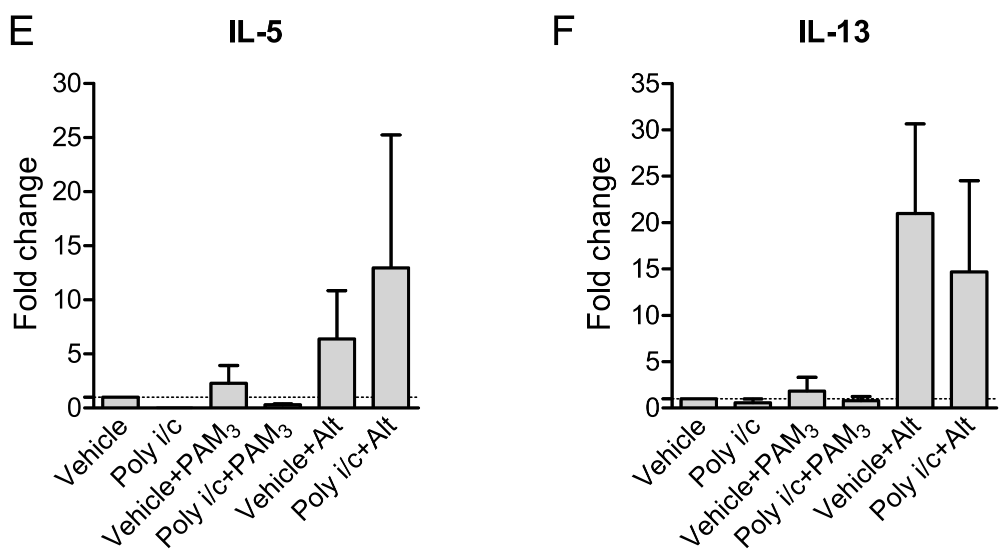
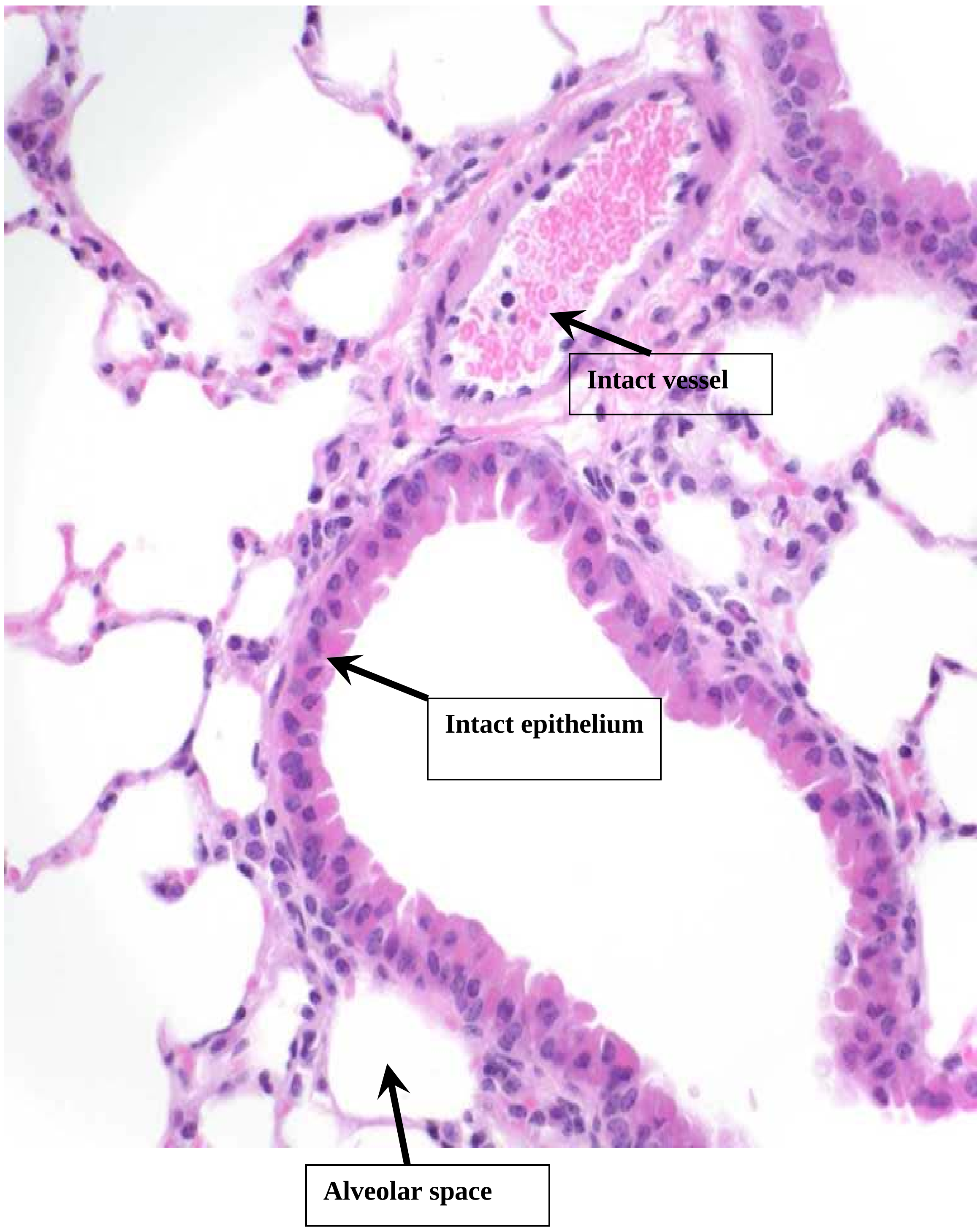
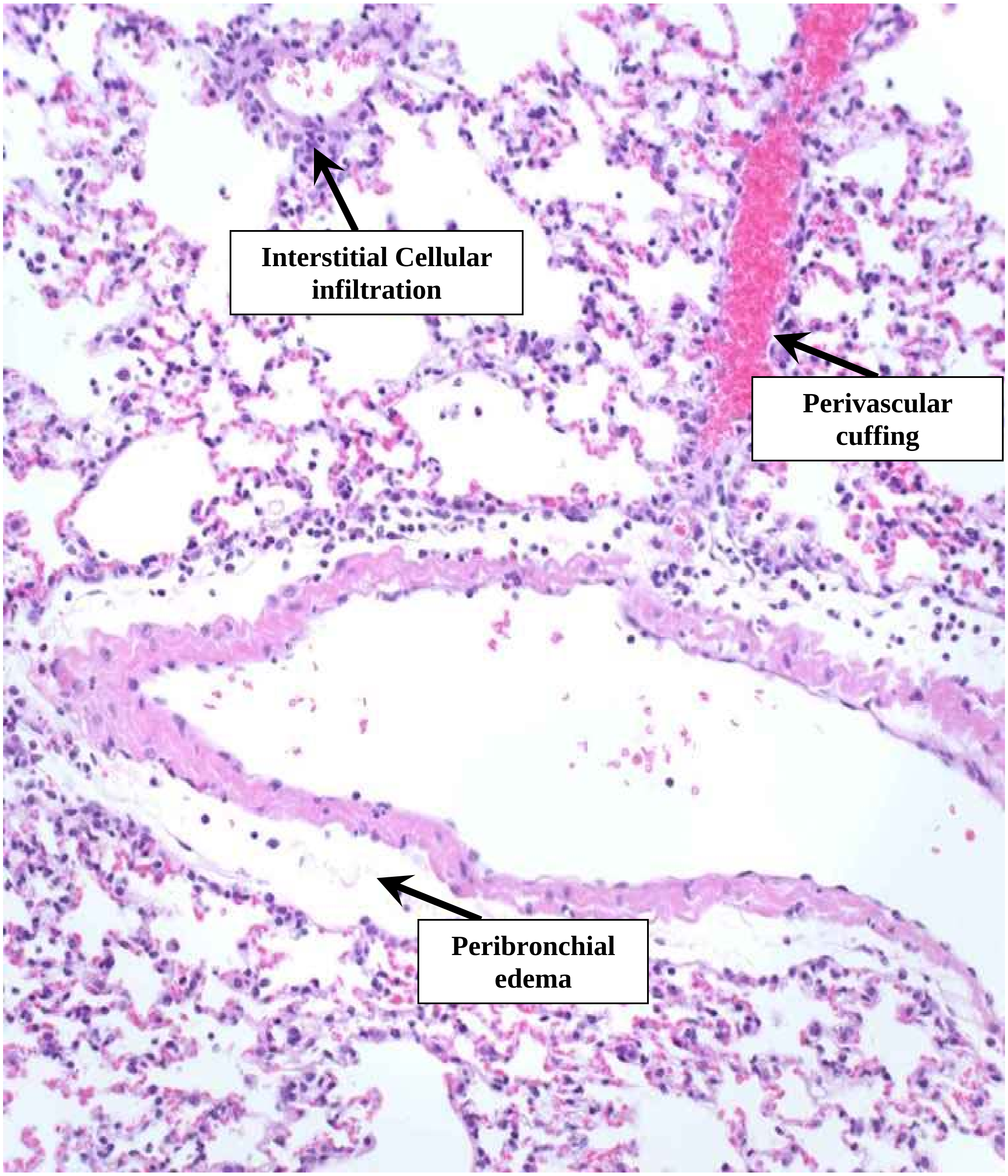

E
IL-5
Fold change
30
25
20
15
10
5
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt
F
IL-13
Fold change
35
30
25
20
15
10
5
0
Vehicle
Poly i/c
Vehicle+PAM3
Poly i/c+PAM3
Vehicle+Alt
Poly i/c+Alt

Figure 7: Histopathological analysis of lung tissue of mice primed with Poly i/c and challenged with specific TLR2 antagonists and *Alternaria Alternata* extract: All treatment conditions evoked mild inflammatory reaction in mice lung, which are patchy—localized peribronchially and perivascularly (B). Though it is mild, this inflammation is easily detectable when observed through histology of mouse lung (A). Apoptosis (as indicated by the arrows) was consistently detected in lung tissue of mice challenged with Poly i/c (C).

A



B


Interstitial Cellular infiltration
Perivascular cuffing
Peribronchial edema

C

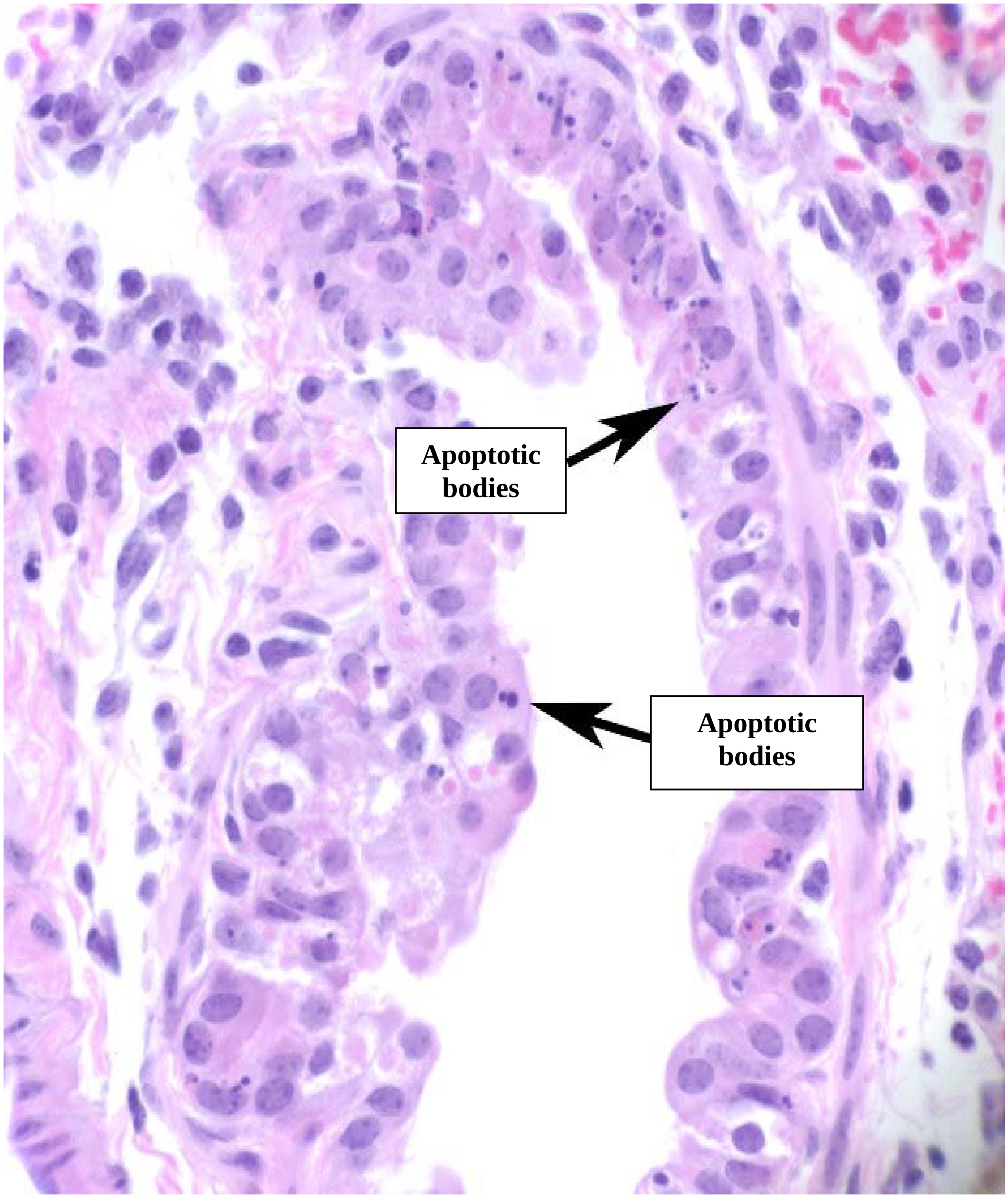


Table 2: Primer sequences for QRT-PCR for Mouse Target Genes

	Primer Sequence (5' to 3')	
Target Gene	*Sense*	*Antisense*
TLR 1	CCCTCATCTTCTACTGTATC	TCACCTTTAGCTCATTGTGG
TLR 2	GTCTTTCACCTCTATTCCCTC	GTCTCTACATTTCCTATCCTG
TLR 3	GGATTCTTCTGGTGTCTTCC	TCGAGTTCTGTCAGGTTCGTG
TLR 4	GAAACTCAGCAAAGTCCCTG	GAAAGGCTTGGTCTTGAATG
TLR 5	CTTCGGCTGTTTTCCTGTGG	CTTCCCTGGATGATGTTGCTG
TLR 6	CCAAAGACCTGCCACCAAGAAC	CACTAAGTCCAGAAGAATGC
TLR 7	CAAACTTCTGTAGACCGTCATGGG	AAGTACCGCAACTCTCTCAACGG
TLR 8	GTTATGTTGGCTGCTCTGGTTCAC	TCACTCTCTTCAAGGTGGTAGC
TLR 9	CTCTCTCCATACACTGAACTC	TGCTCTGCATCATCTGCCTC
IL-1β	TCGCTCAGGGTCACAAGAAA	ATCAGAGGCAAGGAGGAAACAC
IL-5	CTCTGTTGACAAGCAATGAGACG	TGCCCACTCTGTACTCATCACA
IL-6	GAGAGGAGACTTCACAGAGGATAC	GTACTCCAGAAGACCAGAGG
IL-13	CAGCCCACAGTTCTACAGCTC	AATCCAGGGCTACACAGAACC
IFN- β	CCCTATGGAGATGACGGAGA	CTGTCTGCTGGTGGAGTTCA
IFN-γ	GAACTGGCAAAAGGATGGTGA	TGTGGGTTGTTGACCTCAAAC
GAPDH	GTGGAGATTGTTGCCATCAACG	CAGTGGATGCAGGGATGATGTTCTG

Chapter 5: Overall conclusions and Future Direction

Conclusions:

The immune mechanisms associated with virus-triggered exacerbations of chronic inflammatory airway diseases are not fully understood. Evidence suggests that impairment of innate immune responses mediated by Toll-like receptors (TLRs) is involved. Pathogen associated molecular patterns (PAMPs) of viruses bind TLRs, triggering induction of genes involved in innate immune and inflammatory responses. Viral infections compromise the tight regulation of these responses that overwhelm protective anti-inflammatory defenses, leading to amplified reactions. Therefore, understanding the pathways involved in the pathogenesis of these exacerbations should help to facilitate the discovery and implementation of novel interventions to reduce viral exacerbations of airway inflammation. For that reason, this thesis examined the multiple receptor interactions that detect microbial components in order to understand the role of TLRs in exacerbations of chronic airway inflammatory diseases triggered by respiratory viral infections.

The first objective of this thesis was to identify and characterize the basal and PAMP-induced mRNA and protein expression of TLRs in human airway epithelial cells. The second aim was to determine the functional consequence of Poly i/c-dependent differential regulation of TLR2 and TLR5. This was carried out by assessing basal and TLR ligand-modulated patterns of mRNA expression and protein secretion of cytokines and chemokines by airway epithelial cells. The third aim of the study was to investigate the *in vivo* effects of Poly i/c on mouse airway inflammatory responses to TLR2 selective ligand, PAM_3CSK_4, and *Alternaria alternata*, a fungus detected in part by TLR2 and also capable of inducing acute asthmatic episodes. This final aim investigated the effects of enhanced TLR2 expression following TLR3 activation. The findings of the three specific aims of the study are summarized as follows:

1. Of the ten tested TLR mRNAs (TLRs 1-10) all were detected in A549 cells, but only TLRs 1-6 were detected in both Calu-3 and NHBE/HBE cells. In A549 cells, TLR5 was expressed at the highest levels, while TLR3 expression was greatest in both Calu-3 and NHBE/HBE cells.
2. Several cytokines and chemokines were basally secreted by airway epithelial cells. Yet, when treated with TLR ligands, the pattern of secretion was modulated. Of the three tested cell lines (A549, Calu-3 and NHBE/HBE), robust responses, largely to Poly i/c, were seen in NHBE/HBE cells. Cytokines and chemokines important in the recruitment of various leukocytes and dendritic cells, which are relevant to allergic airway inflammatory diseases, were shown to be released from the airway epithelium following stimulation with Poly i/c.
3. Experiments with NHBE cells demonstrated that exposure to synthetic dsRNA as a means to activate TLR3 resulted in up-regulation of TLR2 and certain co-receptors but suppressed TLR5 mRNA level. Poly i/c activation also enhanced TLR2 protein level.

4. This Poly i/c-induced increase in TLR2 mRNA and protein also conferred increased responsiveness to triacyl lipopeptides and to constituents present in extracts from *Alternaria alternata,* showing that activation by Poly i/c also enhanced TLR2 function. TLR2 blocking antibody was effective at inhibiting the enhanced PAM_3CSK_4 response after Poly i/c treatment, but it was not effective in inhibiting the enhanced response evoked by *Alternaria* extract following Poly i/c exposure.This suggests that TLR2 was responsible for the increase in responsiveness to PAM_3CSK_4, but not to *Alternaria* alternata extract.
5. Using TLR3 RNA silencing in HBE, the enhanced level and function of TLR2 by Poly i/c was shown to be TLR3-dependent. Conversely, Poly i/c-mediated down-regulation of TLR5 mRNA was not verified at the protein level. Correspondingly, no functional alteration in cytokine/chemokine and NF-κB activation was observed in Poly i/c-primed airway epithelial cells in which TLR5 was subsequently activated using recombinant flagellin protein and/or wild-type (flagellated) and flagella-mutated *Burkholderia (B.) cenocepacia*, an opportunistic bacteria causing pulmonary infection in individuals with cystic fibrosis.
6. In the *in vivo* study, as observed under *in vitro* conditions, administration of Poly i/c into the lungs enhanced mRNA and protein expression of TLR2. Poly i/c challenge elicited a marked induction of Th1 type cytokines. Poly i/c priming and subsequent challenge with a TLR2 ligand also elicited a marked induction of pro-inflammatory mediators, which was skewed

towards Th1 to a greater extent with the TLR2 specific ligand (PAM_3CSK_4) than with the subsequent *Alternaria* challenge. Interestingly, only BALF from mice challenged with *Alternaria* extract alone exhibited a measurable level of IL-5, and this induction of the Th2 cytokine was abrogated when the mice were primed with Poly i/c before *Alternaria* challenge. Inflammatory cell recruitment into the airways, with neutrophils dominating the cellular milieu, was also observed. Mice challenged with *Alternaria* extract alone exhibited mild eosinophilic infiltration in the lung tissue, which was not observed in Poly i/c-primed and *Alternaria* challenged mice. Apoptotic airway cells were evident in the lung tissue of mice instilled with Poly i/c.

In the past, by employing in vitro and ex vivo human, as well as in vivo mouse studies, various investigators had shown that natural viral infections or a synthetic viral dsRNA analogue (Poly i/c) differentially regulated TLR mRNA expression in airway epithelial/smooth muscle cells or in the whole lung. Similarly, studies conducted as part of this thesis also demonstrated a Poly i/c-dependent differential regulation of TLR mRNA expression, up-regulation of TLR2 and down-regulation of TLR5, *in vitro* in human airway epithelial cells and *in vivo* in mouse lungs. These findings imply that viral infection can differentially alter epithelial sensitivity and response to fungal allergens, bacteria, and other microbial agents sensed by modulated TLRs. The data also revealed that Poly i/c enhanced not only TLR2 mRNA expression but also augmented total protein level in human surface airway epithelial (NHBE/HBE) cells and also in the whole lung of mice. Furthermore, epithelial surface expression of TLR2 protein was boosted. Additionally, our study has revealed for the first time that the enhanced TLR2 mRNA and protein expression following activation of TLR3 by Poly i/c has functional consequence. *In vitro* stimulation of human airway epithelial cells or *in vivo* priming of mouse lung with Poly i/c potentiated the innate immune response of human airway epithelial cells as well as the pulmonary innate immune response of mice against a TLR2 specific synthetic ligand (PAM_3CSK_4) and against a fungus that is detected in part by TLR2 and has a huge significance in causing allergic airway inflammation (*Alternaria alternata*). By silencing TLR3 in airway epithelial cells, we have also demonstrated that Poly i/c-mediated augmentation of TLR2 mRNA and protein, as well as its function, may depend upon TLR3. Altogether, the present study demonstrated that TLR3 activation stimulates TLR2 expression and innate immune responses to TLR2 ligands in airway epithelial cells and potentially in mouse lungs. This represents at least one potential mechanism by which viral infections could exacerbate allergic airway inflammation.

Future Studies:

The studies described in this dissertation demonstrated that Poly i/c activation of TLR3 in airway epithelial cells augments TLR2 mRNA and protein expression, which is associated with enhanced TLR2 function. However, there are several questions that remain unanswered and several future studies are described in the following paragraphs.

1. Determine the mechanisms of Poly i/c-induced up-regulation of TLR2 mRNA and protein expression:

Although it was shown that induction of TLR2 mRNA and protein expression was dependent upon ligation of TLR3 by Poly i/c, a clear understanding of the underlying mechanisms requires further investigation. In the past, *in vitro* and *in vivo* studies in mice revealed that endotoxin-mediated induction of TLR2 mRNA and protein was regulated by activation of NF-κB (Matsuguchi T et al. 2000, Matsumura T et al. 2003). Supportive evidence for a critical role of NF-κB as transcriptional regulator of the TLR2 gene has emerged from the identification of NF-κB-binding sites in the mouse TLR2 promoter region (Mtisikacharoen T et al. 2001, Wang T et al. 2001). Besides NF-κB, other pathways including PKR, STAT5 and p38 kinase were also shown to be involved for the induction of TLR2 in mouse macrophages following LPS or cytokine stimulation (Cebanski M et al. 2008, Matsumura T et al. 2000, Mtisikacharoen T et al. 2001, Wang T et al. 2001, Oshikawa K and Sugiyama Y 2003, Ojaniemi M et al. 2006). Recently, studies in human esophageal epithelial and endothelial cells have also shown that enhanced TLR2 expression is regulated by NF-κB activation (Lim DM et al. 2009, Satta N et al. 2008). Based on these studies, we hypothesize that Poly i/c-mediated and TLR3-dependent induction of TLR2 may be the result of transcriptional activation of the TLR2 gene by NF-κB. We also propose that cytokines induced by Poly i/c ligation of TLR3 may contribute to the enhanced expression and function of TLR2 gene in airway epithelial cells. To test these hypotheses, NHBE/HBE cells can be treated with an irreversible inhibitor of NF-κB that blocks its nuclear translocation and activation by selectively blocking phosphorylation and degradation of its repressor protein, IκB-α. Additionally, NHBE/HBE cells can be transfected with a promoterless pGL2Basic vector (negative control), a wild-type TLR2 promoter construct, and a TLR2 promoter construct containing a site-directed mutation of its NF-κB-binding element. By employing these pharmacological and molecular approaches, the kinetics and intensity of TLR2 and IκB-α expression along with signature cytokines of TLR3 activation (IL-6 and IFN-β) under different treatment conditions (with and without Poly i/c stimulation) could be compared.

Despite the fact that more than 80% of Poly i/c-induced augmentation of TLR2 in NHBE/HBE cells was due to TLR3, there still is a potential role for other dsRNA sensing receptors in the process, particularly if TLR2 induction is elicited by cytokines induced by Poly i/c. Therefore, siRNA-mediated silencing of these RNA helicases should be conducted to determine whether TLR3 is the lone receptor that mediates the response to Poly i/c in NHBE/HBE cells. The *in vitro* role of TLR3 in differentially regulating the level as well as the function of TLR2 could also be verified using an *in vivo* approach, by employing TLR3 knock-out in mice. The *in vivo* role of the alternative dsRNA sensors may also be established using transgenic RIG-I and MDA5 deficient mice.

2. Identify possible mechanisms involved in the processing of TLR2:

In evaluating the effect of Poly i/c stimulation on TLR2 protein expression by immunoblotting, multiple bands with sizes ranging from the intact TLR2 protein to several much smaller bands, presumably representing cleavage products of the receptor with possible regulatory functions, were detected. Moreover, TLR2 labeling of all detected bands was eliminated from the Western blot using peptide competition, implying that the lower molecular weight bands contain TLR2 specific antigen. Earlier reports of soluble forms of TLR2 (sTLR2) in various biological fluids, including human plasma, milk, saliva, amniotic fluid and in blood monocytes has provided direct evidence for TLR2 cleavage (Dulay AT et al. 2009, Kuroishi T et al. 2006, LeBouder E et al. 2003). sTLR2 in body fluids can either be processed fragments of the entire molecule or the extracellular domain of TLR2. Interestingly, recombinant extracellular domains of TLRs were shown to attenuate TLR-mediated cellular activation (LeBouder E et al. 2003). Likewise, the binding of PAM_3CSK_4 to sTLR2 in parotid saliva was weaker than that of intact TLR2. Pre-incubation of salivary sTLR2 with PAM_3CSK_4 can lead to neutralization, without simultaneous activation, enabling sTLR2 to capture and exclude its ligands from the oral cavity, averting inflammatory responses (Kuroishi T et al. 2006).

Furthermore, discovery of ectodomain shedding of TLR7 and TLR9 (Ewald SE et al. 2008, Park B et al. 2008) support the presumption that ectodomain shedding of TLR2 also occurs. Both the full-length and cleaved forms of TLR9 can bind ligand, but it is only the processed form that recruits MyD88 upon activation. Furthermore, pharmacological inhibition of TLR9 receptor proteolysis was shown to render the receptor to be non-functional, indicating that the cleaved form, rather than the full-length form, is functional. Therefore, ectodomain cleavage of TLR7 and TLR9 represents a strategy for restricting receptor activation to endolysosomal compartments and prevents TLRs from responding to self nucleic acids. The likelihood of TLR2 processing is further supported by the discovery of cleavage of the intracellular C-terminal domain of IL-1R1 (that has homologous cytoplasmic Toll/IL-1R (TIR) domain, and analogous signaling mechanisms as TLRs) by γ-secretase with subsequent release of intracellular peptides (Elzinga BM et al. 2009). Cleavage products of type I transmembrane receptors (IL-1R1 and TNFR1) were shown to be structurally incapable of signaling or presenting the agonist to receptor complexes, hence function as decoy receptors (Mantovani A et al. 2001). Similarly, cleavage products of TLR2 might serve as decoy receptor and act as a molecular trap for agonists and other essential components of TLR2 signaling, thereby limiting excessive inflammatory responses.

To test whether Poly i/c-stimulation of NHBE/HBE cells results in intra-membrane proteolysis of TLR2 protein and its fragmentation, pharmacological blockers can be utilized. Using selective protease inhibitors, including metalloproteinase (for extracellular proteolysis) and γ-secretase (for intracellular proteolysis) with and without Poly i/c treatment, cleavage of TLR2 initiated by Poly i/c can be blocked and analyzed by Western blot to detect full-length and the various cleavage products of TLR2.

3. Evaluate the contribution of epithelial cells and other resident cell types (particularly macrophages) to the pathophysiology of airway inflammation either alone or in synergy with other cell types:

Inflammatory processes involve multiple cell types that include macrophages and epithelial cells. However, the degree of interaction of epithelial cells and macrophages in responding to various stimuli and initiating inflammatory reactions within the airways has not been fully characterized. Exacerbation of airway responses in LPS- or virus-infected mice has previously been ascribed to airway macrophages, rather than to epithelial cells (Didierlaurent A et al. 2008). On the other hand, epithelial cells are known to participate in the control of immune-related processes, including differentiation of leukocytes, microbial sensing, peripheral tissue sampling and presentation of antigens to T-cells, thereby regulating the onset of adaptive immune responses (Shaykhiev R and Bals R 2007). Direct evidence regarding the critical role of airway epithelial cells in the innate immune response to pathogens, particularly in controlling viral replication has been recently investigated using the Stat1$^{-/-}$ mouse infection model with paramyxovirus (Shornick LP et al. 2008). Accordingly, it is likely that airway epithelial cells are the initial responders to environmental stimuli and are involved in activation of macrophages.

To assess whether Poly i/c-mediated induction of TLR2 occurs selectively in epithelial cells, frozen lung sections can be subjected to laser capture micro-dissection (LCM). LCM enables the selection of the epithelial cell layer in fixed sections of the airway. Therefore, it may be possible to precisely determine the contribution of epithelial cells on pulmonary inflammation with reduced confounding influences of multiple cell types. In addition, it would be important to explore the effect the epithelium has on the resident immune cells, particularly macrophages. The role of epithelial cells: macrophage direct contact in modulating inflammatory response can be investigated by physically separating the co-cultured cell monolayers by plating the epithelial cells on transwell filters and U937 cells (macrophage surrogate cell model) inside transwell inserts. Macrophage activation could be determined by measuring the surface expression of CD11b, activation of NF-κB and phagocytic activity. Macrophage adherence to the epithelial monolayer can be confirmed by CD68 immunostaining. Relative to a monoculture model, when macrophages are co-cultured with epithelial cells, an increase in the CD11b expression, increased adherence to the epithelial cell layer and enhanced NF-κB activation are often observed. It is anticipated that co-cultured macrophages will be exposed to the inflammatory mediators secreted by epithelial cells, resulting in macrophage activation. This result would suggest that airway epithelial cells are primary generators of inflammatory signals that result in activation of macrophages. To validate the role of epithelial cells, co-culture experiments could also be done between lung fibroblasts cell line (HFL1) and macrophages (U937) and compared with NHBE-U937 co-culture experiments.

4. Elucidate the relationship among the various signaling pathways activated during TLR3 priming and subsequent ligation of TLR2:

Ligation of TLR3 with Poly i/c leads to activation of NF-κB and IRF transcription factors resulting in eventual secretion of pro-inflammatory cytokines and type I interferons. Poly i/c-mediated induction of apoptosis has also been described particularly in transformed epithelial cells. Among our *in vitro* and *in vivo* experiments, we have also recognized that all these potential pathways were active. The balance among these various signaling pathways determines the outcome of inflammation. For instance, induction of IFN-alpha/beta by dsRNA can aid in host defense against many viruses; however, if not tightly regulated, dsRNA may also elevate levels of chemokines, such as IL-8 and RANTES, which are implicated in exacerbation of asthma. Though multiple biological outcomes are possible via dsRNA ligation of TLR3, the mechanism that controls the phenomenon has not been characterized. Furthermore, it is of great interest to assess the conditions that control switching from the canonical antiviral IFN-response, which is mediated by IRF3 pathway, to responses that induce expression and release of proinflammatory cytokines via the NF-κB pathway.

On the basis of the intensity and the rapidity of IL-6 mRNA induction in response to Poly i/c stimulation of NHBE cells, the transcriptional mechanisms of Poly i/c-stimulated induction of IL-6 in airway epithelial cells can be investigated. NHBE cells can be stimulated with Poly i/c following transient transfection with a luciferase reporter construct containing an IL-6 promoter construct containing a site-directed mutation of its NF-κB binding site or its binding element for IRF3. Poly i/c stimulation is expected to exhibit significant transactivation of the IL-6 promoter over unstimulated controls. Mutation of the NF-κB or IRF3 binding site is expected to eliminate this effect with variable intensity. Then the degree and the condition at which that Poly i/c-induced IL-6 promoter activation is dependent on the interaction of NF-κB or IRF3 with the IL-6 promoter can be assessed.

References

1. Abraham CM, Ownby DR, Peterson EL, et al. (2007) The relationship between seroatopy and symptoms of either allergic rhinitis or asthma. J Allergy Clin Immunol; 119:1099-1104.
2. Akira S (2003) Mammalian Toll-like receptors. Curr Opin Immunol; 15: 5-11.
3. Akira S and Takeda K (2004) Toll-like receptor signaling. Nat Rev Immunol; 4:499-511.
4. Alexopoulou L, Holt AC, Medzhitov R, and Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-_B by Toll-like receptor 3. Nature; 413:732-738.
5. Auger F, Gendron M-C, Chamot C, Marano F, and Dazy A-C (2006) Responses of well-differentiated nasal epithelial cells exposed to particles: Role of the epithelium in airway inflammation. Toxicol and Appl Pharmacol; 215:285.
6. Avila PC, Donnelly S, Dolganov G, Lopez-Souza N, and Xu A, et al. (2005) Toll-like receptor response to rhinovirus infection in airway epithelial cells of healthy subjects. J of Allergy and Clinical Immunol; 115 (2):S206.
7. Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C, and Hellman J (2007) MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. J Immunol; 178:1164-1171.
8. Bals R (2000) Epithelial antimicrobial peptides in host defense against infection. Respir Res; 1(3):141-150.
9. Bals R and Hiemstra PS (2004) Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. Eur Respir J; 23:327-333.
10. Barclay AN (2003) Membrane proteins with immunoglobulin-like domains – a master superfamily of interaction molecules. Semin Immunol; 15:215-223.
11. Basu S and Fenton MJ (2004) Toll-like receptors: function and roles in lung disease. Am J Physiol Lung Cell Mol Physiol; 286(5):L887-L892.
12. Beasley R, Coleman ED, Hermon Y, Holst PE, O'Donnell TV, and Tobias M (1988) Viral respiratory tract infection and exacerbations of asthma in adult patients. Thorax; 43:679-683.
13. Belvisi MG (2003) Sensory nerves and airway inflammation: role of A delta and C-fibres. Pulm Pharmacol Ther; 16(1):1-7.
14. Beutler B (2004) Inferences, questions and possibilities in Toll-like receptor signaling. Nature; 430:257-263.
15. Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, Duprex WP, Wagner H, Kirschning CJ, ter Meulen V, and Schneider-Schaulies S (2002) Hemagglutinin Protein of Wild-Type Measles

Virus Activates Toll-Like Receptor 2 Signaling. J Virol; 76(17):8729-8736.

16. Bishop B and Lloyd CM (2003) CC Chemokine Ligand 1 Promotes Recruitment of Eosinophils But Not Th2 Cells During the Development of Allergic Airways Disease. J Immunol; 170:4810-4817.
17. Blease K, Kunkel SL, and Hogaboam CM (2001) IL-18 modulates chronic fungal asthma in a murine model; putative involvement of Toll-like receptor-2. Inflamm Res; 50:552-560.
18. Bodet C, Chandad F, and Grenier D (2006) Inflammatory responses of a macrophage/epithelial cell co-culture model to mono and mixed infections with *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia.* Microbes and Infection; 8:27-35.
19. Brennan S, Cooper D, Sly PD (2001) Directed neutrophil migration to IL-8 is increased in cystic fibrosis: a study of the effect of erythromycin. Thorax; 56:62-64.
20. Brown GD (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol; 6:33-43.
21. Brown GD and Gordon S (2001) Immune recognition: a new receptor for beta-glucans. Nature; 413:36-37.
22. Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S (2003) Dectin-1 mediates the biological effects of beta-glucans. J Exp Med; 197:1119-1124.
23. Burke JM, Ganley-Leal LM, Khatri A, and Wetzler LM (2007) Neisseria meningitidis PorB, a TOLL-like receptor 2 ligand, induces an antigen-specific eosinophil recall response: Potential adjuvant for helminth vaccines? J Immunol; 179:3222-3230
24. Burns JL, Jonas M, Chi EY, Clark DK, Berger A, Griffith A (1996) Invasion of respiratory epithelial cells by Burkholderia (Pseudomonas) cepacia. Infect Immun; 64(10):4054-4059.
25. Buzina W, Raggam RB, Paulitsch A, Heiling B, and Marth E (2008) Characterization and temperature-dependent quantification of heat shock protein 60 of the immunogenic fungus Alternaria alternata. Medical mycology; 46:627-630.
26. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procópio DO, Travassos LR, Smith JA, Golenbock DT, and Gazzinelli RT (2001) Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol; 167:416-423.
27. Carroll MC (1998) The role of complement and complement receptors in induction and regulation of immunity. Annu Rev Immunol; 16:545-568.
28. Chi L, Li Y, Stehno-Bittel L, Gao J, Morrison DC, Stechschulte DJ, and Dileepan KN (2001) Interleukin-6 production by endothelial cells via stimulation of protease-activated receptors is amplified by

endotoxin and tumor necrosis factor-alpha. J Interferon Cytokine Res; 21(4):231-240.
29. Cieri MV, Mayer-Hamblett N, Griffith A, and Burns JL (2002) Correlation between an In Vitro Invasion Assay and a Murine Model of Burkholderia cepacia Lung Infection. Infect Immun; 70(3):1081-1086.
30. Cohen L and Castro M (2003) The role of viral respiratory infections in the pathogenesis and exacerbation of asthma. Semin Respir Infect; 18(1):3-8.
31. Compton T, Kurt-Jones EA, Boehme KW, Belko J, Latz E, Golenbock DT, and Finberg RW (2003) Human Cytomegalovirus Activates Inflammatory Cytokine Responses via CD14 and Toll-Like Receptor 2. J Virol; 77(8):4588-4596.
32. Cook DN, Pisetsky DS and Schwartz DA (2004) Toll-like receptors in the pathogenesis of human disease. Nat Immunol; 5(10):975-979.
33. Covert MW, Leung TH, Gaston JE, and Baltimore D (2005) Achieving stability of lipopolysaccharide-induced NF-kappaB activation. Science; 309(5742):1854-1857.
34. Daeron M (1997) Fc receptor biology. Annu Rev Immunol; 15:203-234.
35. de Kluijver J, Grunberg K, Pons D, et al. (2003) Interleukin-1β and interleukin-1ra levels in nasal lavages during experimental rhinovirus infection in asthmatic and non-asthmatic subjects. Clin Exp Allergy; 33:1415-1418.
36. Dennehy KM, Ferwerda G, Faro-Trindade I, Pyz E, Willment JA, Taylor PR, Kerrigan A, Tsoni SV, Gordon S, and Meyer-Wentrup F (2008) Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. Eur J Immunol; 38:500-506.
37. Diamond G, Legarda D, and Ryan LK (2000) The innate immune response of the respiratory epithelium. Immunol Rev; 173:27-38.
38. Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, Bebien M, Lawrence T, van Rijt LS, Lambercht BN, Sirarid J-C, and Hussell T (2008) Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. J Exper Med; 205(2):323-329.
39. Douwes J, Gibson P, Pekkanen J, and Pearce N (2002) Non-eosinophilic asthma: importance and possible mechanisms. Thorax; 57(7):643-648.
40. Duff C, Murphy PG, Callaghan M, and McClean S (2006) Differences in invasion and translocation of Burkholderia cepacia complex species in polarised lung epithelial cells in vitro. Microb Pathog; 41(4-5):183-192.
41. Dulay AT, Buhimschi CS, Zhao G, Oliver EA, Mbele A, Jing S, and Buhimschi IA (2009) Soluble TLR2 Is Present in Human Amniotic

Fluid and Modulates the Intraamniotic Inflammatory Response to Infection. J Immunol; 182:7244-7253.
42. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, and Bottomly K (2002) Lipopolysaccharide-enhanced, Toll-like Receptor 4-dependent T Helper Cell Type 2 Responses to Inhaled Antigen. J Exp Med; 196:1645-1651.
43. Elzinga BM, Twomey C, Powell JC, Harte F, and McCarthy JV (2009) Interleukin-1 Receptor Type 1 Is a Substrate for γ-Secretase-dependent Regulated Intramembrane Proteolysis. The JBC; 284(3):1394-1409.
44. Erb KJ (1999) Atopic disorders: a default pathway in the absence of infection? Immunol Today; 20:317-322.
45. Ewald SE, Lee BL, Lau L, Wickliffe KE, Shi G-P, Chapman HA, and Barton GM (2008) The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature; 456(4):658-663.
46. Fahey JV, Schaefer TM, Channon JY, and Wira CR (2005) Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. Human Reproduction; 20:1439-1446.
47. Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG, and Adema GJ (2008) Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cell Microbiol; 10(10):2058-2066.
48. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, and Maniatis T (2003) IKKε and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol; 4:491-496.
49. Fleming HE, Little FF, Schnurr D, et al. (1999) Rhinovirus-16 colds in healthy and in asthmatic subjects: similar changes in upper and lower airways. Am J Respir Crit Care Med; 160:100-108.
50. Fraenkel DJ, Bardin PG, Sanderson G, Lampe F, Johnston SL, Holgate ST (1995) Lower airways inflammation during rhinovirus colds in normal and in asthmatic subjects. Am J Respir Crit Care Med; 151:879-886
51. Fransson M, Adner M, Erjefalt J, Jansson L, Uddman R, and Cardell LO (2005) Up-regulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. Respir Res; 6:100-110.
52. Fujii T, Hayashi S, Hogg JC, Mukae H, Suwa T, Goto Y, Vincent R, and van Eeden SF (2002) Interaction of Alveolar Macrophages and Airway Epithelial Cells Following Exposure to Particulate Matter Produces Mediators that Stimulate the Bone Marrow. Am J Respir Cell Mol Biol; 27:34-41.
53. Fung F, Tappen D, and Wood G (2000) Alternaria-associated asthma. Appl Occup Environ Hyg; 15:924-927.
54. Furuta T, Shuto T, Shimasaki S, Ohira Y, Suico MA, Gruenert DC, and Kai H (2008) DNA demethylation-dependent enhancement of toll-ike

receptor-2 gene expression in cystic fibrosis epithelial cells involves SP1-activated transcription. BMC Mol Biol; 9:39-56.
55. Gantner BN, Simmons RM, Canavera SJ, Akira S, and Underhill DM (2003) Collaborative induction of inflammatory responses by Dectin-1 and Toll-like receptor 2. J Exp Med; 197:1107-1117.
56. Gern JE (2004) Viral respiratory infection and the link to asthma. Pediatr Infect Dis J; 23:S78-S86.
57. Gentry M, Taormina J, Pyles RB, Yeager L, Kirtley M, Popov VL, Klimpel G, and Eaves-Pyles T (2007) Role of Primary Human Alveolar Epithelial Cells in Host Defense against Francisella tularensis Infection. Infect Immun; 75:3969-3978.
58. Gern JE, French DA, Grindle KA, Brockman-Schneider RA, Konno S-I, and Busse WW (2003) Double-stranded RNA induces the synthesis of specific chemokines by bronchial epithelial cells. Am J Respir Cell Mol Biol; 28(6):731-737.
59. Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, Diamond MS, and Colonna M (2006) Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. PNAS; 103:8459-8464.
60. Gomariz RP, Arranz A, Juarranz Y, Gutierrez-Canas I, Garcia-Gomez M, Receta J, and Martınez C (2007) Regulation of TLR expression, a new perspective for the role of VIP in immunity. Peptides 28 :1825-1832.
61. Gonzalo J-A, Lloyd CM, Wen D, Albar JP, Wells TNC, Proudfoot A, Martinez-A C, Dorf M, Bjerke T, Coyle AJ, and Gutierrez-Ramos J-C (1998) The Coordinated Action of CC Chemokines in the Lung Orchestrates Allergic Inflammation and Airway Hyperresponsiveness. J Exp Med; 188(1):157-167.
62. Goodrich and Underhill (2008) Fungal Recognition by TLR2 and Dectin-1 in Toll-Like Receptors (TLRs) and Innate Immunity. Handbook of Experimental Pharmacology; 183:87-109. Springer-Verlag Berlin Heidelberg.
63. Grabiec A, Meng G, Fichte S, Bessler W, Wagner H, and Kirschning CJ (2004) Human but not murine toll-like receptor 2 discriminates between tri-palmitoylated and tri-lauroylated peptides. J Biol Chem; 279:48004-48012.
64. Greenberg SB, Allen M, Wilson J, and Atmar RL (2000) Respiratory viral infections in adults with and without chronic obstructive pulmonary disease. Am J Respir Crit Care Med; 162(1):167-173.
65. Grissell TV, Powell H, Shafren DR, et al. (2005) Interleukin-10 gene expression in acute virus-induced asthma. Am J Respir Crit Care Med; 172:433-439.
66. Groskreutz DJ, Monick MM, Powers LS, Yarovinsky TO, Look DC, and Hunninghake GW (2006) Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded

RNA responsiveness in airway epithelial cells. J Immunol; 176:1733-1740.

67. Gross O, Gewies A, Finger K, Schafer M, Sparwasser T, Peschel C, Forster I, and Ruland J (2006) Card9 controls a non-TLR signaling pathway for innate anti-fungal immunity. Nature; 442:651-656.
68. Grunberg K, Smits HH, Timmers MC, de Klerk EPA, Dolhain RJEM, Dick EC, Hiemstra PS, and Sterk PJ (1997) Experimental rhinovirus 16 infection: effects on cell differentials and soluble markers in sputum in asthmatic subjects. Am J Respir Crit Care Med; 156:609-616.
69. Grunberg K, Smits HH, Timmers MC, et al. (1997) Experimental rhinovirus 16 infection: effects on cell differentials and soluble markers in sputum in asthmatic subjects. Am J Respir Crit Care Med; 156:609-616.
70. Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M, and Si-Tahar M (2004) Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. J Biol Chem; 279(4):2712-2718.
71. Haehnel V, Schwarzfischer L, Fenton MJ, and Rehli M (2002) Transcriptional regulation of the human Toll-like receptor 2 gene in monocytes and macrophages. J Immunol; 168:5629-5637.
72. Hamm HE (1998) The many faces of G protein signaling. J Biol Chem; 273:669.
73. Hayashi F, Means TK, and Luster AD (2003) Toll-like receptors stimulate human neutrophil function. Blood; 102(7):2660-2669.
74. Hele DJ and Belvisi MG (2003) Novel therapies for the treatment of inflammatory airway disease. Expert Opin Investig Drugs; 12(1):5-18.
75. Herre J, Gordon S, and Brown GD (2004) Dectin-1 and its role in the recognition of beta-glucans by Macrophages. Mol Immunol; 40:869-876.
76. Hertz CJ, Wu Q, Porter EM, Zhang YJ, Weismuller KH, Godowski PJ, Ganz T, Randell SH, and Modlin RL (2003) Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human β defensin-2. J Immunol; 171:6820-6826.
77. Herz U, Lacy P, Renz H, and Erb K (2000) The influence of infections on the development and severity of allergic disorders. Current Opinion in Immunology; 12:632-640.
78. Hewson CA, Jardine A, Edwards MR, Laza-Stanca V, and Johnston SL (2005) Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. J Virol; 79: 12273-12279.

79. Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, Crozat K, Sovath S, Shamel L, Hartung T, Zähringer U, and Beutler B (2005) CD36 is a sensor of diacylglycerides. Nature; 433(7025):523-527.
80. Holgate ST (2004) Lessons learnt from the epidemic of asthma. QJM; 97(5):247-257.
81. Holgate ST (2007) Epithelium dysfunction in asthma. J Allergy Clin Immunol; 120:1233-1244.
82. Hollingsworth JW, Whitehead GS, Lin KL, Nakano H, Gunn MD, Schwartz DA, and Cook DN (2006) TLR4 Signaling Attenuates Ongoing Allergic Inflammation. J Immunol; 176:5856-5862.
83. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, and Hartmann G (2006) 5'-Triphosphate RNA is the ligand for RIG-I. Science; 314:994-997.
84. Hoshino K, Takeuchi O, Kawai T, et al. (1999) Cutting Edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol; 162:3749.
85. Hou YF, Zhou YC, Zheng XX, Wang HY, Fu YL, Fang ZM, and He SH (2006) Modulation of expression and function of Toll-like receptor 3 in A549 and H292 cells by histamine. Molecular Immunology; 43:1982-1992.
86. Hurd S (2000) The impact of COPD on lung health worldwide: Epidemiology and incidence. Chest; 117:1S-4S.
87. Inouei Y, Matsuwaki Y, Shin SH, Ponikau JU, and Kita H (2005) Nonpathogenic, Environmental Fungi Induce Activation and Degranulation of Human Eosinophils. J Immunol; 175:5439-5447.
88. Jacobs AL, Sehgal PB, Julian J, and Carson DD (1992) Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro. Endocrinology; 131:1037-1046.
89. Jafri HS, Chavez-Bueno S, Mejias A, Gomez AM, Rios AM, Nassi SS, Yusuf M, Kapur P, Hardy RD, Hatfield J, Rogers BB, Krisher K, and Ramilo O (2004) Respiratory syncytial virus induces pneumonia, cytokine response, airway obstruction, and chronic inflammatory infiltrates associated with long-term airway hyperresponsiveness in mice. J Infect Dis; 189(10):1856-1865.
90. Jarjour NN, Gern JE, Kelly EA, Swenson CA, Dick CR, and Busse WW (2000) The effect of an experimental rhinovirus 16 infection on bronchial lavage neutrophils. J Allergy Clin Immunol; 105:1169-1177.
91. Jenssen R, Pennings J, Hodemaekers H, Buisman A, van Oosten M, de Rond L, Ozturk K, Dormans J, Kimman T, and Hoebee B (2007) Host Transcription Profile upon Primary Respiratory Syncytial Virus Infection. J Virol; 81:5958-5967.

92. Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, Huber M, Kalis C, Keck S, Galanos C, Freudenberg M, and Beutler B (2005) CD14 is required for MyD88-independent LPS signaling. Nat Immunol; 6(6):565-570.
93. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, Symington P, O'Toole S, Myint SH, and Tyrrell DA (1995) Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. Br Med J; 310:1225-1229.
94. Kariko K, Ni H, Capodici J, Lamphier M, and Weissman D (2004) mRNA is an endogenous ligand for Toll-like receptor 3. J Biol Chem; 279(13):12542-12550.
95. Kataoka H, Yasuda M, Iyori M, Kiura K, Narita M, Nakata T, and Shibata K (2006) Roles of N-linked glycans in the recognition of microbial lipopeptides and lipoproteins by TLR2. Cell Microbiol; 8:1199-1209.
96. Kauffman HF and van der Heide S (2003) Exposure, sensitization, and mechanisms of fungus-induced asthma. Curr Allergy Asthma Rep; 3:430-437.
97. Kawai T and Akira S (2006) TLR signaling. Cell Death Differ; 13:816-825.
98. Kariko K, Ni H, Capodici J, Lamphier M, and Weissman D (2004) mRNA is an endogenous ligand for Toll-like receptor 3. J Biol Chem; 279:12542-12550.
99. Keig PM, Ingham E, and Kerr KG (2001) Invasion of human type II pneumocytes by Burkholderia cepacia. Microb Pathog; 30:167-170.
100. Kobayashi T, Iijima K, Radhakrishnan S, Mehta V, Vassallo R, Lawrence CB, Cyong J-C, Pease LR, Oguchi K, and Kita H (2009) Asthma-Related Environmental Fungus, Alternaria, Activates Dendritic Cells and Produces Potent Th2 Adjuvant Activity. J Immunol; 182:2502-2510.
101. Krakauer T (2002) Stimulant-Dependent Modulation of Cytokines and Chemokines by Airway Epithelial Cells: Cross Talk between Pulmonary Epithelial and Peripheral Blood Mononuclear Cells. Cl and Diagn Lab Immunol; 9(1):126-131.
102. Kumar A, Zhang J and Yu FSX (2006) Toll-like receptor 3 agonist poly(I:C)-induced antiviral response in human corneal epithelial cells. Immunology; 117(1):11-21.
103. Kunzelmann K and McMorran B (2004) First Encounter: How Pathogens Comprise Epithelial Transport. Physiology; 19.
104. Kuroishi T, Tanaka Y, Sakai A, Sugawara Y, Komine K-I, and Sugawara S (2007) Human parotid saliva contains soluble toll-like receptor (TLR) 2 and modulates TLR2-mediated interleukin-8 production by monocytic cells. Molecular Immunology; 44:1969-1976.
105. Kurt-Jones EA, Popoya L, Kwinn L, Haynes LM, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, and Finberg RW (2000) Pattern recognition receptors TLR4 and CD14

mediate response to respiratory syncytial virus. Nat Immunol; 1:398-401.
106. Lampinen M, Carlson M, Hakansson LD, and Venge P (2004) Cytokine-regulated accumulation of eosinophils in inflammatory disease. Allergy; 59:793-805.
107. Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, Chignard M, and Si-Tahar M (2007) Cutting Edge: Influenza A Virus Activates TLR3-Dependent Inflammatory and RIG-I-Dependent Antiviral Responses in Human Lung Epithelial Cells. J Immunol; 178:3368-3372.
108. LeBouder E, Rey-Nores JE, Rushmere NK, Grigorov M, Lawn SD, Affolter M, Griffin GE,Ferrara P, Schiffrin EJ, Morgan BP, and Labe´ta MO (2003) Plasma and Breast Milk Signaling Are Present in Human (TLR)2 Capable of Modulating TLR2. J Immunol; 171: 6680-6689.
109. Lee HK, Dunzendorfer S, and Tobias PS (2004) Cytoplasmic domain-mediated dimerizations of toll-like receptor 4 observed by beta-lactamase enzyme fragment complementation. J Biol Chem; 279:10564-10574.
110. Lee SY, Palmer ML, Maniak PJ, Jang SH, Ryu PD, and O'Grady SM (2007) P2Y receptor regulation of sodium transport in human mammary epithelial cells. Am. J. Physiol. [Cell Physiology] 293:C1472-C1480.
111. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, and Hoffmann JA (1996) The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell; 86:973.
112. LeVine AM, Koeningsknecht V, and Stark JM (2001) Decreased pulmonary cclearance of Streptococcus pneumoniae following influenza A infection in mice. J Virol Methods; 94:173-186.
113. Li XL, Boyanapalli M, Weihua X, Kalvakolanu DV, and Hassel B (1998) Induction of interferon synthesis and activation of interferon-stimulated genes by liposomal transfection reagents. J. Interferon Cytokine Res. 18:947-952.
114. Liaudet L, Szabo C, Evgenov OV, Murthy KG, Pacher P, Virag L, Mabley JG, Marton A, Soriano FG, Kirov MY, Bjertnaes LJ, and Salzman AL (2003) Flagellin from gram-negative bacteria is a potent mediator of acute pulmonary inflammation in sepsis. Shock; 19:131-137.
115. Lim DM, Narasimhan S, Michaylira CZ, and Wang M-L (2009) TLR3-mediated NF-κB signaling in human esophageal epithelial cells. Am J Physiol Gastrointest Liver Physiol; 297:G1172-G1180.
116. Liu AH and Redmon AH Jr (2001) Endotoxin: friend or foe? Allergy Asthma Proc; 22(6):337-340.

117. Livak KJ, and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods.25:402–408.

118. Lordan JL, Bucchieri F, Richter A, Konstantinidis A, Holloway JW, Thornber M, Puddicombe SM, Buchanan D, Wilson SJ, Djukanov R, Holgate ST, and Davies DE (2002) Cooperative Effects of Th2 Cytokines and Allergen on Normal and Asthmatic Bronchial Epithelial Cells. J Immunol; 169:407-414.

119. Lukacs NW, Standiford TJ, Chensue SW, Kunkel RG, Strieter RM, and Kunkel SL (1996) C–C chemokine-induced eosinophil chemotaxis during allergic airway inflammation. J Leukoc Biol; 60:573-578.

120. Mall MA (2008) Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. J Aerosol Med Pulm Drug Deliv; 21(1):13-24.

121. Mallia P and Johnston SL (2006) How viral infections cause exacerbation of airway diseases. Chest; 130:1203-1210.

122. Manukyan M, Triantafilou K, Triantafilou M, Mackie A, Nilsen N, Espevik T, Wiesmüller KH, Ulmer AJ, and Heine H (2005) Binding of lipopeptide to CD14 induces physical proximity of CD14, TLR2 and TLR1. Eur J Immunol; 35(3):911-921.

123. Marr KA, Balajee SA, Hawn TR, Ozinsky A, Pham U, Akira S, Aderem A, and Liles WC (2003) Differential Role of MyD88 in Macrophage-Mediated Responses to Opportunistic Fungal Pathogens. Infect Immun; 71:5280-5286.

124. Martin TR (2000) Recognition of bacterial endotoxin in the lungs. Am J Respir Cell Mol Biol; 23:128-132.

125. Martin TR and Frevert CW (2005) Innate immunity in the lungs. Proc Am Thorac Soc; 2:403-411.

126. Martinez-Pomares L, Linehan SA, Taylor PR, and Gordon S (2001) Binding properties of the mannose receptor. Immunobiology; 204:527-535.

127. Mascarenhas JO, Goodrich ME, Eichelberger H, and McGee GW (1996) Polarized secretion of IL-6 by IEC-6 intestinal epithelial cells: differential effects of IL-1β and TNF-α. Immunol Invest; 25:333-340.

128. Matsuguchi T, Musikacharoen T, Ogawa T, and Yoshikai Y (2000) Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. J Immunol; 165:5767-5772.

129. Matsukura S, Kokubu F, Kurokawa M, Kawaguchi M, Ieki K, Kuga H, Odaka M, Suzuki S, Watanabe S, Takeuchi H, Kasama T, and Adachi M (2006) Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-κB and/or IRF-3 in airway epithelial cells. Clin Exper Allergy; 36(8):1049.

130. Matsukura S, Kokubu F, Kurokawa M, Kawaguchi M, Ieki K, Kuga H, Odaka M, Suzuki S, Watanabe S, Homma T, Takeuchi H, Nohtomi K, and Adachi M (2007) Role of RIG-I, MDA-5, and PKR on the Expression of Inflammatory Chemokines Induced by Synthetic dsRNA in Airway Epithelial Cells. Int Arch Allergy Immunol; 143(1):80-83.
131. Matsumura T, Ito A, Takki T, Hayashi H, and Onozaki K (2000) Endotoxin and cytokine regulation of toll-like receptor (TLR) 2 and TLR4 gene expression in murine liver and hepatocytes. J Interferon Cytokine Res; 20:915-921
132. Matsumura T, Degawa T, Takii T, Hayashi H, Okamoto T, Inoue J, and Onozaki K (2003) TRAF6-NF-kappaB pathway is essential for interleukin-1- induced TLR2 expression and its functional response to TLR2 ligand in murine hepatocytes. Immunology; 109:127-136.
133. Maus UA, Janzen S, Wall G, Srivastava M, Blackwell TS, Christman JW, Seeger W, Welte T, and Lohmeyer J (2006) Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. Am J Respir Cell Mol Biol; 35(2):227-235.
134. Mayer AK, Muehmer M, Mages J, Gueinzius K, Hess C, Heeg K, Bals R, Lang R, and Dalpke AH (2007) Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells. J Immunol; 178: 3134-3142.
135. McClean S and Callaghan M (2009) Burkholderia cepacia complex: epithelial cell-pathogen confrontations and potential for therapeutic intervention. J Med Microbiol; 58(1):1-12.
136. McGettrick AF and O'Neill LA (2004) The expanding family of MyD88-like adaptors in Toll-like receptor signal transduction. Mol Immunol; 41(6-7):577-582.
137. McGettrick AF, Brint EK, Palsson-McDermott EM, Rowe DC, Golenbock DT, Gay NJ, Fitzgerald KA, and O'Neill LA (2006) Trif-related adapter molecule is phosphorylated by PKC{epsilon} during Toll-like receptor 4 signaling. PNAS; 103(24):9196-9201.
138. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, and Fenton MJ (1999) Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. J Immunol; 163(7):3920-3927.
139. Means TK, Lien E, Yoshimura A, Wang S, Golenbock DT, and Fenton MJ (1999) The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J Immunol; 163(12):6748-6755.
140. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature; 388:394-397.
141. Medzhitov R and Janeway CA Jr (1997) Innate immunity: impact on the adaptive immune response. Curr Opin Immunol; 9:4-9.

142. Meerschaert J, Busse WB, Bertics PJ, and Mosher DF (2000) $CD14^+$ Cells Are Necessary for Increased Survival of Eosinophils in Response to Lipopolysaccharide. Am. J. Respir. Cell Mol. Biol; 23(6): 780-787.
143. Melkamu T, Squillace D, Kita H, and O'Grady SM (2009) Regulation of TLR2 expression and function in human airway epithelial cells. J Membr Biol; 229(2):101-113.
144. Meylan E and Tschopp J (2006) Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. Mol Cell; 22:561-569.
145. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, and Tschopp J (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature; 437(7062):1167-1172.
146. Moretti S, Bellocchio S, Bonifazi P, Bozza S, Zelante T, Bistoni F, and Romani L (2008) The contribution of PARs to inflammation and immunity to fungi. Mucosal Immunol; 1(2):156-168.
147. Morris GE, Whyte MKB, Martin GF, Jose PJ, Dower SK, and Sabroe I (2005) Agonists of Toll-like Receptors 2 and 4 Activate Airway Smooth Muscle via Mononuclear Leukocytes. Am J Respir Crit Care Med; 171(8): 814-822.
148. Muir A, Soong G, Sokol S, Reddy B, Gomez MI, Van Heeckeren A, and Prince A (2004) Toll-like receptors in normal and cystic fibrosis airway epithelial cells. Am J Respir Cell Mol Biol; 30:777-783.
149. Mukhopadhyay S, Herre J, Brown GD, and Gordon S (2004) The potential for Toll-like receptors to collaborate with other innate immune receptors. Immunology; 112(4):521-530.
150. Murray CS, Simpson A, and Custovic A (2004) Allergens, viruses, and asthma exacerbations. Proc Am Thorac Soc; 1:99-104.
151. Nagase H, Yamashita N, Adachi T, Nakano J, and Ohta K (2006) Toll-like Receptor 3 (TLR3) Ligand Exacerbates Pre-existing Allergic Airway Inflammation in Murine Asthma Model. J Allerg Clinical Immunol; 117(2):S1 446: S114.
152. NIAID: national institute of allergy and infectious diseases (2001) Asthma A Concern for Minority Populations, NIAID Fact Sheet: NIAID.
153. Norzila MZ, Fakes K, Henry RL, et al. (2000) Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. Am J Respir Crit Care Med; 161:769-774.
154. Ojaniemi M, Liljeroos M, Harju K, Sormunen R, Vuolteenaho R, and Hallman M (2006) TLR-2 is upregulated and mobilized to the hepatocyte plasma membrane in the space of Disse and to the Kupffer cells TLR-4 dependently during acute endotoxemia in mice. Immunology Letters; 102:158-168.

155. O'Neill LA (2006) How Toll-like receptors signal: what we know and what we don't know. Curr Opin Immunol; 18:3-9.
156. O'Neill LA, Fitzgerald KA, and Bowie AG (2003) The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol; 24:286-290.
157. Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, and Seya T (2003) TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-β. J Biol Chem; 278:49751-49762.
158. Ostrowska E, Sokolova E, and Reiser G (2007) PAR-2 activation and LPS synergistically enhance inflammatory signaling in airway epithelial cells by raising PAR expression level and interleukin-8 release. Am J Physiol Lung Cell Mol Physiol; 293(5):L1208-L1218.
159. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, and Aderem A (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. PNAS; 97:13766-13771.
160. Palfreyman RW, Watson ML, Eden C, and Smith AW (1997) Induction of biologically active interleukin-8 from lung epithelial cells by Burkholderia (Pseudomonas) cepacia products. Infect Immun; 65(2):617-622.
161. Palmer ML, Lee SY, Maniak PJ, Carlson D, Fahrenkrug SC, and O'Grady SM (2006) Protease-activated receptor regulation of Cl- secretion in Calu-3 cells requires prostaglandin release and CFTR activation. Am J Physiol Cell Physiol; 290:C1189-C1198.
162. Palsson-McDermott EM and O'Neill LA (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology; 113(2):153-162.
163. Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, Fabbri LM, and Johnston SL (2006) Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. Am J Respir Crit Care Med; 173:1114-1121.
164. Park S W, Kim DJ, Chang HS, Park SJ, Lee YM, Park JS, Chung IY, Lee JH, and Park CS (2003) Association of interleukin-5 and eotaxin with acute exacerbation of asthma. Int Arch Allergy Immunol; 131:283-290.
165. Parker LC, Prince LR, and Sabroe I (2007) Translational mini-review series on Toll-like receptors: networks regulated by Toll-like receptors mediate innate and adaptive immunity. Clin Exp Immunol; 147(2):199-207.
166. Patel AC, Brett TJ, and Holtzman MJ (2009) The role of CLCA proteins in inflammatory airway disease. Annu Rev Physiol; 71:425-449.
167. Peiser L, Mukhopadhyay S, and Gordon S (2002) Scavenger receptors in innate immunity. Curr Opin Immunol; 14(1):123-128.

168. Peng Q, Matsuda T, and Hirst SJ (2004) Signaling pathways regulating interleukin-13-stimulated chemokine release from airway smooth muscle. Am J Respir Crit Care Med; 169(5):596-603.
169. Perera PY, Mayadas TN, Takeuchi O, Akira S, Zaks-Zilberman M, Goyert SM, and Vogel SN (2001) CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. J Immunol; 166(1):574-581.
170. Pinhal-Enfield G, Ramanathan M, Hasko G, Vogel SN, Salzman AL, Boons GJ, and Leibovich SJ (2003) An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A(2A) receptors. Am J Pathol; 163(2):711-721.
171. Polito AJ and Proud D (1998) Epithelia cells as regulators of airway inflammation. J Allergy Clin Immunol; 102(5):714-718.
172. Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, and Beutler B (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *tlr4* gene. Science; 282:2085-2088.
173. Prince LR, Allen L, Jones EC, Hellewell PG, Dower SK, Whyte MK, and Sabroe I (2004) The role of interleukin-1 beta in direct and toll-like receptor 4-mediated neutrophil activation and survival. Am J Pathol; 165(5):1819-1826.
174. Proud D and Chow CW (2006) Role of viral infections in Asthma and Chronic Obstructive Pulmonary Disease. Am J Respir Cell Mol Biol; 35:513-518.
175. Qureshi ST and Medzhitov R (2003) Toll-like receptors and their role in experimental models of microbial infection. Genes Immun; 4:87-94.
176. Rallabhandi P, Nhu QM, Toshchakov VY, Piao W, Medvedev AE, Hollenberg MD, Fasano A, and Vogel SN (2008) Analysis of proteinase-activated receptor 2 and TLR4 signal transduction: a novel paradigm for receptor cooperativity. J Biol Chem; 283(36):24314-24325.
177. Ramirez RD, Sheridan S, Girard L, Sato M, Kim Y, Pollack J, Peyton M, Zou Y, Kurie JM, Dimaio JM, Milchgrub S, Smith AL, Souza RF, Gilbey L, Zhang X, Gandia K, Vaughan MB, Wright WE, Gazdar AF, Shay JW, and Minna JD (2004) Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. Cancer Res; 64(24):9027-9034.
178. Rassa JC, Meyers JL, Zhang Y, Kudaravalli R, and Ross SR (2002) Murine retroviruses activate B cells via interaction with toll-like receptor 4. PNAS; 99(4):2281-2286.
179. Reddi K, Phagoo SB, Anderson KD, and Warburton D (2003) Burkholderia cepacia-Induced IL-8 Gene Expression in an Alveolar

Epithelial Cell Line: Signaling Through CD14 and Mitogen-Activated Protein Kinase. Pediatric Research; 54 (3):297-305.
180. Reid DM, Gow NA, and Brown GD (2009) Pattern recognition: recent insights from Dectin-1. Curr Opin Immunol; 21(1):30-37.
181. Ritter M, Mennerich D, Weith A, and Seither P (2005) Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. J Inflamm (Lond); 29: 2-16.
182. Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E, Williams DL, Gordon S, Tybulewicz VL, Brown GD, Reis E, and Sousa C (2005) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. Immunity; 22(4):507-517.
183. Rudd BD, Burstein E, Duckett CS, Li X, and Lukacs NW (2005) Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. J Virol; 79(6):3350-3357.
184. Sajjan US, Jia Y, Newcomb DC, Bentley JK, Lukacs NW, LiPuma JJ, and Hershenson MB (2006) H. influenzae potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression. FASEB J; 20(12):2121-2123.
185. Salaun B, Coste I, Rissoan MC, Lebecque SJ, and Renno T (2006) TLR3 can directly trigger apoptosis in human cancer cells. J Immunol; 176(8):4894-4901.
186. Salzet M (2002) Antimicrobial peptides are signaling molecules. Trends Immunol; 23(6):283-284.
187. Sapey E and Stockley RA (2006) COPD exacerbations. 2: aetiology. Thorax; 61:250-258.
188. Sasai M, Shingai M, Funami K, Yoneyama M, Fujita T, Matsumoto M, and Seya T (2006) NAK-Associated Protein 1 Participates in Both the TLR3 and the Cytoplasmic Pathways in Type I IFN Induction. J Immunol; 177: 8676-8683.
189. Sato S, Nomura F, Kawai T, Takeuchi O, Muhlradt PF, Takeda K, and Akira S (2000) Synergy and Cross-tolerance between Toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. J Immunol; 165:7096-7101.
190. Satta N, Kruithof EKO, Reber G, and de Moerloose P (2008) Induction of TLR2 expression by inflammatory stimuli is required for endothelial cell responses to lipopeptides. Mol Immunol; 46:145-157.
191. Schleimer RP (2004) Glucocorticoids suppress inflammation but spare innate immune responses in airway epithelium. Proc Am Thorac Soc; 1:222-230.
192. Seth RB, Sun L, Ea CK, and Chen ZJ (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell; 122(5):669-682.

193. Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, and Schleimer RP (2004) Activation of Airway Epithelial Cells by Toll-Like Receptor Agonists. Am J Res Cell and Mol Bio; 31:358-364.
194. Shen BQ, Finkbeiner WE, Wine JJ, Mrsny RJ, and Widdicombe JH (1994) Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl- secretion. Am J Physiol; 266(5):L493-L501.
195. Shi S, Nathan C, Schnappinger D, Drenkow J, Fuortes M, Block E, Ding A, Gingeras TR, Schoolnik G, Akira S, Takeda K, and Ehrt S (2003) MyD88 primes macrophages for full-scale activation by interferon-gamma yet mediates few responses to Mycobacterium tuberculosis. J. Exp. Med. 198:987-997.
196. Shir A, Ogris M, Wagner E, and Levitzki A (2006) EGF receptor-targeted synthetic double-stranded RNA eliminates glioblastoma, breast cancer, and adenocarcinoma tumors in mice. PLoS Med; 3(1):e6.
197. Shiraishi Y, Asano K, Niimi K, Fukunaga K, Wakaki M, Kagyo J, Takihara T, Ueda S, Nakajima T, Oguma T, Suzuki Y, Shiomi T, Sayama K, Kagawa S, Ikeda E, Hirai H, Nagata K, Nakamura M, Miyasho T, and Ishizaka A (2008) Cyclooxygenase-2/prostaglandin D2/CRTH2 pathway mediates double-stranded RNA-induced enhancement of allergic airway inflammation. J Immunol; 180(1):541-549.
198. Shornick LP, Wells AG, Zhang Y, Patel AC, Huang G, Takami K, Sosa M, Shukla NA, Agapov E, and Holtzman MJ (2008) Airway Epithelial versus Immune Cell Stat1 Function for Innate Defense against Respiratory Viral Infection. J Immunol; 180:3319-3328.
199. Siegle JS, Hansbro N, Herbert C, Rosenberg HF, Domachowske JB, Asquith KL, Foster PS, and Kumar RK (2010) Early-life viral infection and allergen exposure interact to induce an asthmatic phenotype in mice. Respir Res; 11(1):14.
200. Sigurs N, Bjarnason R, Sigurbergsson F, and Kjellman C (2000) Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit Care Med; 161: 1501-1507.
201. Simmons C and Farrar J (2008) Insights into inflammation and influenza. N Engl J Med; 359(15):1621-1623.
202. Singh AM and Busse WW (2006) Asthma exacerbations. 2: aetiology. Thorax; 61(9):809-816.
203. Stark JM, Stark MA, Colasurdo GN, and LeVine AM (2006) Decreased bacterial clearance from the lungs of mice following primary respiratory syncytial virus infection. J Med Virol; 78(6):829-838.
204. Stone P, Konstan MW, Berger M, et al. (1995) Elastin and collagen degradation products in urine of patients with cystic fibrosis. Am J Respir Crit Care Med; 152:157-162.
205. Stowell NC, Seideman J, Raymond HA, Smalley KA, Lamb RJ, Egenolf DD, Bugelski PJ, Murray LA, Marsters PA, Bunting RA, Flavell RA,

Alexopoulou L, San Mateo LR, Griswold DE, Sarisky RT, Mbow ML, and Das AM (2009) Long-term activation of TLR3 by poly(I:C) induces inflammation and impairs lung function in mice. Respir Res; 10:43.

206. Strieter RM, Belperio JA, and Keane MP (2002) Cytokines in innate host defense in the lung. J Clin Invest; 109(6):699-705.
207. Stuart LM, Deng J, Silver JM, Takahashi K, Tseng AA, Hennessy EJ, Ezekowitz RA, and Moore KJ (2005) Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J Cell Biol; 170(3):477-485.
208. Subrata LS, Bizzintino J, Mamessier E, Bosco A, McKenna KL, Wikström ME, Goldblatt J, Sly PD, Hales BJ, Thomas WR, Laing IA, LeSouëf PN, and Holt PG (2009) Interactions between innate antiviral and atopic immunoinflammatory pathways precipitate and sustain asthma exacerbations in children. J Immunol; 183(4):2793-2800.
209. Sukkar MB, Xie S, Khorasani NM, Kon OM, Stanbridge R, Issa R, and Chung KF (2006) Toll-like receptor 2, 3, and 4 expression and function in human airway smooth muscle. J Allergy Clin Immunol; 118(3):641-648.
210. Takeda K and Akira S (2005) Toll-like receptors in innate immunity. Int Immunol; 17:11-14.
211. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, and Akira S (2002) Cutting Edge: Role of Toll-Like Receptor 1 in Mediating Immune Response to Microbial Lipoproteins. J Immunol; 169:10-14.
212. Takizawa H (1998) Airway epithelial cells as regulators of airway inflammation. Int J Mol Med; 1(2):367-378.
213. Tal G, Mandelberg A, Dalal I, Cesar K, Somekh E, Tal A, Oron A, Itskovich S, Ballin A, Houri S, Beigelman A, Lider O, Rechavi G, and Amariglio N (2004) Association between common Toll-like receptor 4 mutations and severe respiratory syncitial virus disease. J Infect Dis; 189(11):2057-2063.
214. Tan WC (2005) Viruses in asthma exacerbations. Cur Opin in Pulmon Med; 11(1):21-26.
215. Taura M, Eguma A, Suico MA, Shuto T, Koga T, Komatsu K, Komune T, Sato T, Saya H, Li JD, and Kai H (2008) p53 regulates Toll-like receptor 3 expression and function in human epithelial cell lines. Mol Cell Biol; 28(21):6557-6567.
216. Thompson JM and Iwasaki A (2008) Toll-like receptors regulation of viral infection and disease. Adv Drug Deliv Rev; 60(7):786-794.
217. Tomich M, Herfst CA, Golden JW, and Mohr CD (2002) Role of Flagella in Host Cell Invasion by Burkholderia cepacia. Infect and Immun; 70(4):1799-1806.

218. Triantafilou M, Gamper FGJ, Haston RM, Mouratis MA, Morath S, Hartung T, and Triantafilou K (2006) Membrane Sorting of Toll-like Receptor (TLR)-2/6 and TLR2/1 Heterodimers at the Cell Surface Determines Heterotypic Associations with CD36 and Intracellular Targeting. J Biol Chem; 281(41):31002-31011.
219. Trinchieri G and Sher A (2007) Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol; 7:179-190.
220. Tulic MK, Fiset PO, Manoukian JJ, Frenkiel S, Lavigne F, Eidelman DH, and Hamid Q. Role of toll-like receptor 4 in protection by bacterial lipopolysaccharide in the nasal mucosa of atopic children but not adults. Lancet; 363(9422):1689-1697.
221. Underhill DM (2003) Macrophage recognition of zymosan particles. J Endotoxin Res; 9:176-180.
222. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, and Aderem A (1999) The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature; 401(6755):811-815.
223. Vabulas RM, Ahmad-Nejad P, da Costa C, Miethke T, Kirschning CJ, Hacker H, and Wagner H (2001) Endocytosed HSP60 Use Toll-like Receptor 2 (TLR2) and TLR4 to activate to activate the Toll/Interleukin-1 Receptor Signaling Pathway in Innate Immune Cells. J Biol Chem; 276(33):31332-31339.
224. van Ewijk BE, van der Zalm MM, Wolfs TFW, and van der Ent CK (2005) Viral respiratory infections in cystic fibrosis. J Cyst Fibro; 4:31-36B.
225. Vercammen E, Staal J, and Beyaert R (2008) Sensing of Viral Infection and Activation of Innate Immunity by Toll-Like Receptor 3. Clin Microbio Rev; 21(1):13-25.
226. Wang Y, Bai C, Li K, Adler K, and Wang X (2008) Role of airway epithelial cells in development of asthma and allergic rhinitis. Respiratory Medicine; 102(7):949-955.
227. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, Holgate ST, and Davies DE (2005) Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. J Exp Med; 201(6):937-947.
228. Weber NR, Morse MA, and Gay NJ (2004) Four N-linked glycosylation sites in human Toll-like receptor 2 cooperate to direct efficient biosynthesis and secretion. J Biol Chem; 279:34589-34594.
229. Welliver RC Sr (2008) The immune response to respiratory syncytial virus infection: friend or foe? Clin Rev Allergy Immunol; 34(2):163-173.
230. Werner SL, Barken D, and Hoffmann A (2005) Stimulus specificity of gene expression programs determined by temporal control of IKK activity. Science; 309(5742):1857-1861.

231. Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A, Tobias PS, and Ulevitch RJ (2001) Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat Immunol; 2(4):346-352.
232. Wilkinson TM, Donaldson GC, Johnston SL, Openshaw PJ, and Wedzicha JA (2006) Respiratory syncytial virus, airway inflammation, and FEV1 decline in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med; 173:871-876.
233. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, and Mathison JC (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science; 249(4975):1431-1433.
234. Xiao W, Hsu YP, Ishizaka A, Kirikae T, and Moss RB (2005) Sputum cathelicidin, urokinase plasminogen activation system components, and cytokines discriminate cystic fibrosis, COPD, and asthma inflammation. Chest; 128(4):2316-2326.
235. Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, and Shu HB (2005) VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol Cell; 19(6):727-740.
236. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, and Akira S (2003) TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol; 4:1144-1150.
237. Yamashita K, Imaizumi T, Taima K, Fujita T, Ishikawa A, Yoshida H, Oyama C, and Satoh K (2005) Polyinosinic-polycytidylic acid induces the expression of GRO-alpha in BEAS-2B cells. Inflammation; 29(1):17-21.
238. Yamazaki K, Suzuki K, Yamada E, Yamada T, Takeshita F, Matsumoto M, Mitsuhashi T, Obara T, Takano K, and Sato K (2007) Suppression of Iodide Uptake and Thyroid Hormone Synthesis with Stimulation of the Type I Interferon System by Double-Stranded Ribonucleic Acid in Cultured Human Thyroid Follicles. Endocrinology; 148(7):3226-3235.
239. Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M Jr, Akira S, Yonehara S, Kato A, and Fujita T (2005) Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol; 175(5):2851-2858.
240. Yoneyama M and Fujita T (2007) Function of RIG-I-like receptors in antiviral innate immunity. J Biol Chem; 282:15315-15318.
241. Zalacain R, Sobradillo V, Amilibia J, Barrón J, Achótegui V, Pijoan JI, and Llorente JL (1999) Predisposing factors to bacterial colonization in chronic obstructive pulmonary disease. Eur Respir J; 13(2):343-348.

242. Zeldin DC, Eggleston P, Chapman M, Piedimonte G, Renz H, and Peden D (2006) How exposures to biologics influence the induction and incidence of asthma. Environ Health Perspet; 114(4):620-626.
243. Zughaier SM, Ryley HC, and Jackson SK (1999) Lipopolysaccharide (LPS) from Burkholderia cepacia is more active than LPS from Pseudomonas aeruginosa and Stenotrophomonas maltophilia in stimulating tumor necrosis factor alpha from human monocytes. Infect Immun; 67:1505-1507.