Interaction between Macrophages and Epithelial Cells in Innate Immune Responses against Adenoviral Vectors

by

Benjamin Haeyul Lee

A thesis submitted in conformity with the requirements for the degree of Philosophy of Doctor

The Institute of Medical Science
University of Toronto

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Doctor of Philosophy
The Institute of Medical Science
University of Toronto
2012

Abstract

Although induction of innate immune responses during viral infection is essential, it can cause acute inflammation and lead to devastating results. The deleterious effect of innate immune responses has been demonstrated in gene therapy where administration of a replication deficient adenoviral vector (Ad) caused fatality during a clinical trial. Despite recent advances in our understanding of the innate immunity, there is a lack of understanding on how different cell types interact to mount inflammatory responses, which may play an important role in regulating immune responses in vivo.

In this study, we investigated the interaction between macrophages and epithelial cells, the two major cell types capable of sensing and responding to viral infection in the airway, in induction of inflammatory responses against replication deficient Ads. We show in Chapter 2 that Ad infection of the macrophage-epithelial cell co-culture resulted in synergistic induction of inflammatory responses. Ad infection of the co-culture compared to macrophages alone resulted in higher cytotoxicity and induction of
significantly higher levels of inflammatory mediators including pro-inflammatory cytokines, chemokines, nitric oxide, and reactive oxygen species. We found that these synergistic responses require macrophages and epithelial cells to be in close proximity suggesting that a novel mechanism regulates the inflammatory responses.

In Chapter 3, we studied whether ATP plays a role in regulating inflammatory responses during acute Ad infection. Using the co-culture system, we found that ATP signaling through P2X7 receptor (P2X7R) is critical as inhibition or deficiency of P2X7R resulted in reduced inflammatory responses. We demonstrate that ATP-P2X7R signaling regulates inflammasome activation and IL-1β secretion. Furthermore, intranasal administration of Ad resulted in high mortality in mice but inhibition of ATP-P2X7R signaling enhanced survival and reduced inflammatory responses. These results suggest that ATP released by the infected cells plays an important role in regulating inflammatory responses during acute viral infection.
I am most grateful to my supervisor, Dr. Jim Hu for his guidance and support during my study. He was always there for me and led me with optimistic encouragement so that I could focus on my research. I wish to thank all the help from lab members who have provided reagents, helped my experiments and shared their knowledge and resources. I also thank my advisory committee, Dr. Sergio Grinstein, and Dr. Dana Philpott for their excellent guidance that allowed me to find answers whenever I was lost.

My study could not have been possible without the collaboration with other research groups, and I am indebted to Dr. David Hwang, Dr. Nades Palaniyar, Dr. Tom Steinberg and Dr. Alberto Matin and the late Dr. Paul Bertics for their assistance and excellent advice during this study. I also thank Dr. Stephen Girardin and Dr. Karen Mossman for serving in the final examination committee. I would like to express my gratitude to the Natural Science and Engineering Research Council for supporting me financially during my study.

Last but definitely not least, I could not have taken on this challenge without my family and friends. I have to say this study is the fruit of their faithful prayers and overflowing love, and I am forever grateful to them.
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<td>adenoviral vector</td>
</tr>
<tr>
<td>AFP</td>
<td>3- (p-aminophenyl) fluorescein</td>
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<td>AIM2</td>
<td>absent in melanoma 2</td>
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<td>ALI</td>
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<td>AM</td>
<td>alveolar macrophage</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>apoptosis-related speck-like protein</td>
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<td>ATPR-B2, P2X7R deficient J774 cell line</td>
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<td>bronchoalveolar lavage fluid</td>
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<td>duffused alveolar damage</td>
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<td>danger associated molecular pattern</td>
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<td>2',7'-dichlorodihydrofluorescein diacetate</td>
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<td>DC-SIGN</td>
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<td>ds</td>
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<td>ERK</td>
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<td>i.n.</td>
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<td>IL</td>
<td>interleukin</td>
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<td>inducible nitric oxide synthase</td>
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<td>IL-1R-associated kinase</td>
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<td>immunoreceptor tyrosine-based activation motif</td>
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<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
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<td>LGP2</td>
<td>laboratory of genetics and physiology 2</td>
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<td>LPS</td>
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<td>lethal toxin</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MDP</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
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<td>MSU</td>
<td>mono-sodium urate</td>
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<td>NK</td>
<td>natural killer cell</td>
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<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NOD</td>
<td>nucleotide oligomerization domain</td>
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<tr>
<td>oATP</td>
<td>oxidized ATP</td>
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<td>P2X7R</td>
<td>P2X7 receptor</td>
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<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<tr>
<td>pDC</td>
<td>plasmocytoid dendritic cell</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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<td>PMA</td>
<td>phorbal-12-myristate-13-acetate</td>
</tr>
<tr>
<td>poly(I:C)</td>
<td>polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>pro-IL-1</td>
<td>precursor of IL-1</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
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<td>PYD</td>
<td>pyrin domain</td>
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<td>PYHIN</td>
<td>pyrin and HIN200 domain</td>
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<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
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<td>Raw</td>
<td>Raw 264.7 mouse macrophage cell line</td>
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<td>RDG</td>
<td>the arginin-glycine-aspartic acid motif</td>
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<td>retinoic acid-inducible gene-I</td>
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<td>RIP</td>
<td>receptor interacting protein</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I like receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SARM</td>
<td>Steruke-alpha and Armadillo motif-conatining protein</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>severe acute respiratory syndrome coronavirus</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SF</td>
<td>P2X7R deficient Raw cell line</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>STAT</td>
<td>singal transducer and activator of transcription</td>
</tr>
<tr>
<td>STING</td>
<td>stimulator of interferon genes</td>
</tr>
<tr>
<td>T3SS/T4SS</td>
<td>type 3/4 secretion system</td>
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<td>TAK1-binding protein</td>
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<tr>
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<td>TFG-β-activated kinase</td>
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<tr>
<td>TBK</td>
<td>TANK binding kinase</td>
</tr>
<tr>
<td>Th</td>
<td>helper T cell</td>
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<tr>
<td>TIR</td>
<td>Toll-interleukin-1 receptor</td>
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<td>TIR adaptor protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNFR-associated death domain</td>
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<tr>
<td>TRAF</td>
<td>TNFR-associated factor</td>
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<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain containing adaptor inducing IFN-β</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>vp</td>
<td>viral particles</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
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Chapter 1
Introduction

1.1 Innate Immune Response

1.1.1 Introduction to innate immunity

The immune system that protects the body from pathogens has two arms, the innate immune system and the adaptive immune system. The adaptive immune system provides antigen specific defensive mechanisms based on somatic recombination that can provide virtually unlimited antigen recognition capacity. In order to acquire adaptive immunity against a certain pathogen, however, the host needs to be exposed to that particular pathogen. Eradication of the infection is achieved by subsequent mobilization of the immune system through clonal expansion, which provides protection against the same pathogen. Due to the requirement of pre-exposure, adaptive immunity is also called acquired immunity and much of its mechanism has been unraveled in the last few decades.

In contrast to the adaptive immune system, the innate immune system provides immediate and relatively broad defensive mechanisms against pathogens. It is through the innate immune system that pathogens are first recognized and its activation leads to various measures to contain the infection and fight the pathogen. Thus, the innate immune system has two main functions; 1) sensing the presence of invading pathogens and 2) providing effector mechanisms to deal with the infection (Beutler, 2004).

Activation of the innate immune system results in induction of antimicrobial mechanisms to thwart the infection but it also mobilizes the adaptive immune response. The innate immune response is required for induction of the adaptive immune response and affects the outcome of the immune response against a particular infection (Palm and Medzhitov, 2009).
Although the innate immune system has been recognized since the 1800’s, its importance has recently resurfaced thanks to several landmark discoveries in this field (Medzhitov, 2009). Specifically, there has been substantial accumulation of knowledge on how pathogens are detected by various innate molecules. Proposed by Dr. Charles Janeway, the idea of pathogen recognition through the unique molecular signature called pathogen associated molecular pattern (PAMP) has been accepted as one of the key features in the innate immune system. Based on his theory, there has been a myriad of studies identifying PAMPs on pathogens and their cellular receptors called pattern recognition receptors (PRRs).

### 1.1.2 Pathogen recognition by the innate immune system

Until the mid-1990, innate immunity was generally conceived as a nonspecific defense mechanism against microbes. However, discovery of Toll-like receptors (TLRs) based on the pattern recognition theory has revolutionized the field. Over the last fifteen years a large number of PRRs that belong to several distinct families have been identified. Understanding the mechanism of how these molecules sense various microbes and respond to infection has become an important topic in the field of innate immunity.

Unlike the antigen specific immune receptors in the adaptive immune system, PRRs are germ-line encoded proteins expressed in innate immune cells. However, studies have clearly shown that PRRs also have ligand specificity and are regulated by underlying mechanisms that have been grossly overlooked. Furthermore, there is a growing interest in the function of PRRs as it became apparent that activation of the innate immune response by PRRs can dictate the outcome of the adaptive immune response downstream (Iwasaki and Medzhitov, 2010).

#### 1.1.2.1 TLRs

TLRs are the first family of PRRs discovered. There are 10 human and 12 murine TLRs presently identified. TLRs are type I transmembrane proteins containing leucine-rich repeat (LRR) ectodomains, transmembrane domains, and intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domains (Takeuchi and Akira, 2010). The LRR domains mediate
the recognition of PAMPs and the TIR domain is required for downstream signal transduction. TLRs can recognize various PAMPs including lipids, lipoproteins, proteins and nucleic acids that are derived from a wide range of microbes such as bacteria, viruses, parasites and fungi (Akira et al., 2006). TLRs are largely divided into two subgroups depending on where the recognition of PAMPs occurs. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on cell surfaces and recognize outer microbial components. The other group of TLRs including TLR3, TLR7, TLR8 and TLR9 are localized in intracellular vesicles such as endosomes and lysosomes and are mostly involved in detecting microbial nucleic acids.

TLR4 was the first PRR to be identified. TLR4 in complex with MD2 on the cell surface binds to bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria that can cause septic shock. Other proteins such as LPS-binding protein and CD14 are also known to be involved in TLR4 binding to LPS. The formation of a receptor multimer composed of two copies of the TLR4-MD2-LPS complex initiates signal transduction through intracellular adaptor molecules. In addition to LPS, TLR4 is involved in the recognition of other PAMPs such as respiratory syncytial virus fusion proteins, envelope protein of mouse mammary tumor virus, and *Streptococcus pneumoniae* pneumolysin (Kawai and Akira, 2010).

TLR2 can recognize a wide range of PAMPs derived from various pathogens including lipopeptides from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, glycoprotein from *Trypanosoma cruzi* and the hemagglutinin protein from measles virus (Kawai and Akira, 2010). TLR1 or TLR6 forms heterodimers with TLR2 to provide specificity to different ligands. Moreover, other co-receptors on the cell surface such as CD36 and dectin-1 are also involved in the recognition of PAMPs by TLR2 (Goodridge and Underhill, 2008; Hoebe et al., 2005).

TLR5 recognizes flagellin, the protein component of bacterial flagella. Lamina propria DCs in the small intestine have high TLR5 expression and it plays important roles in promoting the differentiation of IL-17-producing helper T (Th)17 cells and Th1 cells, as
well as the differentiation of naive B cells (Uematsu et al., 2008). TLR11 in mouse is a relative of TLR5 and is thought to be involved in detection of pathogenic bacteria components (Zhang et al., 2004) and the profilin-like molecule derived from *Toxoplasma gondii* (Yarovinsky et al., 2005).

The intracellular TLRs survey the presence of microbes in cytoplasmic vesicles that contain the cargo from the extracellular environment imported through various mechanisms such as endocytosis, macropinocytosis, and phagocytosis. The intracellular TLRs, including TLR3, TLR7, TLR8, and TLR9 detect nucleic acids within the endolysosomal compartments so that they can distinguish foreign nucleic acids from the host DNA and RNA. These intracellular TLRs are expressed in endoplasmic reticulum (ER) and are rapidly translocated by chaperon molecules such as UNC93B1to endosomes upon cellular stimulation. After reaching the endosomal compartment, TLRs require endolysosomal acidification processing for their activation and ligand binding (Blasius and Beutler, 2010).

TLR3 was originally discovered to recognize a synthetic analog of double-stranded (ds) RNA, poly(I:C) (polyninosinic-polycytidylic acid). In addition to poly(I:C), TLR3 detects dsRNA viruses by recognizing their genomic RNA or single stranded (ss) RNA as well as dsDNA virus through the intermediate dsRNA product during viral replication (Kawai and Akira, 2008). TLR3 is shown to be essential in antiviral immune responses since TLR3-deficiency is associated with susceptibility to viral infection (Blasius and Beutler, 2010).

Identified as a receptor for imidazoquinoline derivatives such as imiquimod and resiquimod, such as R-848, and guanine analogs such as loxoribine, TLR7 recognizes ssRNA. Plasmocytoid dendritic cells (pDCs), which produce large amounts of type I interferon (IFN) after virus infection, express a high level of TLR7. Induction of cytokines by pDCs in response to RNA viruses was shown to be TLR7 dependent (Akira et al., 2006). Conventional DCs (cDCs) also detect RNA species from certain bacteria to induces type I interferon (Mancuso et al., 2009). Recognition of RNA viruses such as HSV (herpes simplex virus) -1, HIV(human immunodeficiency virus) -1, and influenza
by TLR7 occurs in endosomes in a replication-independent manner. On the other hand, vesicular stomatitis virus (VSV) and Sendai virus require cytoplasmic replication and autophagy formation before they can be recognized by TLR7 in the lysosome (Lee et al., 2007). This suggests that autophagy is an important delivery mechanism for cytosolic viral PAMPs to the lysosome, where TLR7 can recognize infection by RNA viruses and initiate antiviral responses. Similar to TLR7, TLR8 recognize imidazoquinoline derivatives such as R-848 and mediates the response to viral ssRNA although mice lacking TLR8 respond normally to these ligands (Kawai and Akira, 2006). TLR8 is expressed in various tissues, with its highest expression in monocytes, and is up-regulated after bacterial infection.

TLR9 was first identified as a receptor for bacterial DNA containing unmethylated CpG motifs, which are more frequent in bacterial or viral DNA than in mammalian genomic DNA (Hemmi et al., 2000). Since then, several DNA viruses as well as genomes of bacteria and protozoa were shown to be recognized by TLR9 (Bauer et al., 2008). Synthetic CpG oligonucleotides containing phosphorothioate linkages have been commonly used to study TLR9-dependent innate immune responses. While studies have addressed sequence specific or modification specific recognition of DNA by TLR9, recently reports challenge the idea that unmethylated CpG is required for DNA recognition. In fact, studies have shown that DNA recognition by TLR9 depends on the 2′ deoxyribose phosphate backbone, and neither phosphorothioate linkages nor specific sequences are necessary to induce a response (Kumagai et al., 2008). In addition, even vertebrate DNA was shown to activate TLR9 when delivered to the endosomes by transfection, suggesting that the cellular localization rather than the sequence or the structure of DNA might be more important in TLR9 activation (Blasius and Beutler, 2010).

Recognition of PAMPs by TLRs leads to activation of signaling cascades through TIR domain-containing adaptor molecules recruited to TLRs in the cytoplasm. So far, five adaptors molecules were identified including myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIRAP (TIR adaptor protein, also known as Mal), TRIF-related adaptor molecule (TRAM), and
Sterile-alpha and Armadillo motif-containing protein (SARM) (Takeuchi and Akira, 2010). In general, TLR signaling is classified as either MyD88 or TRIF-mediated signaling cascade. MyD88 can relay downstream signaling of all TLRs, except TLR3. Some TLRs such as TLR2 and TLR4 require TIRAP to bridge the interaction between the TLR and MyD88. MyD88 interacts with IL-1R-associated kinase (IRAK)-4, a serine/threonine kinase, which, in turn, activates other IRAK family members such as IRAK-1 and IRAK-2. The IRAKs then interact with TNFR-associated factor 6 (TRAF6), which is an E3 ubiquitin ligase. Activation of TRAF6 leads to self-ubiquitination and generation of an unconjugated free polyubiquitin chain linked to Lys63, which bind to TGF-β-activated kinase (TAK) 1-binding protein 2 (TAB2), and TAB3 that regulate TAK1 (Xia et al., 2009). Activated TAK1 phosphorylates IκB kinase (IKK)-β, resulting in the degradation of IκBa and activation of NF-κB. TAK-1 also activates MAP kinase kinase 6 to initiate the MAP kinase cascade to induce formation of transcription factor complex AP-1. Thus, TLR signaling through MyD88 culminates in activation of two pro-inflammatory transcription factors, NF-κB and AP-1, which induce cytokine genes. In addition, type I IFNs can be induced by TLR7 and TLR9 through MyD88 downstream signaling via activation of interferon regulatory factor (IRF) 7 in pDC. IRAK1, IKKα, and TRAF3 are involved in the activation of IRF7 in addition to TRAF6 and IRAK4, which are required in NF-κB activation as well (Kawai and Akira, 2008).

The TRIF-dependent pathway activates IRF3 and NF-κB transcription factors. NF-κB activation in TRIF pathway is similar to that of the MyD88-dependent pathway but RIP1 is also involved in activating TRAF6 utilizing ubiquitination-dependent mechanisms. Also, TNFR-associated death domain (TRADD) and Pellino-1 are adaptor molecules shown to be involved in RIP1 activation (Kawai and Akira, 2010). The multi-protein complex consisting of TRAF6, TRADD, Pellino-1 and RIP1 activates TAK1, which in turn activates the NF-κB and mitogen-activated protein kinase (MAPK) pathways. In addition to NF-κB activation, the TRIF-dependent pathway leads to IRF3 activation, which results in induction of type I IFNs. This signaling pathway is mediate by TRAF3 as well as TANK binding kinase (TBK)-1 and IKKi (IKKe), which catalyze the phosphorylation of IRF3 and induce its nuclear translocation.
1.1.2.2 Retinoic acid-inducible gene-I (RIG-I) like receptors (RLRs)

Although recognition of nucleic acids by TLRs in pDCs provides an important innate immune response against viruses, studies have suggested that there were TLR-independent mechanisms for RNA recognition. Subsequently, RIG-I was identified as a gene capable of inducing type I IFNs upon stimulation with poly (I:C) in various cell types (Nakhaei et al., 2009). RIG-I belongs to the DExD/H box-containing RNA helicase family and has two caspase recruitment domains (CARDs), a central helicase domain and a C-terminal regulatory domain. RIG-I is maintained in an inactive monomer state but upon viral RNA binding it changes the conformation that allows dimerization and interaction with the downstream adaptor molecule IPS-1 (also known as MAVS, VISA, or CARDIF).

The interaction between RIG-I and IPS-1 is mediated by CARD–CARD homotypic interactions. IPS-1 is localized on the outer membrane of mitochondria suggesting that mitochondria are the sites for RLR-mediated signaling. The interaction between activated RIG-I and IPS-1 induces further recruitment of other downstream signaling molecules. TRAF3 directly interacts with IPS-1 and induces downstream protein kinases and IKKs to activate transcription factors such as IRF3, IRF7 and NF-κB. TRAF2 or TRAF6 were also shown to interact with the IPS-1 and they are likely to be responsible for NF-κB activation (Yoneyama and Fujita, 2009). Two death domain containing molecules, Fas-associated death domain (FADD) and RIP1, interact with IPS-1, which leads to NF-κB activation via caspase-8 and caspase-10. Recently, it has been shown that TRADD plays an important role as an adaptor molecule in forming a multi-protein complex consisting of IPS-1, TRAF3, TANK, FADD, and RIP1 (Nakhaei et al., 2009). Another protein called stimulator of interferon genes (STING, also known as MITA or MPYS) was identified as a new component to the antiviral pathway. STING is localized on the outer mitochondrial membrane and forms a complex with RIG-I and IPS-1, which leads to recruitment of TBK1 and IRF-3 activation.
Melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) are two other members of the RLR family. They show significant homology to RIG-I, particularly in their helicase domain, but MDA5 lacks the C-terminal regulatory domain and LGP2 is completely devoid of the N-terminal CARD domain. Functional analyses indicated that MDA5 plays an essential role in virus-induced innate immunity. In contrast to RIG-I and MDA5, LGP2 is known as a negative regulator in innate immune responses, possibly by disrupting the homotypic interaction of RIG-I with its downstream molecules. However, a recent report indicated that LGP2 can play a positive role in the innate immune response against encephalomyocarditis virus, requiring further studies to determine the physiological role of LGP2 (Yoneyama and Fujita, 2009).

Interestingly, knockout (KO) mouse models showed that RIG-I and MDA5 detect different types of viruses. For example, hepatitis C virus, Sendai virus, influenza virus, VSV, Newcastle disease virus, rabies virus, and Japanese encephalitis virus are recognized by RIG-I, whereas MDA-5 detects picornaviruses such as EMCV and responds to poly I:C stimulation. This specificity is related to preferential recognition of RNA structures by RIG-I or MDA5. In general, RIG-I recognizes ssRNA and shorter dsRNA (<1 kb) while recognition of longer dsRNA is mediated by MDA5 (Nakhaei et al., 2009). Furthermore, biochemical studies demonstrated that RIG-I distinguishes viral RNA from cellular RNA by its 5’-triphosphate modification.

1.1.2.3 Nucleotide oligomerization domain (NOD)-like receptors (NLRs)

NLRs comprise a large family of intracellular PRRs that have a common structural design; a variable N-terminal protein-protein interaction domain, a central NOD domain, and a C-terminal LRR domain (Chen et al., 2009). The variable N-terminal domain of a NLR may contain a CARD domain, pyrin (PYD) domain, acidic transactivating domain, or baculovirus inhibitor repeat, and these domains mediate interaction with downstream signaling molecules. In the recent nomenclature, NLRs have been classified into four subfamilies based on their N-terminal domains (NLRA, NLRB, NLRC, and NLRP);
NLRX is an orphan NLR, whose N-terminal domain does not show a strong homology with any of the subfamilies. The central NOD domain regulates self-oligomerization during activation of NLRs and the C-terminal LRR domain detects various PAMPs.

Currently, there are 22 human and 33 murine NLR genes discovered. Although many studies demonstrated that the NLRs are involved in detection of various pathogens, there are only a few NLRs whose ligands were identified. NOD1 and NOD2 are the first NLRs identified to detect distinct moieties of bacterial peptidoglycan. NOD1 senses peptidoglycan containing meso-diaminopimelic acid (meso-DAP) and NOD2 detects muramyl dipeptide (MDP) (Fritz et al., 2006). In vitro studies showed activation of NOD1 and NOD2 by several pathogenic bacteria but their role in vivo is still under intense investigation (Chen et al., 2009).

NOD1 and NOD2 downstream signaling pathways are similar to TLR signaling and utilize common target molecules (Franchi et al., 2009b). Upon recognition of their PAMP, they self-oligomerize and recruit the adaptor protein receptor interacting protein (RIP) 2 (also known as RICK) via CARD-CARD interaction. RIP2 is a serine-threonine kinase that becomes polyubiquitinated upon activation, which is essential for recruitment and activation of TAK1. Activated TAK1 in complex with TAB1, TAB2, and TAB3 promotes phosphorylation of IκKβ and the subsequent phosphorylation and degradation of IκBα, to allow nuclear translocation of NF-κB. RIP2 can also directly bind to IKKγ and promote its polyubiquitination, resulting in NF-κB activation. In addition to activation of NF-κB, stimulation of NOD1 and NOD2 results in the activation of MAPKs, including p38, extracellular signal–regulated protein kinase (ERK), and c-Jun N-terminal kinase (JNK). Although the detailed interactions in MAPK activation are not well defined, it is suggested that RIP2 and TAK1 might be involved in these pathways (Chen et al., 2009).

NLRC4 (also known as Ipaf) is known to recognize bacterial flagellin and responds to Salmonella, Pseudomonas, and Legionella. On the other hand, NLRC4 plays a role in responding to Shigella, a non-flagellated bacteria, suggesting that it may recognize yet another PAMP (Franchi et al., 2009b). NLRP1 is the first member identified in the large
NLRP subfamily that contains an N-terminal PYD domain. NLRP1 has been studied as a susceptibility factor to *Bacillus anthracis* lethal toxin (LT) *in vivo*. LT induces caspase-1 activation through the inflammasome pathway (see section 1.2) and results in macrophage cell death. NLRP1 polymorphisms have been associated with susceptibility to LT.

NLRP3 is a member of the NLRP subfamily that has been extensively studied for the last several years. NLRP3 has been identified as a sensor for various PAMPs such as LPS, MDP, DNA, RNA and RNA analogs such as poly I:C as well as the imidazoquinoline antiviral compounds, R837 and R848 (Franchi et al., 2009b). In addition to these PAMPs, NLRP3 has been implicated in inducing inflammatory responses to non-microbial molecules and particles such as uric acid, asbestos, silica and alum. Studies have shown that infection with viruses such as Sendai virus, influenza virus and adenovirus activates NLRP3, which results in activation of the inflammasome pathway. However, given the fact that NLRP3 is involved in sensing such a variety of stimuli, it has been proposed that NLRP3 might be a general sentinel for cellular distress (Martinon, 2008).

### 1.1.2.4 Other PRRs

C-type lectin receptors (CLRs) form a large superfamily that share structural homology in their high-affinity carbohydrate-recognition domains. They are classified to 17 groups based on their domain organization and phylogeny and have diverse biological functions. Among them, mannose receptor, Dectin-1, Dectin-2, dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) and the collectins are implicated in immune functions. Recent studies have shown that these CLRs play an important role as PRRs by recognizing PAMPs, modulating TLR signaling, and inducing inflammatory gene expression. Dectin 1 and dectin 2 detect β-glucan from *Candida albicans* and MINCLE is responsible for detection of fungal infection as well as an endogenous protein, SAP130, released during necrosis (Takeuchi and Akira, 2010). Activation of CLRs induces signaling pathways through immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecules, such as Fc receptor, or DAP12, or through protein...
kinases or phosphatases that either directly or indirectly interact with their cytoplasmic domains (Geijtenbeek and Gringhuis, 2009). The downstream signaling is carried out by Syk tyrosine kinase as it interacts with the ITAM motif to activate MAP kinases. Ultimately, CLR-induced signal transduction results in activation of NF-κB and induction of pro-inflammatory cytokines.

DNA from bacteria, virus, as well as non-microbial sources has been known to trigger innate immune responses that result in secretion of pro-inflammatory cytokines, such as IL-1, induction of type I interferons, and macrophage cell death (Hornung and Latz, 2010). Moreover, it was found that intracellular delivery of DNA induced TLR independent pathway indicating that there are other intracellular DNA sensors (Ishii et al., 2006; Stetson and Medzhitov, 2006). The cytosol should be normally DNA free but infection by virus or bacteria can introduce DNA in the cytoplasm that would be detected by PRRs. Subsequent efforts to find cytosolic DNA sensors resulted in the discovery of a number of new PRRs that are important in detecting DNA in the cytoplasm and induction of innate immune responses.

One of the first cytoplasmic DNA sensors discovered was DNA-dependent activator of IRFs (DAI, also known as DLM1 or ZBP1) (Takaoka et al., 2007). It was shown that DAI is essential in induction of type I IFNs in response to dsDNA and HSV-1 infection in vitro. The DNA-DAI complex physically interacts with IRF3 and TBK1, suggesting a possible downstream signaling mechanism to activate inflammatory responses. However, cells from DAI deficient mice showed normal responses to poly (dA:dT) and plasmid DNA transfection as well as to DNA virus infection, suggesting that there are multiple DNA sensing molecules in the cytosol (Hornung and Latz, 2010).

PRRs responsible for RNA recognition such as RIG-1 were regarded as possible cytoplasmic DNA sensors because knockdown of their downstream adaptor molecule, IPS-1, reduced response to poly(dA:dT) (Ishii et al., 2006). Although there is no evidence that RIG-I is involved in DNA recognition in the cytoplasm, recent studies have suggested that it might play an indirect role. The proposed pathway explains that the transfected DNA can be transcribed by RNA polymerase III in the cytoplasm to produce
dsRNA, which then activates RIG-I-mediated responses to induce type I IFNs. Indeed, functional RNA polymerase III was identified from cytoplasmic extracts and it produced RNA intermediate transcribed from poly(dA:dT), indicating the proposed pathway as one of the DNA sensing mechanisms through RNA detecting machineries (Ablasser et al., 2009). Another cytoplasmic RNA sensor, MDA5, was also shown to be involved in DNA sensing to induce type I IFN responses but the mechanism is independent of RNA polymerase III (Rathinam and Fitzgerald, 2011).

More cytoplasmic DNA sensors have been recently identified. LRRFIP1 has been shown to recognize RNA as well as DNA in the cytosol (Yang et al., 2010). Interestingly, LRRFIP1 enhanced IFN-β via activation of β-catenin. Interferon-induced protein 16 (IFI16) is a member of the pyrin and HIN200 domain (PYHIN) containing protein family that recognize DNA through its HIN domain and activates IRF-3 through STING (Unterholzner et al., 2010). Although IFI16 is localized in the nucleus the study has shown that a fraction of IFI16 entered in the cytoplasm to interact with transfected DNA, suggesting more PYHIN proteins might be involved in DNA recognition. DHX36 and DHX9 are helicases belonging to the DExD/H box helicase subfamily that sense CpG-A and CpG-B DNA, respectively, in the cytosol (Kim et al., 2010). Unlike other DNA sensing molecules, which activate IRF3, binding of DNA to DHX36 and DHX9 leads to phosphorylation of IRF7 via recruitment of MyD88.

Intracellular delivery of DNA not only induces type I IFNs but also leads to IL-1β secretion via the inflammasome pathway, but the upstream molecules that may directly interact with DNA to activate this pathway is not clear. In 2009, four research groups independently identified an IFN-inducible protein, absent in melanoma 2 (AIM2), as a DNA receptor that can activate the inflammasome pathway (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). AIM2 is a PYHIN containing protein that can interact with apoptosis-related speck-like protein (ASC, also known as PYCARD, CARD5 and TMS1), an important adaptor molecule for inflammasome activation. AIM2 binds to DNA through the HIN200 domain whereas its pyrin domain associates with ASC to activate both NF-κB and caspase-1. Studies have shown that AIM2 preferentially binds to dsDNA rather than ssDNA and does not
discriminate the source or the sequence of the DNA as viral and bacterial infection as well as transfection of synthetic DNA induced secretion of IL-1β (Hornung and Latz, 2010). In addition to IL-1β secretion, activation of inflammasome AIM2 resulted in formation of ASC pyroptosome (see section 1.3.3) and cell death (Fernandes-Alnemri et al., 2009). In mice, another HIN200 protein, p202, was found to bind to dsDNA but had inhibitory effects on inflammasome activation. Since p202 can heterodimerize with AIM2 but lacks pyrin domains it has been proposed that the inhibitory effect of p202 is caused by interfering with the AIM2-mediated inflammasome pathway (Roberts et al., 2009).

1.1.2 Inflammatory responses

Inflammation is a protective response to injury or irritation of tissues by means of destroying, diluting, or sequestering the injurious agent and the injured tissue. Wounds and infection are the two common causes of inflammation and successful inflammatory responses result in elimination of the injurious agent and restoration of normal tissue functions followed by repair processes. Inflammation has several distinct physiological characteristics; redness and swelling with heat and pain as well as loss of physiological function (Medzhitov, 2010). These signs of inflammation are consequences of vascular and cellular responses mediated by various inducible factors, whose expression is tightly controlled as any aberration of the processes could cause devastating consequences to the host.

The initial inflammatory responses cause vasodilation rendering increased blood flow and infiltration of leukocytes into the affected tissue. The increased blood flow manifests redness, heat, and swelling and causes pain due to increased pressure. More importantly, the increased blood vessel diameter slows down the speed of blood flow, facilitating infiltration of leukocytes into the tissue. Chemokines expressed by the cells in the injured or infected tissue further help leukocytes home-in to the affected area. Among the infiltrates neutrophils, macrophages, monocytes and DCs are the major players that orchestrate the inflammatory responses and restoration processes. Although each of these
cells has its own distinctive functions, inflammatory responses are sequential events mediated by network of signals among various cell types, which include cells from the affected local tissue such as epithelial cells.

### 1.1.2.1 Cells involved in inflammatory responses

Neutrophils are the most prominent inflammatory cells that are rapidly recruited by chemokines such as IL-8 (Nathan, 2006). Activation of neutrophils by microbial products and pro-inflammatory mediators initiates potent antimicrobial responses. Pathogens are engulfed by neutrophils and killed within the phagosomes containing reactive oxygen species (ROS) and proteases. Neutrophils can release cytotoxic agents through degranulation mechanism to destroy extracellular pathogens but this can also cause substantial bystander tissue damage. In addition, neutrophils produce chemokines that can recruit and activate other immune cells to aid clearance of the pathogen. Most neutrophils recruited into the inflamed tissue have a short lifespan and die by apoptosis, which plays an important role in the resolution of inflammation and tissue repair.

Monocytes also infiltrate into the tissue during inflammation. Although monocytes have been known to develop into macrophages or DCs, it is yet to be determined whether those infiltrating monocytes during inflammatory situations also function as macrophages or DCs (Yona and Jung, 2010).

Macrophages and DCs are important resident innate immune cells. They are located at the ports of entry for pathogens such as the mucous linings in the respiratory, intestinal, and genital tracts and constantly probe the presence of invading pathogens (Kohlmeier and Woodland, 2009). The strategic localization and expression of various PRRs allow these immune cells to efficiently detect any infection and initiate inflammatory responses. Their activation at the site of injury or infection leads to secretion of cytokines and chemokines that allow mobilization and migration of other inflammatory cells such as neutrophils and monocytes. Although macrophages and DCs exert phagocytic and microbicidal activity during inflammatory responses, their destructive effects on the host are less severe compared to neutrophils (Dale et al., 2008). In addition to their innate
immune functions, DCs and macrophages, to some extent, are potent antigen presenting cells (APCs). Therefore, DCs and macrophages are important as sensors and sentinels in innate immunity but also play a critical role in initiating adaptive immune responses.

pDCs are distinguished from cDCs for their specialized role in innate immune responses. Despite the small number of pDCs, constituting only 0.2-0.8% of human blood cells, they play an important role in detecting viral infection through their unique repertoire of PRR expression including TLR7 and TLR9. Once they are activated, pDCs rapidly produce large amount of type I IFNs, which induce potent antiviral responses in various types of immune cells. Although pDCs were found in peripheral tissues during viral infection and in inflammatory diseases, they mostly reside in lymphoid organs under steady state conditions suggesting that they might be recruited by inflammatory stimuli (Lande and Gilliet, 2010). However, the mechanism of pDC migration from lymphoid tissue to peripheral tissue is poorly understood. In addition to the cells mentioned above other types of immune cell such as natural killer (NK) cells, mast cells, T cells, and B cells reside in the peripheral tissues and can be involved in innate immune responses against various pathogens.

In addition to the professional immune cells, there is considerable evidence that epithelial cells are also involved in the early immune response and inflammation. The epithelial cells lining the airway, intestine, and genital tract are exposed to the environment and they are at the best position to sense invading pathogens. Unfortunately, the role of epithelial cells in innate immune response has been generally overlooked. Until recently, epithelial cells were merely considered as cells providing physical barriers against potentially harmful substances and microbial pathogens or cells involved in non-specific defense mechanisms such as secretion of antimicrobial molecules. For this reason, most of the studies on pathogen recognition were focused on immune cells such as macrophages, DCs, and monocytes and the contribution of epithelial cells in inflammatory response has been mostly neglected. However, it is becoming clear that epithelial cells play an important part in pathogen recognition and regulation of immune responses as they express PRRs and respond to pathogens by induction of cytokines and chemokines.
Studies have shown that epithelial cells express various PRRs allowing detection of microbes through relevant PAMPs (Kato and Schleimer, 2007). Once a pathogen is detected by PRRs, downstream signaling pathways are activated to induce various genes involved in inflammatory responses. Although it is not clear whether the downstream signaling pathways are the same in epithelial cells as in the innate immune cells, NF-κB and AP-1 are commonly activated and often regarded as an indicator of inflammatory responses in epithelial cells. Activation of these transcription factors leads to induction of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and GM-CSF. In addition to cytokines, epithelial cells produce and secrete antimicrobial molecules upon activation. These molecules are enzymes, permeabilizing peptides, opsonins, protease inhibitors and toxic molecules that directly affect pathogens (Kato and Schleimer, 2007). The importance of these antimicrobial molecules in innate immunity has been highlighted in studies showing their deficiencies cause microbial colonization in the epithelial layers.

Although epithelial cells can provide important host defense mechanisms directly against pathogens there is an increasing interest in their regulation of immune responses through interaction with other immune cells in the tissue. It has been shown that epithelial cells can influence DCs, T cells and B cells in their immune responses and the consequent outcomes. Recent studies have shown that activated epithelial cells can dictate DC control of Th differentiation via production of the DC activator, thymic stromal lymphopoietin (TSLP) (Lambrecht and Hammad, 2010). TSLP is an IL-17 like cytokine secreted by epithelial cells that induces Th2 differentiation by stimulating DCs to produce IL-4, IL-13 and TNF-α, but not IL-10 and IFN-γ. TSLP binding to the TSLP receptor on DCs induces expression of OX40L, which is considered the key regulator of Th2 differentiation. Over-expression of TSLP in epithelial cells has been related to chronic inflammatory conditions such as asthma and atopic dermatitis, highlighting its role in deriving Th2 immune response (Swamy et al., 2010). Induction of TSLP is dependent on activation of TLRs and induction of their downstream signaling pathways, suggesting that epithelial cells may be important decision makers as they sense various invading pathogens and send the relevant signal to DCs that would direct an appropriate immune response.
It has been known that epithelial cells are the main source of chemokines that attract lymphocytes to the site of infection. Epithelial cells can produce chemokines that are specific for Th1, Th2, or B cells so that appropriate immune cells are mobilized and required immune responses are mounted. Once T cells are recruited by chemokines, epithelial cells secrete cytokines that would further support them to carry out their immune functions. For example, IL-33 and IL-25 are produced by epithelial cells and enhance the Th2 response (Kato and Schleimer, 2007). Epithelial cells can also influence B cell immune functions. Activated epithelial cells secrete BAFF and APRIL, which are co-stimulatory signals that can induce class switching from IgG to IgA independent of T cell stimulation (Fritz et al., 2008). Taken together, activation of epithelial cells by pathogens at the first line of exposure can be the focal point where the immune response is subsequently amplified by recruitment and activation of professional immune cells (Swamy et al., 2010).

Despite the evidence that epithelial cells may play a key role in initiating and directing immune responses, the specific mechanisms of their responses to pathogens have been understudied compared to those for leukocytes. It is expected that epithelial cells express a host of PRRs but only a few studies have addressed the expression profiles in epithelial cells (Kato and Schleimer, 2007). In order to understand how epithelial cells initiate immune responses, more studies are needed to examine the repertoire of PRRs expressed in epithelial cells. It should be noted that the expression profile for PRRs in epithelial cells could be different from one organ to another and their expression might change depending on the particular inflammatory context. Furthermore, how inflammatory pathways are induced in epithelial cells and what kinds of signals are generated at the cellular level requires close scrutiny.

1.1.2.2 Macrophages in innate immune responses

Macrophages are mononuclear leukocytes derived from monocyte precursors developed in the bone marrow. It has been generally believed that the macrophages in the tissue are constantly replenished from the blood monocyte reservoir, but recent studies
demonstrated that tissue resident macrophages may be able to maintain their populations through local proliferation (Yona and Jung, 2010). The tissue resident macrophages provide two major functions. First, macrophages have a high phagocytic capacity that is essential in removing toxic substances and debris as well as dead cells. Secondly, they are equipped with various mechanisms that can detect and respond to pathogens either by producing cytotoxic agents or by releasing cytokines and chemokines to recruit other immune cells into the infected tissue. In addition to these main functions, macrophages can function as APCs and play a role in the resolution of inflammation and tissue remodeling after elimination of pathogens.

In general, it has been accepted that macrophage activation can be classified to either classical activation or alternative activation depending on the stimuli from the environment and the acquired phenotypes and functions (Mosser and Edwards, 2008). The classical activation of macrophages requires combination of two signals; IFN-γ and TNF-α or PAMPs that can induce TNF-α (Mosser and Edwards, 2008). The classically activated macrophage (so called M1 macrophage) is well characterized as an effector cell producing pro-inflammatory mediators in Th1 cellular immune responses. The M1 macrophages show significant changes in their gene expression pattern with a potent cytotoxic capacity. In addition to PAMPs, there is accumulating evidence that endogenous molecules can activate macrophages. These endogenous molecules, generally termed as danger signals, alarmins or danger associated molecular patterns (DAMPs), are released by stressed or dying cells in response to various insults and activate innate immune cells like macrophages (Zhang and Mosser, 2008). The alternatively activated macrophages (M2 macrophages) occur in the presence of IL-4 and IL-13, which are known to be induced by parasites and some fungi (Ma et al., 2003). M2 macrophages produce much less pro-inflammatory cytokines and toxic radicals compared to M1 macrophages. M2 macrophages are believed to be involved in wound healing and remodeling as they are known to secrete components of the extracellular matrix. Although macrophage activation is often classified according to M1 and M2 classification, recent studies suggest that macrophage activation can result in spectrum of different phenotypes depending on the environmental cues allowing them to perform dynamic functions (Mosser and Edwards, 2008).
The tissue resident macrophages develop and differentiate into specialized population in different anatomical locations. One of the most unique macrophage populations is alveolar macrophages (AMs). The lung has a special challenge to protect the large surface area of the respiratory tract against constantly inhaled particles, toxins, allergens and microbes. In order to eradicate harmful particles and prevent infection, the lung is equipped with several biochemical defense mechanisms such as surfactants, lysozyme and anti-microbial peptides as well as the mucociliary system to prevent pathogens from reaching the lower airways. When pathogens penetrate these barriers, AMs play a critical role at the first line of defense to efficiently detect and respond to the infection. AMs are the most numerous immune cells in the normal lung and they are localized at the interface between the air and epithelial cell lining throughout the airway but more predominantly in the alveolar space and conducting airways (Opitz et al., 2010). Pathogens such as bacteria and viruses invading into airways are detected by AMs through various PRRs on the cell surface or in the cytoplasm, which activates macrophages to produce cytokines and inflammatory mediators. Many studies demonstrated that deficiency or depletion of macrophages can exacerbate the outcome of pathogenic infection in the lung, underscoring the essential role of AMs in the immune response (Peters-Golden, 2004).

1.1.2.3 Pro-inflammatory cytokines

Recognition of pathogens and activation of downstream signaling pathways commonly lead to induction of cytokines. Cytokines can be produced by most cells upon stimulation and they can produce inflammatory effects even at very low concentrations. There are functional redundancies among cytokines as different cytokines can cause similar effects and the same cytokine can induce various effects depending on the circumstances. In general, cytokines have four major functions; promoting lymphocyte proliferation and differentiation, polarizing immune response, inducing pro-inflammatory responses or producing anti-inflammatory responses (Dinarello, 2007a). The expression of cytokines is critical in mounting proper immune responses against pathogens but their regulation is also important in resolving inflammation without significant damage to the host.
TNF-α, IFN-γ, IL-6, and IL-1 are some of the most potent pro-inflammatory cytokines. Primarily produced by macrophages, TNF-α has been associated with endotoxin induced septic shock. It has a potent chemoattractant activity, induces the expression of adhesion molecules, promotes phagocytosis, and enhances production of other inflammatory molecules such as IL-1 and prostaglandin E2 (PGE2) (Schottelius et al., 2004). A local increase in concentration of TNF-α causes inflammation in the tissue, whereas high concentrations of TNF-α induce shock-like symptoms and the prolonged exposure to TNF-α can result in cachexia. Although IFN-γ is known as a key cytokine that leads to cytotoxic T cell responses and antiviral activity, it is also considered a pro-inflammatory cytokine that augments TNF-α activity and induces nitric oxide (NO) (Schroder et al., 2004).

IL-6 is a pleiotropic cytokine with a wide range of biological activities. It can be produced by both lymphoid and nonlymphoid cells and its secretion can be stimulated by TNF-α and IL-1. IL-6 mediates a variety of biological activities in immune regulation, hematopoiesis, inflammation and oncogenesis (Kishimoto, 2010). In addition, other members of the IL-6 related cytokines such as leukemia inhibitory factor, ciliary neurotrophic factor and oncostatin M can induce similar activities. This pleiotropy and redundancy of IL-6 function can be partly explained by the common cytokine signal transducer, gp130, which mediates two downstream pathways; the JAK–STAT (Janus family tyrosine kinase–signal transducer and activator of transcription) pathway and the Ras–MAPK pathway. Expression of IL-6 is regulated through negative feedback mechanism by SOCS (suppressor of cytokine signals), a downstream target for JAK-STAT pathway (Kishimoto, 2010). IL-6 is rapidly induced during acute inflammatory reactions and has been associated with sepsis. IL-6 has been recognized as a useful marker for inflammatory responses because it persists in the plasma much longer than TNF-α or IL-1β. More recently, it has been shown that IL-6 has an essential role in the differentiation of Th17 cells.

IL-1 (IL-1α and IL-1β) is one of the first interleukins to be identified and studied in detail. Its discovery and initial functional analyses are driven by its potent fever
generating (pyrogenic) property (Dinarello, 2007a). Since the molecular identification of
IL-1α and IL-1β in the mid 1980’s, cytokines in the IL-1 family has grown to 11
members, which share a highly conserved gene structure. Although some members of the
IL-1 family, such as IL-1Ra, are known to provide anti-inflammatory functions, most of
them have potent pro-inflammatory effects. IL-1 regulates inflammatory responses by
indirect means of inducing gene expression. For example, IL-1β induces cyclooxygenase
type-2 (COX-2), phospholipase A, and inducible nitric oxide synthase (iNOS), which
then generate PGE2, platelet activating factor and NO as secondary inflammatory
effectors to induce fever, vasodilation and hypotension (Dinarello, 2009). Furthermore,
IL-1 enhances expression of extracellular adhesion molecules to facilitate infiltration of
immune cells from the circulation into the tissue and promote expression of other pro-
inflammatory cytokines and chemokines such as IL-6 (Dinarello, 2007b). In addition to
its inflammatory functions, studies have shown that IL-1 is involved in various immune
responses including stimulation of lymphocytes as well as Th2 and Th17 polarization
(Sims and Smith, 2010). The critical role of IL-1 in inflammation and immune responses
has been demonstrated by IL-1 or IL-1 receptor (IL-1RI) deficient mouse models.
Although these KO mouse models showed no phenotype under normal conditions, when
challenged with local or systemic inflammatory insults, they exhibited reduced
inflammatory responses and were more susceptible to infections.

IL-1α and IL-1β both bind to IL-1RI and similar biological actions in the target cells.
However, there is a substantial difference in the post-translational processing of these two
molecules. Both IL-1α and IL-1β are produced in precursor forms and post-
translationally processed to their mature forms. IL-1α is cleaved by a plasma membrane
enzyme, calpain, but its precursor form is as active as the mature molecule. Produced by
a wide range of cell types including epithelial and endothelial cells, IL-1α is rarely found
in circulation and believed to act locally as it is generally associated with the plasma
membrane or released during cell death (Chen et al., 2007). On the other hand, IL-1β is
mainly produced and secreted by monocytes, macrophages, and DCs. More importantly,
the precursor form of IL-1β (proIL-1β) is inactive and has to be cleaved by caspase-1 to
the mature form (mIL-1β). This process can occur in the cytoplasm or in the specialized
secretory lysosomes and IL-1β is exported by various mechanisms such as exocytosis of secretory lysosomes or exosomes, microvesicle shedding, or direct export by transporters (Dinarello, 2009). The post-translational processing and secretion of IL-1β has drawn much interest as it provides an important regulatory mechanism in inflammatory responses. Recently, activation of inflammasomes has been shown to be the main mechanism that activates caspase-1 and IL-1β secretion. IL-18 is closely related to IL-1β and it is also processed by the inflammasome. IL-18 is known to induce IFN-γ in activated T cells and NK cells and contributes in Th1 polarization. Whereas IL-18 does not have pyrogenic activity, it shares similar inflammatory functions with IL-1β, such as induction of other pro-inflammatory cytokines, chemokines and NO (Lamkanfi and Dixit, 2009).

Although expression of pro-inflammatory cytokines was considered as the hallmark of tissue inflammation, it is common that the levels of these cytokines do not correlate with the inflammatory phenotype (Dinarello, 2007a). Most of the pro-inflammatory cytokines are expressed at the early stage of infection but are down-regulated in the later stage of inflammation in order to avoid deleterious effects. Therefore, the levels of pro-inflammatory cytokines often may not always correlate with their physiological effects. Also, the cytokines may act on a particular tissue or micro-environment and their concentration might be much lower at the systemic level. Therefore, it is important to consider when and where the cytokines are measured in order to fully understand their role in inflammation.

1.1.2.4 NO and ROS

In addition to cytokines, NO and ROS are known to be important inflammatory mediators (Laskin et al., 2011). Although at low levels they are utilized as signaling molecules in maintaining tissue homeostasis, these reactive molecules are produced in large quantities by innate immune cells such as macrophages during inflammatory responses. Due to the unstable nature they can readily react with oxygen and water to produce other radicals and intermediates. The reactive secondary products provide microbicidal function against
pathogens, but excessive generation of these reactive species during inflammation also causes serious tissue injury to the host.

NO is produced in cells by three subtypes of nitric oxide synthase (NOS). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed in cells and become activated upon stimulating signals such as calcium and calmodulin to generate low levels of NO. As the name implies eNOS and nNOS regulate the function of blood vessels and neurons as their catalytic product, NO, plays a role as a regulatory molecule. On the other hand, iNOS is expressed in various cell types including epithelial cells and macrophages in response to inflammatory signals such as microbial products and pro-inflammatory cytokines (Aktan, 2004). NO has been shown to be involved in regulation of various signaling pathways during inflammation through transcription factor activation, gene expression, and post-translational modification of inflammatory mediators. It has been suggested that NO might regulate these functions by activating guanylyl cyclase, which catalyzes reactions to generate an important intracellular signaling molecule, cyclic GMP (Bove and van der Vliet, 2006). Also, NO can change protein conformation and affect protein functions by S-nitrosylation on cysteine residues. However, overproduction of NO in the presence of superoxide results in generation of peroxynitrite, which is a strong oxidizing and nitrosating agent. Therefore, generation of NO during inflammation leads to alteration of various proteins and disruption of normal cellular functions which exert cytotoxic or cytostatic effects against pathogens but also may cause deleterious effects on the host.

ROS is a collective term for the chemical species with strong oxidizing power generated by incomplete reduction of oxygen. Superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (HO-) are some of the ROS found in the biological system that can cause damage on proteins and DNA. Although the ROS generated in the cell has been regarded as accidentally-occurring harmful byproducts of aerobic metabolism, it has been shown that ROS plays an important role in host defense. In fact, defects in ROS generating mechanisms result in impaired responses against bacterial and fungal infection and can cause a severe immunodeficiency disorder (Guerra et al., 2007). In phagocytes, a multi-subunit complex containing a NADPH oxidase, NOX2, is mainly responsible for ROS
generation although ROS can be generated from other cellular sources, such as mitochondria or xanthine oxidase. Activation of the NOX2 complex requires two steps: priming by pro-inflammatory cytokines or microbial products and activation by additional signals, such as an increase in intracellular Ca\textsuperscript{2+} concentration, which lead to further phosphorylation and translocation of the subunits to the plasma membrane (Sareila et al., 2011). The activated NOX2 complex facilitates electron transfer from NADPH to oxygen to generate a large amount of superoxide in the phagolysosome, a process known as the respiratory burst. It is generally believed that superoxide and other ROS derived from superoxide then oxidize protein, DNA, lipid and carbohydrate to destroy engulfed pathogens, although activation of proteases by the polarized condition in the phagolysosome during the respiratory burst has also been suggested as a cytotoxic mechanism (Lambeth, 2004).

In addition to its cytotoxic effects, there is evidence that ROS may have other biological functions. In non-phagocytic cells, several NOX homologues were discovered and it has been shown that they can produce ROS at lower levels in various cells suggesting that ROS may act as an intracellular signaling molecule. Although the role of ROS as signaling molecules has been received with skepticism due to their strong reactivity, recent studies showed that ROS may have specific reactivity toward certain biological molecules. For example, it has been shown that at lower levels ROS oxidize protein tyrosine phosphatases and PTEN at their catalytic cysteine residues to inhibit their enzymatic function (Lambeth, 2004). As another example of ROS playing a role as a signaling molecule, hydrogen peroxide can activate anti-oxidant response pathways through NFE-related factor 2 to regulate its own intracellular concentration (D'Autreaux and Toledano, 2007). Although these studies suggest that ROS can function as an inflammatory mediator it is not clear how these mechanisms are implemented during inflammatory responses. Recently, studies have indicated that ROS may be a crucial component in activation of the NLRP3 inflammasome (Tschopp and Schroder, 2010).

1.1.2.5 Acute respiratory distress syndrome (ARDS)

ARDS is a serious condition of acute respiratory failure that presents with progressive arterial hypoxemia, dyspnea, and pulmonary dysfunction (Matthay and Zemans, 2010). It
is often associated with acute lung injury (ALI) caused by infectious and non-infectious clinical disorders of either pulmonary or non-pulmonary origin. For example, ARDS is most often caused by primary pneumonia from bacterial, viral and fungal infection but also by lung injuries due to severe sepsis, including non-pulmonary infection, such as peritonitis. In addition, ALI/ARDS can arise from non-pulmonary disorders such as hemorrhage and shock following major trauma, severe acute pancreatitis, transfusion-associated lung injury, and drug reactions. Conversely, many ARDS patients also develop complications in non-pulmonary organs leading to multiple organ failure (Matthay and Zemans, 2010). Therefore, ARDS can be viewed as a condition associated with overwhelming local or systemic inflammation that results in excessive and damaging immune responses.

The common pathological features of ARDS are diffuse alveolar damage (DAD), lung capillary endothelial injury and accumulation of protein-rich neutrophilic pulmonary edema (Ware and Matthay, 2000). Neutrophils appear to play a major role in lung injury and their infiltration and activation are dependent on chemokines and other pro-inflammatory stimuli. As activated neutrophils infiltrate into the alveoli, they release toxic mediators such as proteases, ROS, and pro-inflammatory cytokines, which cause damage to the epithelium and form edema in the alveoli. There is evidence that platelets may also contribute to lung injury by interacting with neutrophils to produce pro-inflammatory cytokines (Looney et al., 2009). If patients survive the acute phase some of the edema is reabsorbed and repair processes begin with infiltration of fibroblasts as well as proliferation of epithelial cells. The neutrophilic infiltrate is gradually resolved and replaced with more mononuclear cells and alveolar macrophages in the alveoli but often results in fibrosis.

Although the complicated mechanisms involved in ARDS are mostly unknown, studies on sepsis have provided valuable information in understanding the pathogenesis of the disease. Sepsis is defined as a systemic inflammatory response syndrome caused by bacterial infection or by administration of LPS in animal models which trigger hyperactivation of innate immune responses and excessive inflammation (Rittirsch et al., 2008). During sepsis, neutrophils and macrophages produce and respond to cytokines,
chemokines, complement-activation products and other mediators such as lipid factors and ROS which further amplify the inflammatory process. The clinical features include abnormal body temperature (hyperthermia or hyperthermia), tachycardia, tachypnea, and leukocytopenia (Cai et al., 2010). The uncontrolled inflammation accompanied by the massive cytokine response known as a “cytokine storm”, eventually leads to host damage in various organs including the lung while the severe form of sepsis results in septic shock and fatality. Although the primary cause of sepsis is the host response against bacterial PAMPs such as LPS, DAMPs released from the damaged tissue may also play a role in over-stimulation of immune cells. For example, a high level of high mobility group B protein-1 (HMGB1) is released in the lung after LPS instilled mice (Lin et al., 2005). HMGB1 is a transcription factor but it is known to be released from necrotic cells as a danger signal. It has been shown that extracellular HMGB1 can interact with PRRs to further promote inflammatory responses (Castellheim et al., 2009).

The emergence of new pandemic viruses such as severe acute respiratory syndrome coronavirus (SARS-CoV) and H5N1 influenza virus has become a global health issue. Some of the patients infected with these viruses have developed fatal pneumonia with ARDS. In severe cases, patients also show lymphopenia and multiple organ failure, but without much evidence of viral replication in non-respiratory organs, these pathological features may be consequences of dysregulated immune responses (La Gruta et al., 2007). There are many common features in the pathologies of SARS and H5N1 influenza infection but the most prominent is DAD in the lung that leads to pulmonary dysfunction and fatality. In addition, infections by these viruses induce significant pro-inflammatory cytokines and chemokines, although type I IFN or TNF-α were absent in patients with SARS (Chen and Subbarao, 2007). As in sepsis, inflammatory mediators are known to play a significant role as tissue damaging agents but the mechanism for virus induced ARDS has not been well studied. It is interesting that infection by the pandemic viruses showed varied clinical outcomes with only a fraction of patients developing ARDS. Although the clinical variations might be partly influenced by the individual variations of the host such as the age as well as the immunological and genetic background, the dynamics of the early events of infection including the dose and route of infection can be
an important factor producing varying degrees of immunopathological effects (Rouse and Sehrawat, 2010).

Unfortunately, virus induced ARDS has been often studied with limited clinical cases and the data obtained from these end point studies may not provide adequate information for investigating the early events of pathogenesis. In order to investigate how viral infection causes ARDS, animal models were developed. Although it has been proven difficult to replicate the same clinical features of ARDS in animals as they rarely progress to fatal outcome, studies with the animal models have provided valuable insights of the pathogenesis of ARDS. For example, intranasal infection with SARS-CoV in aged mice caused prolonged viral replication, and pneumonitis with DAD and edema while showing clear signs of illness including significant weight loss, dehydration, and ruffled fur (Roberts et al., 2008). By comparing different KO mouse strains, studies have demonstrated the critical role of the innate immune response against SARS-CoV and its over-activation has been suggested as the mechanism of the pathogenesis and tissue damage (Chen and Subbarao, 2007).

Similar to SARS-CoV, highly virulent influenza virus strains, such as H5N1, also caused ARDS like disease with substantial weight loss, lethargy, and respiratory dysfunction in animal models (Maines et al., 2008). Using KO mouse models, the postulation that the excessive induction of innate immune response during viral infection causes acute lung injury and high mortality has been tested. However, single gene knockout studies often do not show significant differences in the pathological phenotypes or mortality as it is believed that there are many overlapping mechanisms to compensate for the loss of a single protein in the innate immune system. In support of this notion, several KO mouse models deficient in key pro-inflammatory cytokines and their signaling pathways produced similar disease outcomes as the wild type mice, exhibiting only minor improvement including delay of morbidity (Belser et al., 2009). Interestingly, a study with IL-1R KO mice demonstrated that IL-1 plays an important role in induction of acute immunopathology during early influenza infection whereas its expression might be necessary for survival against the viral infection suggesting that the innate immune
responses against viral infection can be either friend or foe to the host (Schmitz et al., 2005).
1.2 Inflammasomes

2.1.1 Introduction to inflammasomes

Since its first cloning in 1984, many studies have demonstrated IL-1β as one of the most important cytokines regulating various inflammatory responses. However, it is only recently that the most unique feature about IL-1β has been revealed. Unlike most other cytokines, both IL-1α and IL-1β are produced in a precursor form and without the signal peptide required for secretion. While the proIL-1α is biologically as active as mIL-1α, proIL-1β needs to be further processed by enzymatic cleavage to the mature form in order to provide its biological activity. The mystery of how IL-1β is processed and secreted had been intensely studied and the discovery of the inflammasome not only provided a breakthrough in our understanding of post-translational modification of IL-1β but also opened a door to new insights into the molecular mechanisms of inflammatory responses.

The inflammasome was discovered by Tschopp and colleagues in 2002 (Martinon et al., 2002). The first inflammasome described was a large multiprotein complex consisted of caspase-1, caspase-5, ASC, and NLRP1. The term ‘inflammasome’ was coined in analogy to the apoptosome, where cytochrome c released from mitochondria forms a complex with Apaf-1, which then recruit and activate apoptotic caspases to induce apoptotic cell death. Caspases are cysteine proteases that are produced as zymogens and following proteolytic activation, they control cellular processes such as apoptosis, cell survival, proliferation, differentiation, and inflammation (Broz and Monack, 2011). Apoptotic caspases are classified as either initiator or effector caspases. Initiator caspases have homotypic protein–protein interaction motif that belongs to the death domain superfamily, namely CARD or DED (death effector domain) (Taylor et al., 2008). Upon stimulation, the initiator caspases are recruited through these domains to form multiprotein complexes called the apoptosome. The complex formation allows dimerization of the initiator caspases, which become activated by auto-proteolytic
processing. Once activated, the initiator caspases cleave downstream effector caspases to propagate and amplify the signal.

Although the inflammatory caspases have not been characterized as comprehensively as the apoptotic caspases, several caspases have been identified to be involved in inflammatory processes, including caspase-1, 4, and 5 in humans and caspase-1, 11, and 12 in mice (Martinon and Tschopp, 2004). Caspase-1, also known as IL-1β converting enzyme or ICE, is responsible for the processing of the 33 kDa pro-IL-1β to the 17 kDa mIL-1β. In addition to IL-1β, caspase-1 also processes IL-18 in the same manner while IL-1α is cleaved by a membrane protease, calpain. Caspase-5, along with caspase-1, was identified in the NALP1 inflammasome, suggesting that different inflammatory caspases may be involved in the formation of different inflammasomes. Sequence comparison analysis revealed that both human caspase-4 and caspase-5 might have evolved from a mouse caspase-11 ancestor gene (Martinon and Tschopp, 2004).

ASC, the adaptor molecule in the inflammasome complex, was first identified as a 22 kDa protein that formed a perinuclear speck in apoptotic cells from which its name originated. ASC is composed of a PYD and a CARD domain so that it can interact with the PYD domain of cytoplasmic PRRs and the CARD domain of caspase-1 (Schroder and Tschopp, 2010). Studies with ASC deficient mice showed that ASC is a crucial component in NLRP3 and AIM2 inflammasomes and it appears that NLRC4 and murine Nlrp1b, which may directly interact with caspase-1 through their CARD domain, also require ASC for inflammasome activity (Broz and Monack, 2011).

In addition to caspase-1 and ASC, the last critical component in inflammasome complexes is the cytoplasmic PRRs. In macrophages, ASC and caspase-1 are constitutively expressed in large amounts but inflammasome forming PRRs such as NLRP3 are highly inducible by inflammatory stimuli indicating that they play a key role in regulating inflammasome function (Gross et al., 2011). Currently, four PRRs are known to form inflammasomes: three members of the NLR family (NLRC4, NLRP1/Nlrp1b, and NLRP3) and a PYHIN protein (AIM2).
The ultimate function of inflammasomes appears to be to activate caspase-1, and thereby allow processing of its main substrates, pro-IL-1β and pro-IL-18. The process is thought to occur when inflammasome-forming PRRs bind to their respective PAMPs and become activated. The activated PRRs then recruit ASC via their PYD domain and the CARD domain of ASC, which in turn interacts with the CARD domain on pro-caspase-1. Once the inflammasome components form the complex, proximity-induced autoactivation cleaves the 45 kDa pro-caspase-1 into active fragments. The active caspase-1 is an oligomeric enzyme of two subunits, 10 kDa and 20 kDa which processes the 33 kDa inactive pro-IL-1β into the carboxyl-terminal active fragment by cleaving between amino acid residues Asp116 and Ala117.

1.2.2 Inflammasome complexes

1.2.2.1 NLRP1 inflammasome

The human NLRP1 was the first PRR identified to form an inflammasome complex containing ASC, caspase-1, and caspase-5. The molecular analysis of human NLRP1 showed that it has the N-terminal PYD, the central NACHT and LRR domains, and a unique C-terminal extension of the FIIND domain with unknown function in addition to the CARD domain. NLRP1 can directly interact with caspase-5 and induce caspase-5 processing, whereas the interaction with caspase-1 requires ASC (Martinon et al., 2002). In the mouse, three NLRP1 paralogs were found in the genome (Nlrp1a, Nlrpb, Nlrp1c). However, they lack the N-terminal PYD, raising speculation that murine NLRP1 could have a different role (Martinon et al., 2009).

Studies have identified that MDP, a bacterial cell wall component, and anthrax lethal factor (LF) of the B. anthracis lethal toxin (LeTx) can induce the NLRP1 inflammasome (Boyden and Dietrich, 2006; Faustin et al., 2007). The molecular mechanism of the NLRP1 inflammasome has been studied using cell-free reconstitution systems to show that caspase-1 and NLRP1 were sufficient for MDP-dependent NLRP1 activation.
whereas ASC was required for a robust activation (Faustin et al., 2007). The study also suggested a two-step mechanism of NLRP1 activation. First, binding of MDP to the LRR domain induce a conformational change, which allows subsequent binding of ATP and self oligomerization through the NACHT domain, forming a higher order structure similar to the APAF-1 apoptosome (Faustin et al., 2007). Despite the evidence from the cell-free experiments, however, direct binding and activation of NLRP1 by MDP is currently being questioned. In fact, a recent study suggested that NOD2 might be involved in the NLRP1 inflammasome in response to both MDP and anthrax lethal toxin \textit{in vivo} (Hsu et al., 2008). NOD2 is known as an intracellular sensor for MDP and their binding induces activation of NF-κB and MAPK kinase pathways via RIP2 (Girardin et al., 2003). Therefore, NOD2 may play a central role in innate immune response to MDP both in NF-κB-dependent induction of pro-IL-1β and in the processing of IL-1β by playing a role in activation of the NLRP1 inflammasome.

In addition to MDP, anthrax lethal factor (LF) from \textit{B. anthracis} has been shown to activate the NLRP1 inflammasome. When LF is delivered into the cytoplasm it activates MAPK kinase signaling pathways and induces rapid necrosis in macrophages, which is a major pathological feature of anthrax disease. It has been shown that the susceptibility for LT in mice is related to a NLRP1 paralog, Nlrp1b. Caspase-1 was shown to be required for LT induced macrophage death, suggesting that the NLRP1 inflammasome is responsible for the LF-mediated innate immune response (Lamkanfi et al., 2007b). However, NLRP1 may not directly bind to LF, as a catalytically inactive mutant form of LF that has an almost identical structure to the active LF failed to activate caspase-1, supporting a model that LF may indirectly affect NLRP1 activation by degrading its inhibitor or activating other factors involved in NLRP1 inflammasome activation (Fink et al., 2008).

NLRP1 has been associated with several autoimmune diseases, such as generalized vitiligo, vitiligo-associated type I diabetes, and Addison’s disease (Lamkanfi et al., 2011). Generalized vitiligo is a disease causing depigmentation of skin, hair, and mucous membranes due to the loss of melanocytes. Elevated levels of IL-1β were detected in patients with generalized vitiligo, indicating that the disease is related to IL-1β-
processing and the inflammasome pathway. In addition, studies have shown that single nucleotide polymorphisms (SNPs) in the coding region of NLRP1 are linked to vitiligo (Lamkanfi et al., 2011). More studies on the structural and functional analyses of these SNPs could provide clues to unravel the NLRP1 inflammasome activation.

1.2.1.2 NLRC4 inflammasome

NLRC4, also known as IPAF or Card12, contains an N-terminal CARD, a central NBD, and a C-terminal LRR domain. NLRC4 has been shown to activate the inflammasome in response to infection with a number of Gram-negative bacteria, including *Salmonella typhimurium*, *Legionella pneumophila*, *Shigella flexneri*, and *Pseudomonas aeruginosa*. The macrophages deficient in NLRC4 showed reduced caspase-1 activation and less IL-1β secretion in infections with these bacteria, and the mice deficient in NLRC4 or caspase-1 were more susceptible to the bacterial infection suggesting that it is an important player in inflammasome activation (Horvath et al., 2011).

Flagellin is the major structural component of flagella that allows bacterial motility. Studies have shown that bacterial strains like *S. typhimurium* deficient in flagellin are unable to activate caspase-1 in macrophages (Miao et al., 2006). In addition, delivery of flagellin into the cytosol resulted in caspase-1 activation in an NLRC4-dependent manner, indicating that flagellin might be responsible for NLRC4 inflammasome activation. However, specific virulence factor transport systems were also required for NLRC4 inflammasome activation, as flagellin produced within the intracellular compartment of the infected host cell had to be exported to the cytosol. For example, in *Salmonella*, type 3 secretion systems (T3SSs) are required for caspase-1 activation and IL-1β secretion, whereas other bacteria require type 4 secretion systems (T4SSs) (Miao et al., 2006). On the other hand, the flagellated Gram-positive bacterium such as *Listeria monocytogenes* that escapes the phagosome to replicate in the cytosol can also trigger NLRC4 activation, suggesting that the exposure of bacterial flagellin in the cytosol is a critical step in NLRC4 inflammasome activation (Warren et al., 2008). A recent report showed that NLRC4 can detect the rod component of the T3SS apparatus through the
sequence motif that is similar to the C-terminal part of flagellin in several Gram-negative bacteria (Miao and Rajan, 2011). These data demonstrate that NLRC4 can detect conserved structural features of flagellin and virulence factors in the cytosol.

Despite the numerous studies showing that bacterial flagellin triggers activation of the NLRC4 inflammasome, its detailed molecular mechanism is still unclear. Direct binding of flagellin to NLRC4 has not been demonstrated and the possibility of other NLRs involved in the inflammasome formation has also been suggested. In particular, \textit{L. pneumophila} requires NAIP5 as well as NLRC4 to activate caspase-1. NAIP5 is a NLR that shares a similar domain structure to NLRC4 but contains a BIR domain instead of a CARD domain. It has been shown that NAIP5 and NLRC4 can physically interact and that a C-terminal region of Legionella flagellin is responsible for inflammasome activation (Lamkanfi and Dixit, 2009). However, there are conflicting reports showing that NAIP5 is not involved in the activation of caspase-1 but may have other indirect functions in inflammasome activation such as promoting phagosome–lysosome fusion (Lamkanfi et al., 2007a).

In addition to the flagellin-NLRC4 interaction, the interaction between NLRC4 and caspase-1 has not been fully elucidated. Although it is possible for NLRC4 to directly bind to pro-caspase-1 through CARD–CARD interactions, some studies have shown that ASC is required in the NLRC4-mediated caspase-1 activation (Mariathasan et al., 2004). The possible role of ASC in NLRC4 inflammasome might be to facilitate the physical binding of NLRC4 and pro-caspase1 or to recruit another NLR protein with a PYD domain via PYD–PYD interaction, so that the NLR protein and NLRC4 can cooperate in caspase-1 activation. Further studies are required to understand the interaction between NLRC4 and caspase-1.

1.2.1.3 NLRP3 inflammasome

NLRP3, also known as NALP3, cryopyrin, or PYPAF1, is the most extensively studied NLR family member due to the diversity of the stimuli it can respond to. NLRP3 has
been shown to recognize various microbial PAMPs from virus and bacteria, but it can also sense various danger signals. The versatile function of NLRP3 has been related to different physiological and pathological conditions including sterile and infective inflammatory responses, adjuvanticity, and autoinflammatory diseases. NLRP3 contains an N-terminal PYD, a central NBD, and a C-terminal LRR domain. NLRP3 binds to the ligand via its LRR domain, which leads to ATP-dependent self-oligomerization (Duncan et al., 2007). Activated NLRP3 requires the adaptor molecule ASC via homotypic PYD–PYD interactions in order to interact with the effector molecule caspase-1.

The role of NLRP3 in inflammation was first recognized in studies linking mutations in NLRP3 to an autoinflammatory disease, Muckle–Wells syndrome (Leemans et al., 2011). Other autoinflammatory syndromes such as familial cold autoinflammatory syndrome and neonatal-onset multisystem inflammatory disease were also found to be related to mutations in NLRP3, and these autoinflammatory syndromes are now collectively known as cryopyrin-associated periodic syndrome (CAPS). Patients with these hereditary syndromes present with cold-induced fevers, urticaria-like skin rash, arthritis, aseptic meningitis, and other types of localized inflammation in varied severity (Lamkanfi et al., 2011). More than 70 NLRP3 mutations related to CAPS have been identified, with a large number of them localized within the NACHT domain. Peripheral blood mononuclear cells from the patients with NLRP3 mutations secrete higher levels of IL-1β in response to LPS compared with healthy controls, suggesting that these mutations generate constitutively active forms of NLRP3, resulting in an increased inflammasome activation and elevated IL-1β secretion (Lamkanfi et al., 2011). The significance of the NLRP3 inflammasome pathway in CAPS has been shown in therapies using recombinant human IL-1 receptor antagonist (IL-1Ra; anakinra) that successfully reduced disease severity (Dinarello, 2007b).

NLRP3 induces inflammasome activation against a plethora of stimuli of microbial origin (bacteria and viruses), exogenous nonmicrobial origin (crystalline particles), and endogenous origin (endogenous danger signals). The NLRP3 inflammasome is activated by a number of microbes, including viruses such as Sendai virus, influenza A virus, and adenoviruses, and bacteria like *Staphylococcus aureus, L. monocytogenes, E. coli,*
Mycobacterium marinum, S. flexneri, Neisseria gonorrhoe, and Candida albicans. In some cases, the NLRP3 inflammasome activation is associated with specific microbial products. A number of bacterial toxins known to form pores on the plasma membrane can activate the NLRP3 inflammasome. Also, an ion channel protein from influenza A virus was shown to cause NLRP3 inflammasome activation (Ichinohe et al., 2010). Bacterial RNA (Kanneganti et al., 2007) and hemozoin from malaria-causing parasites (Dostert et al., 2009) were also identified to trigger the NLRP3 inflammasome. However, for most of the microbes the specific PAMP molecule responsible for NLRP3 inflammasome activation has not been clearly defined. In fact, it is still unclear whether NLRP3 activation is caused by the PAMPs or by the damaging side effects of the infection or a combination of multiple factors.

Another class of stimuli that can activate NLRP3 is crystal-like particles. Monosodium urate (MSU) crystals that cause gout disease was the first of its kind to be linked to the NLRP3 inflammasome activation (Martinon et al., 2006). Patients with hyperuricemia can accumulate uric acid in joints to form MSU crystals. MSU has been known to induce IL-1β in monocytes and the discovery of MSU-induced inflammasome activation revealed the pathogenic mechanism of gout disease. Numerous subsequent studies have demonstrated that other crystals, particles, and protein aggregates can also induce NLRP3 activation and IL-1β release. For example, inorganic crystalline materials such as silica and asbestos, which are known to cause progressive pulmonary fibrotic disorders, were shown to activate the NLRP3 inflammasome (Hornung et al., 2008; Martinon et al., 2006). Furthermore, the NLRP3 inflammasome pathway is triggered by aluminum salt (alum), the most commonly used vaccine adjuvant (Eisenbarth et al., 2008; Hornung et al., 2008). Using NLRP3, ASC, and caspase-1 deficient in vitro and in vivo models, these studies demonstrated that the crystals imported into the phagocytes can cause activation of the NLRP3 inflammasome.

Another crystalline particle that can activate NLRP3 originates from malaria parasite. Hemozoin, a product of Plasmodium spp., forms insoluble heme crystals that can induce inflammation through NLRP3 activation (Dostert et al., 2009). The study suggests that exogenous inorganic materials as well as organic molecules that are capable of forming
crystal-like structure can be sensed by NLRP3. In line with this view, endogenous peptide aggregates were also shown to cause NLRP3 activation. In Alzheimer’s disease, β-amyloid plaque in the brain is a well-recognized pathologic feature. The fibrillar aggregates of β-amyloid can be sensed by microglia cells via NLRP3 which then leads to production of pro-inflammatory cytokines including elevated secretion of IL-1β (Halle et al., 2008). Although these studies indicate that various crystals and aggregates can act as stimuli for inflammasome activation, further studies are required to address whether they directly trigger activation of the NLRP3 inflammasome or indirectly create conditions to produce other agents that promote inflammasome activation.

Even before the discovery of the inflammasome pathway, ATP had been known to induce IL-1β secretion in macrophages and studies have revealed that a high concentration of ATP is a potent NLRP3 inflammasome activator (Mariathasan et al., 2006; Perregaux and Gabel, 1994). Extracellular ATP can bind to a group of cell surface receptors called P2 purinergic receptors (see section 1.3.2). One of the P2 purinergic receptors called P2X7 receptor (P2X7R) is the major ATP receptor on macrophages and other immune cells and it has been implicated in NLRP3 inflammasome activation. The affinity of P2X7R toward ATP is unusually low with the EC50 in millimolar range. Since such high concentrations of ATP can only be attained when cells release their cytosol content, it has been proposed that ATP might function as a danger signal during cell death (Di Virgilio, 2007). P2X7R is a cation channel and binding of ATP to P2X7R leads to ion flux mediated by pannexin-1 (Pelegrin and Surprenant, 2006). Extensive activation of P2X7R can result in pore formation and ultimately cell death (Di Virgilio et al., 2001). The observation that both ATP and the bacterial toxins can lead to pore formation and stimulate the NLRP3 inflammasome has led to the proposal that a sudden change in ion balance might activate NLRP3 (see section 1.2.3). In fact, a high extracellular potassium level to reduce the concentration gradient across the plasma membrane can prevent NLRP3 activation (Petrilli et al., 2007).
1.2.1.3 AIM2 inflammasome

The AIM2 inflammasome is the most recently identified inflammasome that has a number of distinctive properties compared to the other three inflammasomes. First of all, AIM2 is the only inflammasome-forming PRR that does not belong to the NLR family. AIM2 detects DNA in the cytoplasm and activates the inflammasome pathway (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). Since DNA does not fit the strict definition of a PAMP, the strategy for the AIM2 inflammasome is to detect misplaced DNA in the cytosol compartment. However, because AIM2 can respond to any DNA, including DNA from the host, there is speculation that AIM2 might be involved in pathogenesis of auto-inflammatory disease such as systemic lupus erythematosus (Choubey et al., 2010).

Currently, AIM2 is the only inflammasome-forming PRR that has been shown to directly bind to the relevant ligand. AIM2 binds dsDNA through its HIN200 domain while the PYD domain allows interaction with ASC to activate caspase-1 (Hornung and Latz, 2010). The AIM2 inflammasome activation has been reported in studies using DNA viruses such as vaccinia virus and mouse cytomegalovirus (CMV) as well as bacteria such as Francisella tularensis, L. monocytogenes. Also, in vivo studies using KO mouse models demonstrated that the AIM2 inflammasome plays an important role in the clearance of F. tularensis and murine CMV infections (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010). Intriguingly, AIM2 does not contain a NACHT domain that facilitates self-oligomerization required for inflammasome activation. Although the detailed mechanism of the AIM2 inflammasome activation is yet to be revealed, it is believed that multiple AIM2 proteins can bind to a dsDNA molecule that would allow ASC recruitment to form active inflammasomes.

1.2.2 Mechanism of the NLRP3 inflammasome activation

Although there has been a significant advance in our understanding of inflammatory responses mediated by inflammasomes over the last decade, most of the studies were focused on identifying the PRRs and the PAMPs that induce inflammasome activation.
Among the identified inflammasomes, the NLPR3 inflammasome has been the most intensely studied. However, the specific mechanisms of how the NLRP3 inflammasome is activated by various PAMPs of different structures remain largely unknown. Initially, the C-terminal LRR domain of NLRP3 was thought to be the ligand binding domain that directly interacted with its ligand. Although the LRR domain can be utilized by PRRs for binding microbial products, it is unlikely that such a broad range of molecules can all bind to NLRP3. Alternatively, it has been suggested that NLRP3 might be a signaling molecule downstream of an actual receptor or a co-receptor for other PRRs.

The detailed mechanism of the NLRP3 inflammasome activation is currently under investigation, and recent studies have provided some insights into how the NLRP3 inflammasome activation may be modulated through regulation of NLRP3 expression by the PAMPs (Bauernfeind et al., 2009; Franchi et al., 2009a). It is a common practice to prime macrophages to a semi-stimulated state before applying PAMPs for the actual inflammasome activation. LPS has been typically used for this priming purpose and it leads to enhanced transcription of NLRP3 via the transcription factor NF-κB. Since macrophages constitutively express high levels of ASC and caspase-1, the increased NLRP3 expression by the priming step is critical for NLRP3 inflammasome activation. Indeed, macrophages over-expressing NLRP3 did not require a priming step (Bauernfeind et al., 2009).

If the main function of priming is to increase NLRP3 expression through induction of the NF-κB pathway, various PAMPs that activate TLR pathways can serve as priming agents. In this view, some of the PAMPs that were known to induce the NLRP3 inflammasome such as microbial RNA, peptidoglycans, and CpG DNA (Kanneganti et al., 2007) might actually function as priming agents that induce TLR pathways rather than as activators. In fact, studies using macrophages deficient in TLRs, TLR adaptor proteins, and NLRs showed that inflammasome activation by these PAMPs is dependent on TLRs and TLR signaling (Bauernfeind et al., 2009). The two-step activation of the NLRP3 inflammasome is seen as a mechanism to safeguard against accidental activation of inflammatory responses that could have devastating consequences for the host. This two-step activation of the NLRP3 inflammasome agrees with the Danger Model (see
section 1.3.1) that a signal from sensing of microbial products and a signal from the environment are required to commit to immune responses. However, the earlier studies have mostly regarded the priming as a necessary step to induce pro-IL-1β production and overlooked its effect on the induction of NLRP3 expression. Thus, it is important to distinguish the factors affecting priming from the factors that directly affect assembly and catalytic activities of the NLRP3 inflammasome in order to unravel the mechanism of NLRP3 activation.

Once NLRP3 is induced, the actual NLRP3 inflammasome activation leading to caspase-1 cleavage is a rapid process independent of transcription. Currently, three possible mechanisms are proposed as the NLRP3 inflammasome activators: potassium ion (K+) efflux-inducing stimuli (pore-forming toxins, ion channels), stimuli inducing lysosomal disruption (crystals, protein aggregates, bacteria, viruses) or ROS-inducing stimuli. K+ efflux has been shown to be required for NLRP3 activation after various stimuli (Petrilli et al., 2007). The evidence for this mechanism was first demonstrated by applying a high concentration of extracellular K+ to inhibit its efflux from the cell, which reduced inflammasome activation. Recent studies have found that other inflammasomes were also inhibited by higher extracellular K+ concentrations at varying degrees (Arlehamn et al., 2010; Poeck et al., 2010). Although these observations highlight the importance of K+ efflux, the underlying mechanisms of how it affects the inflammasome activation remained unclear. Some of the suggested mechanisms are that the reduced intracellular K+ might directly activate NLRP3 or that it might provide a favorable environment to form the inflammasome complex.

The production of ROS is another proposed pathway that has been associated with the activation of the NLRP3 inflammasome. Macrophages are known to generate ROS after engulfing particles or when stimulated with ATP, and the production of ROS is likewise commonly observed during the NLRP3 inflammasome activation (Dostert et al., 2008). Studies have shown that inhibition of ROS by anti-oxidant such as N-acetyl-l-cysteine can reduce the amount of IL-1β secretion (Cruz et al., 2007; Dostert et al., 2008; Petrilli et al., 2007). Furthermore, inhibition of ROS production by treating with NOX inhibitors or NOX knockdown reduced IL-1β secretion. On the contrary, macrophages deficient in
NOX2 showed normal response to ATP or crystals (Hornung et al., 2008). In addition, macrophages deficient in the ROS detoxifying enzyme SOD1 showed decreased caspase-1 activity (Meissner et al., 2008). Therefore, the role of ROS in NLRP3 inflammasome activation is currently controversial and more studies are needed to clarify whether ROS directly affects NLRP3 activation.

The results from the studies examining inflammasome activation by crystals and particles suggest another possible mechanism for the NLRP3 activation. When macrophages engulf crystals into intracellular compartments by phagocytosis, the crystal-containing phagosome matures and converges to the lysosomal pathway. The crystal-containing cargo causes lysosome destabilization, which leads to lysosomal swelling and leakage of lysosomal contents into the cytosol. MSU, silica, and aluminum salt activate the NLRP3 inflammasome following phagocytosis and lysosomal rupture demonstrating the link between lysosomal damage and NLRP3 activation (Hornung et al., 2008). Inhibition of lysosomal pathways or acidification process significantly reduces inflammasome activation. Other treatments that cause lysosomal disruption can also induce NLRP3 activation, suggesting that lysosomal disruption, not the crystal itself, mediates the activation. The lysosome contains a plethora of proteolytic enzymes which are activated during acidification of the lysosomal pathway. Cathepsin B is one of the lysosomal enzymes that has been shown to be involved in NLRP3 activation as its inhibition or deficiency leads to greatly reduced caspase-1 activation in response to silica or MSU crystals (Halle et al., 2008; Hornung et al., 2008). However, the specific target molecules of cathepsin B that are related to the NLRP3 inflammasome activation have not been identified, nor has the possible involvement of other lysosomal proteases been investigated. Overall, our current knowledge of the NLRP3 activation is incomplete and more studies are needed to fully understand its mechanism.

Once activated the inflammasome components form oligomers between 500 and 700 kDa in size (Martinon et al., 2002). In addition, a much larger molecular assembly called “pyroptosome” or “ASC focus” has been identified in macrophages upon stimulation (Fernandes-Alnemri et al., 2007). Analogous to the apoptosome that functions as the multi-protein complex that activates apoptotic caspases, the pyroptosome is believed to
be the platform for caspase-1 activation and IL-1β processing. Supporting this idea, both pro-caspase-1 and catalytically active caspase-1 were found in the pyroptosomes, while the mutant caspase-1 accumulated in the pyroptosome (Broz et al., 2010). Furthermore, pro-IL-1β accumulated in the pyroptosome upon caspase-1 inhibition. These results suggest that pro-caspase-1 is recruited to the pyroptosome and cleaved to the active caspase-1, which then processes pro-IL-1β to its active form.
1.3 Danger signals in innate immune responses

1.3.1 The Danger Model

In the traditional view of immunity, T cell and B cell populations with unlimited number of antigen specificity have the ability to distinguish self from non-self so that they would induce pathogen specific adaptive immune responses. However, the role of antigen presenting cells in induction of adaptive immune responses through the expression of co-stimulatory molecules clearly shows that innate immune cells without antigen specificity are involved in regulating the process of generating immune responses. In order to explain how innate immunity contributes to distinguishing self and non-self, Dr. Janeway pioneered the concept of pathogen recognition by PRRs. Although the concept was sufficient to explain how foreign antigens or microbes activate the immune system through the induction of PRR-mediated signal transduction, questions were raised for other situations where immune responses were activated in the absence of exogenous pathogens. For example, transplants, some tumors, autoimmune diseases, and severe trauma and burn, in which the pathogens are not the major factor, also induce robust immune responses.

To address how immune responses are activated in the absence of exogenous stimuli, the Danger Model was introduced. Proposed by Dr. Polly Matzinger in 1994, the Danger Model suggests that the function of the immune system is to recognize and prevent harmfulness in the context of "danger signals" (Matzinger, 1994). According to the Danger Model, it is not the exogenous PAMPs but the danger signals released from dying or stressed cells which determine whether the immune system responds. Cell death inevitably occurs during infection or other pathogenic conditions and the immune system senses the abnormal cell death as a universal sign of danger. In this model, the mode of cell death can affect the immune response as apoptotic cell death is immunosuppressive and necrotic cell death is immune-stimulatory.
The Danger Model was proposed purely on theoretical grounds and supported by little evidence at the time of conception. Since then, the experimental evidence that dying cells generate immunostimulatory signals has been demonstrated in many studies. Also known as endogenous adjuvants, alarmins, or DAMPs, several molecules released by dying cells have been identified as danger signals that can promote immune responses. Inflammasome-activating molecules such as MSU, DNA/ RNA, and ATP are considered as endogenous danger signals and the mechanisms of their action are believed to be related to the inflammasome pathways. In addition, HMGB1, heat shock proteins (Hsps), S100 proteins, hyaluronan, galectins, biglycan, versican, and heparan sulphate have been suggested as danger signals often released during inflammatory conditions caused by infection or in sterile injury (Chen and Nunez, 2010).

HMGB1 is a non-histone nuclear protein that binds to DNA to stabilize nucleosome formation and facilitate transcription (Lotze et al., 2007). When cell membrane integrity is lost due to tissue injury or cell necrosis, HMGB1 is released into the extracellular space and functions as a danger signal to the surrounding cells. Extracellular HMGB1 has potent cytokine-like effects. It has been shown that HMGB1 can activate immune cells, induce pro-inflammatory mediators, and attract DCs (Kono and Rock, 2008). Furthermore, blocking HMGB1 using antibodies reduced inflammation and provided some protection against the damaging effects in various injury models providing a rationale that neutralizing HMGB1 could potentially be beneficial in preventing injury induced systemic inflammation (Tsung et al., 2005). Hsps are intracellular chaperone proteins that play important roles in protein folding and trafficking as well as maintenance of protein integrity under normal and stressed conditions (Asea, 2006). Stressful environment such as elevated temperatures triggers massive increase of Hsps to respond to the heat-denatured proteins and block consequent caspase-dependent apoptosis. In more extreme conditions, however, Hsps are released from damaged cells and affect the function of other cells in the environment or enter the bloodstream to exert systemic effects. Specifically, Hsp60, Hsp70, Hsp90, and gp96 were shown to be capable of acting as danger signals by promoting inflammatory responses (Kono and Rock, 2008).
Although the inflammatory effects of HMGB1 and Hsps have been reported, the pro-inflammatory mechanisms of these endogenous molecules are poorly defined. It has been shown that HMGB1 as well as S100 proteins binds to receptor for advanced glycation end products (RAGE) on the cell surface, which can induce various pro-inflammatory signaling pathways such as the NF-κB, JAK-STAT, and MAP kinase pathways (Chen and Nunez, 2010). Several purified danger signal molecules including HMGB1, Hsps, hyaluronan and soluble extracellular matrix proteins have been reported to activate TLR2 and TLR4 (Kono and Rock, 2008). The inflammatory effect of Hsp70 might be exerted by activation of the NF-κB pathway through interaction with TLR2 and TRL4, although it is not clear whether Hsp70 directly binds to these cell surface PRRs (Calderwood et al., 2007). However, mice lacking TLR2 and TLR4 showed only slight reduction in inflammatory response suggesting that the role of these PRRs in inducing inflammation might be relatively small. In general, our understanding of how danger signals induce inflammatory responses is incomplete and more studies are needed to elucidate their pro-inflammatory mechanisms.

1.3.2 ATP and P2X7R

The nucleotide ATP is the main intracellular energy currency and ubiquitously present in every living cell. In normal conditions, intracellular ATP concentration is maintained at a high level (3-10 mM) but ATP is mostly scarce in the extracellular space (Bours et al., 2006). The biological function of ATP other than that of the energy metabolite was first recognized in the 1970’s from a study on the stimulation of sensory nerve, which led to the proposal that ATP could be utilized as a neurotransmitter or a signaling molecule (Burnstock, 1972). Unfortunately, this unorthodox theory at the time was met with broad skepticism and ignored for more than 20 years until the cloning and characterization of purinergic receptors expressed on the cell surface. It is now known that ATP plays an important role as an intercellular signaling molecule in various biological systems including cardiac function, neurotransmission, muscle contraction, vasodilation, bone metabolism, and liver function (Surprenant and North, 2009). In addition, there have been
a number of significant advances that shed light on the critical role of ATP in inflammation.

Extracellular ATP and ADP as well as pyrimidines such as UTP and UDP are sensed by a family of cell surface receptors called P2 purinergic receptors. Adenosine nucleoside, on the other hand, binds to P1 receptors. The P2 receptor family consists of two subfamilies, namely P2X and P2Y receptors. P2X receptors are ligand-gated ion channels and P2Y receptors are G-protein coupled receptors. Currently, seven P2X receptors (P2X1-7) and eight P2Y receptors (P2Y1,2,4,6,11-14) were identified and they show different affinities toward ATP and other nucleotides (Bours et al., 2006). While there are the purinergic receptors that bind to nucleotides, several ecto-enzymes are also expressed on the cell surface that are capable of rapidly hydrolyzing extracellular ATP. Therefore, ATP released from cells has excellent properties to be an effective intercellular signaling molecule with cellular receptors that can transduce signals inside the cell and efficient mechanisms to control its concentration.

A wide variety of cell types co-express several P1 and P2 receptors. Immune cells including macrophages, monocytes, neutrophils, DCs and T cells express most of the P1 and P2 receptors, providing mechanisms to regulate cell functions through purinergic receptor-mediated signaling. Considering that adenosine, mostly through P1 receptors, provides immunosuppressive effects whereas ATP has pro-inflammatory effects via P2 receptors, the balance between the expression of P1 and P2 receptor as well as the ecto-enzymes can produce different cellular responses toward purinergic stimuli (Bours et al., 2006). Moreover, the expression of purinergic receptors is regulated at the transcriptional level according to the immunological stimuli in the microenvironment. For example, the expression of P2X7-R in monocytes and macrophages is reduced by anti-inflammatory cytokines such as IL-4 and IL-10, whereas pro-inflammatory cytokines and LPS up-regulate its expression (Di Virgilio et al., 2001). In addition to their expression levels, the sensitivity of each receptor toward the different purinergic ligands can also confer the complexity and fidelity to the purinergic receptor signaling mechanism.
Once activated by their natural ligand ATP, P2X receptors function as nonselective cation channels. On the other hand, P2Y receptors, which bind to ATP, ADP, UTP, UDP, and UDP-glucose at varying affinity, are G protein-coupled receptors that activate phospholipase C, leading to a rise in intracellular Ca\(^{2+}\) that mediates various cellular responses. Most P2 receptors are activated by micro-molar concentrations of ATP except for P2X\(_7\)R which requires milli-molar concentrations of ATP for its activation. For this unusual property, P2X\(_7\)R has been regarded as the receptor responsible for sudden increase in extracellular ATP that can occur during cell damage or cell death (Bours et al., 2006). Non-lytic mechanisms allowing the release of ATP into the extracellular space via various mechanisms such as vesicular release, ABC transporters, connexin or pannexin hemi-channel, maxi-ion channels and P2X\(_7\)R-mediated releases have been suggested (Burnstock, 2011). Although these non-lytic ATP releasing mechanisms are still under investigation, it is believed that the ATP release from cells is most often a consequence of cell damage or cell death (Di Virgilio et al., 2001).

The effect of ATP on immune cells was first recognized from an experiment showing that cell injury can promote IL-1\(\beta\) post-translational processing and secretion in LPS-treated murine peritoneal macrophages (Hogquist et al., 1991). The study found that post-translational processing of IL-1\(\beta\) required a high concentration of ATP (5 mM), indicating that P2X\(_7\)R is likely to be involved. The ability of ATP to promote IL-1\(\beta\) post-translational processing was also demonstrated in an in vivo model (Griffiths et al., 1995). Subsequently, it was confirmed that P2X\(_7\)R was responsible for this process by using a KO mouse model (Solle et al., 2001). The study compared IL-1\(\beta\) secretion from the peritoneal macrophages obtained from wild-type and P2X\(_7\)R deficient mice. Both macrophages were capable of generating similar amounts of proIL-1\(\beta\) at LPS challenge, but only the wild-type macrophages released large quantities of mature IL-1\(\beta\) when treated with 5 mM ATP. This suggested that P2X\(_7\)R activation by ATP can provide a signal that leads to the post-transcriptional processing and secretion of IL-1\(\beta\).

However, the intracellular mechanism of how P2X\(_7\)R activation leads to the post-translational processing and secretion of IL-1\(\beta\) is only partially understood. Ligation of ATP to the P2X\(_7\)R resulted in phosphorylation of a number of intracellular signaling
molecules such as MAP kinases that would lead to activation of various transcription factors (Lenertz et al., 2011). In addition, other significant cellular changes such as enhanced production of ROS, mitochondrial membrane depolarization, and activation of phospholipase D have also been associated with P2X<sub>7</sub>R activation (Chen and Brosnan, 2006). Although these observations show that ATP-P2X<sub>7</sub>R signaling induces significant cellular responses, further studies are needed to clarify how these changes are related to IL-1β post-translational processing.

The P2X<sub>7</sub>R deficient macrophages fail to response to ATP but treatment of LPS-stimulated macrophages with an ionophore nigericin resulted in release of mature IL-1β without the ATP-P2X<sub>7</sub>R stimulation (Solle et al., 2001). Moreover, maitotoxin and other membrane pore forming toxin produced a similar effect as ATP (Schilling et al., 1999). From these results, it was suggested that activation of ion channels or formation of pores that allows ion flux across the membrane might regulate IL-1β processing. As a ligand-gated cation channel, activation of P2X<sub>7</sub>R results in transmembrane ion flux, notably Ca<sup>2+</sup> and Na<sup>+</sup> influx and K<sup>+</sup> efflux. The intracellular rise of Ca<sup>2+</sup> concentration after ATP stimulation is mainly due to the ion influx but it is also augmented by Ca<sup>2+</sup> release from the intracellular storages. The increase of intracellular Ca<sup>2+</sup> is required in the secretion process of mIL-1β (Brough et al., 2003), but its role in the post-translational processing is not clear. On the other hand, K<sup>+</sup> efflux has been shown to play a key role in regulating P2X<sub>7</sub>R dependent IL-1β post-translational processing as a higher K<sup>+</sup> concentration in the medium inhibited ATP-mediated generation of IL-1β (Ferrari et al., 2006; Kahlenberg and Dubyak, 2004). The detail mechanism of how these changes in the ion homeostasis trigger IL-1β processing and the inflammasome activation is still under investigation.

A short exposure to ATP causes temporary and reversible opening of the P2X<sub>7</sub>-R channel that allows passage of cations. However, a prolonged ATP exposure leads to complete membrane depolarization which involves the formation of membrane pores large enough for molecules of significant molecular weights (≤ 900 kDa) such as ethidium bromide and YO-PRO-1 dye to become permeable, providing a simple assay method. Currently, the mechanism regulating this bi-phasic permeability is not clear but two possible mechanisms were proposed: progressive oligomerization of the P2X<sub>7</sub>-Rs leading to
dilation homomeric channel, and secondary activation of pannexin-1 hemi-channel by P2X7R (Coddou et al., 2011). Related to the latter hypothesis, it has been shown that activation of P2X7R leads to pannexin-1 recruitment and the formation of membrane pores, which is required for inflammasome activation and IL-1β secretion (Pelegrin and Surprenant, 2006). However, contradicting evidence was recently presented using pannexin-1 KO mice that the function of pannexin-1 might be limited to ATP release mechanism (Qu et al., 2011).

Macrophages that are exposed to ATP repeatedly or for an extensive period of time ultimately die. The cell death is likely to result from the loss of membrane potential and permeablisization of the cell membrane. The cell death caused by ATP exposure has been ascribed to either necrosis or apoptosis since both caspase-dependent and –independent processes were observed (Chen and Brosnan, 2006). The observed difference in the pattern of cell death may be attributed to disparate experimental conditions of the ATP exposure such as the incubation time, ATP concentration, and the cell type. To make matters more complex, another newly defined cell death pathway called pyroptosis, which is characterized by cell death associated with caspase-1 activation, might be prevalent in the ATP-mediated cell death. It should be noted that ATP-mediated P2X7R activation leads to death of the target cell, which would generate more extracellular ATP in the microenvironment to amplify the signal. This positive feedback of ATP-mediated cell death might be a possible mechanism that causes tissue damage and elicits acute inflammation.

ATP-mediated cell death is believed to be one of the mechanisms of mIL-1β release. Release of a cytoplasmic marker enzyme lactose dehydrogenase (LDH) was observed along with mIL-1β when LPS-activated macrophages and monocytes were exposed to milli-molar concentrations of ATP (Hogquist et al., 1991; Perregaux and Gabel, 1998). The results from these studies suggested that release of mIL-1β might be a passive process caused by permeablisization of the plasma membrane. However, there is evidence that release of mIL-1β is a facilitated process. Studies have demonstrated that ATP-activated macrophages form membrane blebs and shed microvesicles containing mIL-1β (MacKenzie et al., 2001), although how the contents of microvesicles are released...
remains a question. Others have suggested that mIL-1β is externalized through secretory lysosomes when stimulated by ATP and K⁺ depletion (Andrei et al., 2004). How these different mechanisms contribute to the release of IL-1β in vivo remains to be addressed.

Although there are many in vitro studies demonstrating that the ATP-stimulated IL-1β post-translational processing occurs via P2X7-R, evidence that P2X7-R functions in this manner in vivo is only beginning to emerge. Currently, two P2X7-R deficient mouse lines have been generated and used in in vivo experiments (Chessell et al., 2005; Solle et al., 2001). These mice have no phenotype under normal conditions but they display defective inflammatory responses when challenged with various immunologic insults. For example, the P2X7-R deficient mice produced less IL-1β compared to the wild-type controls after administration of Freund’s complete adjuvant into their paws (Chessell et al., 2005). In an anti-collagen antibody-induced arthritis model, the P2X7-R deficient mice showed reduced inflammation and less cartilage damage (Labasi et al., 2002). These studies demonstrate that P2X7-R plays an important role in induction of inflammation and provided a rationale that blocking its function may offer a novel therapeutic approach for the treatment of various inflammatory disorders.

Pursuing the rationale, several studies have recently demonstrated that ATP-mediated P2X7-R activation is involved in inflammatory responses in different immunopathological models. Idzko and colleagues have shown in an ovalbumin-sensitized asthmatic mouse model that extracellular ATP-mediated purinergic signaling plays a key role in allergic inflammation (Idzko et al., 2007). Inflammatory responses including inflammatory cell infiltration and cytokine production were inhibited when pre-treated with apyrase, an ATP hydrolyzing enzyme, or KN-62, a broad P2 receptor antagonist. Moreover, the study shows increased ATP levels in bronchoalveolar lavage fluid (BALF) from the ovalbumin-sensitized asthmatic mice as well as from asthmatic human subjects after an allergen challenge. The elevated ATP levels were correlated with increased airway inflammation. In their subsequent study, the authors have demonstrated significant reduction in inflammatory responses in P2X7-R deficient mice or in treatment with P2X7-R inhibitors, attesting to the importance of ATP-P2X7-R signaling in manifesting allergic airway inflammation (Muller et al., 2011).
Similar results have also been obtained in an allergic dermatitis model, where P2X$_7$R deficient mice did not develop hypersensitivity responses against skin allergen treatment (Weber et al., 2010). In the study, apyrase, KN-62, or IL-1 receptor antagonist treatment also prevented contact hypersensitivity, while IL-1$\beta$ injection restored allergic response in P2X$_7$R deficient mice. These experiments suggest that ATP-P2X$_7$R activation triggers IL-1$\beta$ release to promote allergic responses. Interestingly, P2X$_7$R deficient DCs, which failed to produce mIL-1$\beta$ upon allergen stimulation, were able to induce mIL-1$\beta$ and exhibited restored sensitization when pretreated with alum. Since alum is known to activate the NLRP3 inflammasome and may allow bypassing of the P2X$_7$R pathway, this result indirectly indicates that ATP-P2X$_7$R activation is an essential signal for the NLRP3 inflammasome activation and IL-1$\beta$ maturation during allergic responses.

There are a number of studies showing that ATP released from injured cells can contribute to inflammation. Using an immune complex-mediated renal injury model, Taylor et al. reported that deficiency of P2X$_7$R or treatment with the P2X$_7$R-specific inhibitor A-438079 significantly reduced development of inflammation and attenuated tissue damage after a nephrotoxic insult (Taylor et al., 2009). Riteau et al. have shown similar results in a bleomycin-induced mouse lung injury model (Riteau et al., 2010). The effects of lung inflammation and fibrosis caused by bleomycin were reduced when mice were treated with apyrase, and the signs of lung inflammation were diminished in P2X$_7$R deficient mice. The study also revealed that bleomycin treatment caused lung epithelial cells to release ATP in a pennexin-1 dependent manner without significant cell death, suggesting ATP released by epithelial cells can induce inflammatory responses. Since the extracellular ATP is controlled by ecto-ATPases, defects in the ATP-hydrolyzing mechanism can lead to more severe inflammation. In fact, studies have shown in the lung injury models that the mice deficient in ecto-ATPases (CD39 and CD73) exhibit enhanced inflammatory responses (Eckle et al., 2007; Reutershan et al., 2009), although the increased inflammation in these KO mice might be partially due to the inability to generate adenosine, which would promote an anti-inflammatory reaction.

ATP is also found to be elevated in lungs of animals exposed to cigarette smoke and of human smokers with chronic obstructive pulmonary disease (COPD) (Lommatzsch et al.,
2010; Mortaz et al., 2009). One study showed that exposure of neutrophils to cigarette smoke extract caused release of ATP, which induced production of IL-8 and elastase, two key mediators in the pathogenesis of lung emphysema and COPD (Mortaz et al., 2009). The study demonstrated that the inflammatory response mediated by smoke-exposed neutrophils was inhibited by apyrase and suramin, a broad P2 receptor antagonist. Cicko et al. also showed that apyrase treatment reduced smoke-induced lung inflammation in mice (Cicko et al., 2010). Furthermore, the role of P2X7R in smoke-induced lung inflammation was demonstrated in mice as both treatment with its inhibitor and its gene ablation reduced inflammation and prevented the development of emphysema (Lucattelli et al., 2011). These studies provide evidence that ATP plays an important role in the pathogenesis of cigarette smoke-induced COPD and that P2X7 mediates the inflammatory effects of ATP in this process.

It has been shown that ATP-P2X7R activation is also involved in the regulation of systemic inflammation during graft-versus host disease (Wilhelm et al., 2010). The study demonstrated that there is an increase in ATP after allogenic tissue graft as the result of tissue destruction mediated by immune rejection. The increase in ATP correlated with elevated pro-inflammatory events as well as the activation and expansion of T cells. More importantly, deficiency or inhibition of P2X7R or apyrase treatment reduced cytokine production and T cell expansion by DCs in vitro and increased mouse survival after allogenic cell transfer. The study provides evidence that ATP released from dying cells induces APC activation through P2X7-R, which in turn can provoke a cascade of events leading to systemic inflammation. Although the associated molecular mechanisms were not discussed in the study, the impaired secretion of IL-1β and IL-18 in the P2X7-R deficient APCs along with the clinical evidence that the levels of these cytokines correlate with the disease severity suggest that ATP-P2X7R-mediated inflammasome activation plays a key role in generating host-damaging systemic inflammation.

Another inflammation related function of ATP that has recently drawn interest is its chemotactic effect. During apoptosis, ATP as well as UTP are released by cells through membrane pores formed by pannexin-1, which has a cytoplasmic domain with a caspase-dependent cleavage site (Chekeni et al., 2010; Qu et al., 2011). The current model for
ATP-mediated chemotaxis suggests that apoptotic cells release ATP through pannexin-1 upon caspase activation in the early stage of cell death, which creates an ATP gradient. Macrophages and monocytes can respond to this ATP gradient mediated by P2Y2R. Since P2Y2R has significantly higher affinity toward ATP (EC50=1–30 µM) compared to P2X7R, whether the extracellular ATP exerts pro-inflammatory effects or the chemotactic function would be dictated by the local ATP concentration (Ravichandran, 2011).

Despite the large volume of studies showing the important functions of ATP in regulating inflammatory responses, it remains difficult to dissect the molecular mechanism of cellular responses induced by ATP. This is not only due to the multiple receptors with varying affinities but also due to the transient nature of ATP-mediated signaling. In this regard, the current method of studying ATP-mediated responses requires careful interpretation, as treating cells with a high concentration of extracellular ATP for an extended period of time in vitro may not represent the same stimuli that cells encounter in vivo and cause aberrant effects on the cells.

1.3.3 Cell death pathways and pyroptosis

Cell death represents the presence of potential threats that can affect other cells, local tissue, and the organism itself. Therefore, cell death is closely monitored by the immune system and the nature of cell death is recognized so that an appropriate response to the type of threat is elicited. An inappropriate response can lead to ineffective immune responses and undesired immunological consequences. Historically, cell death has been classified as apoptotic or necrotic depending on the distinct morphological characteristics as well as the immunological consequences. Also known as programmed cell death, apoptosis is distinguished by well-defined features such as cell shrinkage, membrane blebbing, and chromatin condensation, which lead to breakdown of the cell into apoptotic bodies to be engulfed by phagocytes. It is generally believed that apoptotic cell death is immunologically silent or anti-inflammatory.
‘Oncosis’ is a recently coined term that describes the process of necrotic cell death characterized by cell and organelle swelling followed by the loss of cell membrane integrity and release of the cellular contents (Fink and Cookson, 2005). Although necrosis has been considered as an accidental and uncontrolled cell death, there is accumulating evidence that it can be induced by activation of intracellular signaling pathways, where serine/threonine kinases RIP1 and RIP3 have been shown to play a key role (Lamkanfi and Dixit, 2010). This programmed necrosis called ‘necroptosis’ can be triggered by not only the activation of death receptors such as TNFR, Fas, and TRAIL-R but also by various PRRs. A number of cellular changes such as production of intracellular ROS, calpain activation, and lysosomal destabilization have been identified as possible mechanisms of necroptosis (Duprez et al., 2009). Since the cellular content released during necrosis contains various endogenous danger signal molecules, the process is generally considered pro-inflammatory and results in extensive tissue damage. Necrotic cell death has been observed in ischemia/reperfusion and other injury models as well as in infection by pathogens such as *Shigella*, HIV, West Nile virus and Coxsackievirus B (Vanlangenakker et al., 2008).

Another cell death pathway that has been investigated is autophagic cell death. Autophagy is a recently discovered intracellular catabolic pathway that allows cells to degrade and recycle proteins and organelles. A specialized vesicle called the autophagosome is assembled to sequester the target proteins or organelles, which is then transferred to lysosome for degradation. A large number of molecules cooperate to form autophagosome and the process is regulated by mTOR (Scarlatti et al., 2009). While its main function is to maintain intracellular homeostasis, autophagy is involved in diverse cellular processes, such as the cellular stress response, differentiation, development, and the immune response. Autophagic vacuoles often appear in dying cells prompting debates over whether excessive autophagy causes cell death or it is only one of the phenomena accompanying cellular demise (Duprez et al., 2009). Although infection by various bacterial pathogens is known to induce autophagy, it appears that autophagic cell death is contained within the cell and, therefore, non-inflammatory (Labbe and Saleh, 2008).
Pyroptosis is a newly identified cell death pathway induced by microbial infection and other non-infectious stimuli. Pyroptosis is morphologically and mechanistically distinct from other cell death pathways and is defined by activation of caspase-1, which is not involved in apoptosis. Apoptotic caspases including caspase 3, 6 and 8 are not involved in pyroptosis and the key features of apoptosis such as the loss of mitochondrial integrity and release of cytochrome c do not occur in pyroptosis (Bergsbaken et al., 2009). In addition to the post-translational processing of IL-1β and IL-18, activation of caspase-1 has been associated with rapid cell death process characterized by pore formation and eventual rupture of the plasma membrane. Cells dying by pyroptosis undergo swelling and lysis that result in the release of inflammatory intracellular contents. Consequently, as the term pyroptosis (in Greek 'pyro' means ‘fire’ or ‘fever’) implies, this cell death pathway is inherently inflammatory due to the secretion of pro-inflammatory cytokines and release of endogenous danger signals.

Pyroptosis has been mostly described in macrophages and DCs and it appears to be a cell death pathway restricted to myeloid cells. First demonstrated by Shigella flexneri, macrophages showed pyroptic cell death upon infection by a number of bacteria such as Salmonella typhimurium, Psuedomonas aeruginosa, Legionella pneumophila as they activated caspase-1 through the NLRC4 inflammasome that senses flagellin in the cytosol (Lamkanfi and Dixit, 2010). Francisella tularensis and Listeria monocytogenes induce AIM2 inflammasome-mediated pyroptosis. Shigella flexneri and Straphylococcus aureus infection also cause pyroptosis via a yet unknown inflammasome pathway (Lamkanfi and Dixit, 2010). In addition, treatment of macrophages and DCs with LT from Bacillus anthracis induces caspase-1 dependent pyroptosis (Fink et al., 2008). It has been noted that the infected macrophages form pores of 1-2 nm in the plasma membrane at the early time points of pyroptosis, which leads to cell swelling and eventually cell lysis. A cytoprotective agent such as glycine that blocks ion fluxes prevents the cell swelling and lysis, demonstrating that pyroptotic cell death is mediated by the disruption of osmotic polarity across the plasma membrane (Fink and Cookson, 2006). Since the activation of inflammasome pathways and membrane pore formation are common features for both pyroptosis and ATP-P2X7R activation, it is possible that they are related cellular phenomena during infection-induced inflammation.
Although it is now well accepted that apoptosis and pyroptosis are distinct cell death pathways, there are several overlapping morphological and biochemical features which have made it difficult to distinguish the two cell death pathways in the earlier investigations. First of all, cells dying by pyroptosis show DNA fragmentation and become positive in the TUNEL assay. However, the typical pattern of DNA laddering observed in apoptosis or the processes required for DNA fragmentation such as inactivation of poly-ADP ribose polymerase-1 and activation of inhibitor of caspase-activated DNase are absent in pyroptosis (Brennan and Cookson, 2000). Moreover, while there is chromatin condensation, the nucleus remains intact in pyroptosis whereas apoptosis causes the nucleus to partition into smaller membrane bound bodies. Secondly, cells in pyroptosis and apoptosis commonly show positive staining with annexin V. However, unlike apoptotic cells which become annexin V positive due to translocation of phosphotidylserine to the outer leaflet of the plasma membrane, annexin V staining is restricted to the inner leaflet of the membrane in pyroptic cells suggesting that annexin V permeates into the cell through membrane pores (Fink and Cookson, 2006). Thirdly, pyroptosis generates membrane blebs as in apoptosis, but they are produced as a result of increasing osmotic pressure that often leads to bleb rupture and lysis, a process similar to necrotic cell death (Miao et al., 2011).

In addition to activation of caspase-1 and secretion of pro-inflammatory cytokines, these morphological and biochemical characteristics clearly show that pyroptosis is a distinct cell death pathway. However, the mechanism of how pyroptosis occurs in a caspase-1 dependent manner is largely unknown. Caspase-1 has been the main focus in unraveling the mechanism of pyroptosis. Activation of caspase-1 also regulates post-translational processing and secretion of IL-1β and IL-18 but the inflammatory effect of these cytokines is not required for the execution of pyroptosis (Sarkar et al., 2006). A recent study has indicated that the caspase-1 involved in pyroptosis might function in a distinct mechanism to that of the inflammasome. The study has examined the activation of the NLRC4 inflammasome where NLRC4 can associate with caspase-1 with or without ASC. The study show that caspase-1 activated by NLRC4 in an ASC-independent interaction had catalytic activity without proteolytic processing, possibly by conformational change, and caspase-1 was diffusely located in the cytoplasm, which demonstrated that this
alternatively activated caspase-1 might be responsible for driving pyroptosis during inflammation (Broz et al., 2010).

Despite the evidence presented in the study, it still remains to be determined whether other factors besides caspase-1 are also involved in the induction of pyroptosis. In fact, activation of NLRP3 and AIM2 inflammasomes, where NLRP3 and AIM2 cannot directly associate caspase-1, also have shown pyroptic cell death (Broz and Monack, 2011). Therefore, it is possible that other events upstream of caspase-1 activation, such as activation of the ATP-P2X7R pathway, might be critical in inducing pyroptosis. Furthermore, studies have shown that there is a striking similarity between the morphological changes during pyroptosis and ATP-mediated cell death in macrophages (Brough and Rothwell, 2007). Considering that ATP is one of the danger signals, which can activate caspase-1 through the inflammasome pathway, the significance of the P2X7R-ATP pathway in pyroptosis requires further scrutiny.
1.4 Innate immune response against adenoviral vectors

1.4.1 Adenovirus

Adenovirus is a non-enveloped DNA virus with a genome of 30-40 kb linear dsDNA. The family of adenoviridae includes over 50 different human serotypes and numerous non-human serotypes from various species. The human adenoviruses have a relatively large size of ~150 MDa with a diameter of ~90 nm. The viral capsid has an icosahedral structure comprised of 240 trimeric hexon capsomeres (Smith et al., 2010). In addition to hexon, other structural proteins such as the penton base, shaft and fiber knob proteins are among the 40 viral gene products that form the fiber structure which protrudes out from the viral particle and plays an important role in binding to the host cell during the infection process.

Although adenovirus can infect various organs in humans it most often infects the respiratory tract, more specifically, the airway epithelia and alveoli. Adenoviral infection can cause a wide spectrum of symptoms from mild pharyngitis to fatal pneumonia, but in most cases the infection is self-limiting in immunocompetent adults. Human adenoviruses are classified into six groups (group A-F) and the majority of studies have been performed with two of the most common serotypes from group C, serotype 2 and 5. Also, the cellular interaction with adenovirus has been mainly studied in its primary target cells, the epithelial cells. Adenovirus interacts with epithelial cells via multiple receptors that promote cell surface binding, internalization, and endocytosis. Coxsackievirus B and Adenovirus Receptor (CAR) has been identified as the high-affinity cell surface receptor for serotype 2 and 5 adenovirus. The c-terminal domain of fiber knob protein has been shown to mediate the association with CAR on the cell surface (Campos and Barry, 2007). The initial binding of CAR-fiber knob facilitates a lower affinity interaction between the cellular $\alpha_v$ integrins and the arginine-glycine-aspartic acid (RDG) motif on the penton base which is followed by receptor-mediated
endocytosis through clathrin-coated vesicles. After internalization, the adenovirus escapes from the endosome during the endosomal acidification and within 30-40 min of infection the partially uncoated virus reaches the nucleus where its genome replicates (Liu and Muruve, 2003).

In addition to the well-characterized CAR-mediated adenovirus cell entry, other mechanisms may also contribute to the infection process. For example, heparan sulfate proteoglycans are known to promote virus attachment and adenovirus can trigger micropinocytosis to gain entry to the cell (Meier and Greber, 2004). Although the adenovirus infection is well understood in vitro, much less is known about the mechanism of adenovirus infection in vivo. Recent studies have identified several blood factors which can promote adenoviral infection. Factor VII, IX, X and complement C4-binding protein, for example, can bind to the fiber knob and facilitate alternative virus uptake through cellular receptors other than CAR (Campos and Barry, 2007). Adenovirus in the blood stream binds to these blood factors, which redirect the virus primarily to the liver (Shayakhmetov et al., 2005a).

1.4.2 Adenoviral vectors

Adenovirus has been genetically engineered as gene delivery vectors. The recombinant adenoviral vector (Ad) is one of the most widely used gene therapy vectors and it offers many advantages, including high transduction efficiency, broad cell tropism, ability to transduce non-dividing as well as dividing cells, and high levels of transgene expression. Furthermore, Ads are relatively easy to produce in high titers and accommodate a large size transgene. On the other hand, adenoviral vectors do not provide long term gene expression as they do not integrate into the host genome and they may elicit immune responses. Because of their ability to efficiently infect airway epithelial cells, Ads have been utilized in lung gene therapy targeting lung diseases such as cystic fibrosis.

First generation adenovirus vectors (FG-Ad) were generated by deleting the early gene regions, namely the E1 region or both the E1 and E3 regions from the viral genome.
These non-structural elements are the first to be actively transcribed after entry into the nucleus and they encode gene products that are essential for activation of other viral transcription units. Deletion of the E1 region in FG-Ad abolishes subsequent viral gene expression as well as replication. Moreover, the deleted region can be replaced with a therapeutic gene of interest. FG-Ads are produced in human cell lines, such as 293S, that express the E1 protein \textit{in trans}. Second generation adenovirus vectors were later developed to further prevent viral gene expression by deleting the E2 or E4 region. The helper-dependent adenovirus vector (HDAd) is the latest generation of adenoviral vectors that are devoid of all viral genes. The absence of viral genes improves the safety of the vector by further eliminating accidental viral gene expression and provided capacity to accommodate foreign genes and transcription regulatory elements of sizes up to 36 kb. To maintain the size of the genome for viral packaging, the rest of the construct contains stuffer DNA consisting of non-coding DNA segments from the human genome.

Earlier \textit{in vivo} studies with adenoviral vectors using various animal models and in human clinical trials have shown that transgene expression does not persist for more than a few weeks. Ad administration at high doses induced inflammation followed by activation of potent CD8+ and CD4+ T cell responses against viral antigens as well as the transgene. In particular, a strong cytotoxic T lymphocyte (CTL) response was induced after systemic delivery of FG-Ads, suggesting that leaky viral gene expression in the transduced cells might be responsible for the loss of gene expression (Campos and Barry, 2007). Re-administration of the same vector was mostly ineffective due to the generation of neutralizing antibodies against viral capsids. Although the second generation adenoviral vectors showed longer duration of transgene expression and reduced cellular immune responses, they did not completely resolve the problems of immune response (Driskell and Engelhardt, 2003). HDAds, which do not contain any viral genes, have further reduced adaptive immune responses and provided prolonged transgene expression. However, it has been recently shown that these vectors also induce DC maturation and T cell response in mice including CD8+ T cell responses, presumably through cross-presentation of viral proteins (Kushwah et al., 2008).
Ad administration in mice, nonhuman primates, and human causes secretion of pro-inflammatory cytokines and chemokines as well as type I IFNs within a few hours, even before any viral or transgene transcription can occur, suggesting that Ad infection itself induced innate immune responses (Muruve, 2004). The innate immune response against Ads not only reduces the efficacy of gene therapy but also has become a cause of concern for acute toxicity in Ad gene therapy. In fact, the cause of the unfortunate human death during gene therapy clinical trial was attributed to acute systemic inflammation. The 18-year-old patient, who intravenously received a high dosage FG-Ad immediately showed signs of systemic inflammatory response syndrome culminating in multiple organ failure that resembled pathological features of ARDS (Raper et al., 2003). The incident and other animal studies showing acute systemic inflammation have raised the safety concerns for the viral vectors in gene therapy. Prompted by these studies, efforts were made to understand the molecular mechanisms behind Ad-induced innate immune responses for the last decade.

1.4.3 Innate immune response against Ads

Based on the theory that the innate immune response is mediated by pathogen recognition through PAMPs, numerous studies were performed to identify PAMPs associated with Ad and their cellular receptors. Since adenovirus is a non-enveloped dsDNA virus, the capsid proteins and the viral DNA were considered as potential PAMPs that could be sensed by innate mechanisms. Several studies have suggested that Ad binding to the cell may be responsible for inducing innate immune responses. TLR2 has been shown to mediate innate immune responses against various dsDNA viruses such as HSV and mouse and human CMV. One study has indicated that TLR2 is involved in the innate immune response against Ad in vivo through MAPK activation that regulates cytokine and chemokine expression, although it is not clear whether TLR2 directly interacts with Ad (Appledorn et al., 2008). Recently, Di Paolo et al. demonstrated that Ad interaction with β3 integrins triggers innate immune responses in vivo (Di Paolo et al., 2009). Interestingly, this pathway induced IL-1α instead of IL-1β and was independent of nucleic acid recognition. However, the study was restricted to the response in the spleen.
and more studies are required to fully understand how Ad interaction with β3 integrins mediates the induction of IL-1α.

It is widely accepted that TLR9 is the primary sensing mechanism in the endosomal compartment for various DNA viruses and several studies have shown that Ad recognition is partly dependent on TLR9. As Ad enters the cell through endocytosis, the viral particle is partially disassembled during endosomal maturation process to expose the viral DNA. It is believed that TLR9 senses the presence of the exposed viral DNA in the endosome. Recognition of Ad DNA by TLR9 leads to MyD88-mediated NF-κB activation and the induction of pro-inflammatory cytokines and type I IFNs (Rathinam and Fitzgerald, 2011). Type I IFNs play an important role in inducing pro-inflammatory cytokines and eliciting adaptive responses against Ad by promoting DC maturation through upregulation of costimulatory molecules (Zhu et al., 2007). However, the TLR9-dependent response appears to be cell type-specific, as type I IFN induction was reduced in pDCs derived from TLR9 or MyD88 deficient mice but the response from the cDCs were independent of this pathway. Another study demonstrated that induction of IL-6 and IL-12 in DCs was TLR9-dependent whereas the IL-6 production from the peritoneal macrophages was not affected by deficiencies in TLR9 or MyD88, despite the similar expression levels of TLR9 and MyD88 in DCs and macrophages (Yamaguchi et al., 2007). On the other hand, Cerullo et al. showed that TLR9 deficiency attenuated IL-6 production in macrophage and reduced the overall in vivo responses against Ad administration (Cerullo et al., 2007). These studies show that TLR9 plays a role in triggering the innate immune response against Ad infection, but they also recognized that additional mechanisms are likely involved.

Recently, several studies have identified novel DNA-sensing PRRs in the cytosol that may provide important innate immune mechanisms against DNA viruses. Accumulation of viral genome after wild type virus infection should be recognized as a clear sign of imminent danger to the cell. For replication deficient viral vectors, the vector DNA can be detected by cytosolic PRRs during the endosomal escape. Studies have shown that DAI, DHX36, DHX9, RIG-1, RNA polymerase III, and MDA5 play a role in induction of inflammatory responses against DNA viruses such as HSV-1, as their knockdown or
deficiency reduce the production of type I IFNs (Rathinam and Fitzgerald, 2011). Activation of these PRRs results in the production of type I IFNs via activation of IRF3 and IRF7. Particularly for Ads, it has been shown that RNA polymerase III mediates inflammatory responses, as its inhibition resulted in significantly reduced IFN-β production (Chiu et al., 2009). The study demonstrates that viral DNA exposed in the cytosol during Ad infection can be sensed and transcribed by RNA polymerase III to generate RNAs which activate RNA sensors such as RIG-1. However, due to the multiple mechanisms that can respond to cytosolic DNA, deficiency of a single pathway often does not affect the host response against infection by DNA viruses (Ishii et al., 2008).

Another major innate immune response induced by viral DNA in the cytosol is activation of the inflammasome which results in release of IL-1 family cytokines. NLRP3 and AIM2 are known to be responsible for inflammasome activation against cytosolic DNA. While it is unlikely that NLRP3 directly interacts with DNA, Muruve et al. have shown that NLRP3 and ASC are essential for IL-1β processing during Ad infection (Muruve et al., 2008). The study demonstrated that replication deficient adenoviral vectors as well as wild type adenovirus and herpes virus can activate inflammasomes in phorbal-12-myristate-13-acetate (PMA) stimulated, LPS primed human macrophages. Interestingly, transfection of DNA from microbes and human genome also resulted in caspase-1 activation and IL-1β secretion but NLRP3 was not required for this process, suggesting that other cytoplasmic DNA sensors are involved. The study also found that inhibition of K+ efflux reduced inflammasome activation, which indicates that additional signals mediated by changes in ionic balance might be necessary for the NLRP3 inflammasome activation. In vivo, mice deficient in ASC or NLRP3 produce reduced amounts of pro-inflammatory cytokines including IL-1β after intra-peritoneal injection of Ad but the partial reduction suggests redundant innate immune mechanisms in response to DNA viruses.

Although the study demonstrated that the NLRP3 inflammasome is involved in the innate immune response against Ad infection, understanding the underlying mechanisms was confounded by the use of LPS-primed macrophages in their in vitro experiments. A recent study indicates that ROS is involved in activation of the NLRP3 inflammasome
during Ad infection (Barlan et al., 2011). In their subsequent study, the authors suggested that ROS generation is related to mitochondrial membrane disruption which might be caused by lysosomal cathepsin B released during the Ad-mediated endosomal membrane rupture (McGuire et al., 2011). Although these studies reveal that ROS plays a role during Ad induced inflammation in vitro, no in vivo evidence has been presented. Furthermore, the role of ROS in activation of the NLRP3 inflammasome still remains unclear and controversial (Martinon, 2010).

Unlike NLRP3, AIM2 has been shown to directly bind to DNA in the cytosol to activate the inflammasome. Mice deficient in AIM2 were recently developed and used to study the role of AIM2 in innate immune responses. Rathinam et al. showed that the macrophages and DCs obtained from the AIM2-KO mice did not respond to DNA transfection and they were unable to activate the inflammasome and process IL-1β (Rathinam et al., 2010). Furthermore, the pyroptic cell death caused by DNA transfection was significantly reduced in the AIM2 deficient macrophages. Although the study did not investigate Ad infection, it demonstrated that DNA viruses, including mouse CMV and vaccinia virus, can induce the AIM2 inflammasome. On the other hand, they found that IL-1β secretion was independent of AIM2 in HSV-1 infection. In vivo infection with mouse CMV also resulted in significantly reduced IL-18 induction and higher viral titers in the AIM2 deficient mice. Unfortunately, the effect on IL-1β induction was not reported. While these results indicate that AIM2 plays an important role in innate immune responses against DNA viruses, the study also utilized LPS-primed macrophages which can alter the inflammatory responses. In fact, the study found that infection with F. tularensis does not require LPS priming for inflammasome activation, suggesting that artificial LPS treatment of macrophages may affect the inflammatory response.

Despite these recent findings, more studies are needed to fully understand the mechanisms involved in the Ad-induced innate immune responses. Notably, it remains unclear how Ad infection causes inflammatory responses in vivo. Over the years, numerous studies were conducted to test the safety of Ad vectors in gene therapy and
they show that tissue resident macrophages play a critical role in induction of inflammatory responses during Ad infection (Zhang et al., 2001). The tissue resident macrophages, such as Kupffer cells in the liver and AMs in the lung are the first to recognize invading microbes. Even though macrophages do not express CAR, it has been shown that the majority of Ad is rapidly eliminated by the tissue resident macrophages; for example, 90% of the Ad was eliminated by Kupffer cells after intravenous injection and 70% by AMs after intratracheal administration (Worgall et al., 1997a; Worgall et al., 1997b). Consequently, depletion of macrophages prior to Ad administration significantly increased the efficacy and safety of Ad administration in vivo (Kuzmin et al., 1997).

Similarly, Ad internalization in AMs after intratracheal administration was followed by pro-inflammatory cytokine production within minutes, indicating that macrophages play a key role in the early inflammatory responses (Zsengeller et al., 2000). Taken together, it is believed that tissue resident macrophages uptake viral particles during Ad infection and initiate inflammatory responses by generating pro-inflammatory cytokines and chemokines that are essential in promoting infiltration of other immune cells and modulating their inflammatory functions.

Despite the fact that the tissue resident macrophages are one of the major players in the innate immune response against Ad infection in vivo, there are indications that macrophages by themselves may not generate significant responses. For example, Ad infection of human alveolar macrophages in vitro did not produce IL-8, IL-6, or IL-1β (Noah et al., 1996). Moreover, it is often found that resident macrophages such as AMs do not easily become activated by mere presence of pathogens in vitro, suggesting that regulation of macrophage activation is a complex process. On the other hand, it has been shown that epithelial cells and endothelial cells are capable of inducing inflammatory responses by producing various inflammatory mediators upon stimulation. Therefore, it has been suggested that tissue resident macrophages might interact with other cell types in the tissue during innate immune responses against pathogens (Hippenstiel et al., 2006).

In particular, the airway epithelial cells and AMs in the alveoli have physical interaction and the crosstalk between these cells might play an important role in the airway inflammation during infection. A study has demonstrated that direct cell-cell interactions
between these two cell types through cell surface molecules can regulate innate immune responses against pathogens (Takabayshi et al., 2006). Studies on air pollution particles and ischemic injury have also suggested that the interactions between airway epithelial cells and AMs are essential in inducing inflammation (Fujii et al., 2002; Ishii et al., 2005; Sharma et al., 2007; Tao and Kobzik, 2002). Therefore, more studies are needed to decipher the interaction between macrophages and epithelial cells in order to fully understand the mechanisms of innate immune responses during Ad infection.
Hypotheses and Objectives

Introduction

This thesis describes the mechanism of innate immune responses during adenoviral infection. The research is focused on understanding the interaction between macrophages and epithelial cells that plays an important role in induction of inflammatory responses against the virus. The research was conducted with the following two goals: 1. To examine the inflammatory responses generated by the macrophage-epithelial cell interaction. 2. To identify the mechanisms and the mediators involved in regulating the inflammatory responses elicited by interaction between macrophages and epithelial cells during an Ad infection.

Hypotheses

1. The interaction between epithelial cells and macrophages plays an important role during Ad infection.

2. ATP secreted during Ad infection mediates activation of the inflammasome pathway to regulate inflammatory responses.

Objectives

1. To establish an *in vitro* model system to study the interaction between macrophages and epithelial cells during an Ad infection.

2. To analyze the inflammatory responses mediated by the interaction between macrophages and epithelial cells during an Ad infection.
3. To determine whether ATP secreted by Ad-infected cells is involved in the induction of inflammatory responses during an Ad infection.

4. To analyze the role of P2X7R activation by ATP in Ad-induced inflammatory responses including inflammasome activation.

5. To assess the effect of ATP-P2X7R activation during an acute Ad infection in vivo.
Chapter 2

The synergistic inflammatory responses mediated by the interaction between macrophages and epithelial cells during an adenoviral vector infection

The contents of this chapter have been published in Immunology Letters:

2.1 Rationale and summary

Although Ads have been widely utilized for gene delivery, their clinical application has been hampered by host immune responses. It has been shown that macrophages can induce inflammatory response against Ads \textit{in vivo}, but they are not easily activated by Ads \textit{in vitro}, suggesting their activation requires interaction with other cells.

**Objective of Study:** In this study, we investigated the interaction between macrophages and epithelial cells during Ad infection.

**Summary of Results:** Ad infection of the macrophage-epithelial cell co-culture resulted in rapid and drastic changes in the cell culture such as decrease in pH within 24 h, indicating macrophage activation. Ad-infected co-culture showed several characteristics of inflammation including cytotoxicity, induction of pro-inflammatory cytokines, and generation of nitric oxide and reactive oxygen species. These signs of macrophage activation and inflammation were observed exclusively in the co-culture and were absent or significantly weaker in the macrophage mono-culture suggesting that there was a synergistic response by the interaction between macrophages and epithelial cells. We found that inhibition of NF-κB activation significantly reduced the inflammatory responses in the co-culture. Furthermore, we show that only the macrophages adjacent to epithelial cells were activated during Ad infection demonstrating that the interaction between macrophages and epithelial cells are crucial for Ad-induced inflammatory response.

**Conclusions:** The results indicate that Ad infection of the macrophage-epithelial cell co-culture induced synergistic inflammatory responses, which requires interaction between the macrophages and epithelial cells in the proximity.
2.2 Study background

Adenovirus vectors (Ads) have been widely used in various gene therapy applications and have been utilized in clinical trials more than any other vectors. They are considered one of the most efficient vehicles for lung gene delivery because of the natural infectivity toward the airway cells (Flotte et al., 2007). Despite many experimental and preclinical successes, there are concerns about the safety of the Ad vectors due to host immune responses (Raper et al., 2003). Development of helper-dependent adenovirus vectors (HDAd), in which all viral genes have been eliminated from its genome, has significantly reduced vector-induced adaptive immune responses, allowing prolonged gene expression in animal studies (Brunetti-Pierri and Ng, 2008; Toietta et al., 2005). However, HDAd retains the same viral capsid as other Ad vectors and contains DNA which can be detected by pattern recognition receptors (PRRs) to elicit innate immune responses (Brunetti-Pierri and Ng, 2008).

It has been shown that Ad infection of epithelial cells, the main target cell type in gene therapy as well as for the wild type virus, can cause various cellular changes including activation of inflammatory signaling pathways, expression of cell adhesion molecules, and induction of cytokines and chemokines both \textit{in vivo} and \textit{in vitro} (Liu and Muruve, 2003). These changes occur very early during the infection and are partly associated with binding of Ad to its receptor (CAR), suggesting that the viral interaction during the cell entry can initiate inflammatory responses by epithelial cells (Tibbles et al., 2002). In addition, several studies have investigated global gene expression profiles to show that Ad infection of non-immune cells can induce significant changes in gene expression (Hartman et al., 2008). Cumulatively, these studies illustrate that Ad infection of even non-immune cells such as epithelial cells may contribute to innate immune responses.

Typical immune cells such as dendritic cells, macrophages and peripheral blood mononuclear cells produce inflammatory cytokines upon Ad infection and the internalization of Ad has been correlated with inflammatory responses in these cells (Higginbotham et al., 2002; Zsengeller et al., 2000). The importance of the innate
immune cells in Ad-induced inflammation has been highlighted by the experiments showing that macrophage depletion during Ad gene delivery can significantly reduce inflammatory response and enhance gene delivery (Kuzmin et al., 1997; Zhang et al., 2001). These studies provide evidence that Ad induced inflammatory responses are mediated by innate immune cells in vivo. In contrast to in vivo studies, however, infection of the innate immune cells by Ad in vitro often did not induce significant cytokine expression (Korst et al., 2002; Noah et al., 1996). The lack of response by the innate immune cells to Ad infection in vitro implies that innate immune cells may require additional stimuli from other cells in the tissue to initiate inflammatory responses.

To gain mechanistic insights on macrophage activation in the context of airway inflammation during Ad administration, we examined how macrophages and epithelial cells interact to induce inflammatory responses. These two cell types are important in airway inflammation since they are the first to engage pathogens on the lung epithelial surface. We utilized a co-culture system where mouse lung epithelial cells and macrophages were cultured in contact with each other to examine Ad-induced inflammation. We found that Ad infection of the co-culture synergistically augmented its inflammatory responses compared to the epithelial cells or macrophages alone. The results from our study indicate that the interactions between these two cell types are critical for Ad-induced innate immune response. In addition, we found that inhibition of NF-κB can reduce Ad-induced inflammation in the co-culture indicating that it may be possible to alleviate inflammation during vector administration in gene therapy using NF-κB inhibitors.
2.3 Materials and methods

Reagents and antibodies
Caffeic acid phenethyl ester (CAPE) and genistein were obtained from Sigma (St. Louise, MO, USA). The monoclonal antibodies against mouse IRF3, phospho-IRF3, and phospho-κB-α were from Cell Signaling Technology (Danvers, MA, USA) and FITC conjugated Annexin V was from Biotium (Hayward, CA, USA). The ELISA detection kits for mouse IL-6 and IL-1α were purchased from R&D Systems (Minneapolis, MN, USA).

Helper-dependent adenoviral vectors
The HDAd vector used in this study, HDAd-CMV-LacZ and C4HSU, were prepared as described previously (Palmer and Ng, 2003). HDAd-CMV-LacZ contains bacterial lacZ under control of the CMV immediate-early promoter whereas C4HSU contains no transcribable gene. The final concentration of the virus stock was 1×10^{13} viral particles/ml.

Cell culture and Ad infection
MLE-15 (MLE) cell line was a gift from Dr. Marin Post from the Hospital for Sick Children (Toronto, ON, Canada). Raw 264.7 (Raw) cell line was obtained from American Type Culture Collection (Manassas, VA, USA). We also utilized EGFP- or mCherry-fused actin labeled Raw cell line to distinguish macrophages from epithelial cells (Scott et al., 2005). The cell lines were maintained in DMEM (Invitrogen, Burlington, ON, Canada) supplemented with 10% heat-inactivated FBS (Invitrogen, Burlington, ON, Canada) and cultured at 37 °C in 5% CO₂. The MLE and Raw co-culture was prepared on 6 well plates in most of the experiments according to the following procedure. First, MLE
cells were seeded at 25% confluency and cultured over night. Next day, Raw cells were seeded at 25% confluency on the same plate. On the third day the co-culture was about 80~90% confluent with MLE and Raw in 50:50 ratio. The mono-cultures of MLE and Raw were also grown to 80~90 % confluency before Ad infection. The cell culture was replaced with 1 ml of fresh medium and Ad was directly inoculated to the medium at approximately 20 multiplicity of infection (MOI). For Transwell experiments, MLE was seeded in the insert and Raw cells in the bottom well and each compartment was infected at 20 MOI.

Measurement of cytotoxicity by LDH assay

The cytotoxicity in the culture was assessed by LDH assay using a commercially available assay kit (Promega, Madison, WI, USA). The assay procedure was modified from the manufacturer’s instructions. Briefly, at the designated time after Ad infection, the medium was collected and cells and debris were removed by centrifugation. Fifty µl of medium was added to 50 µl of the substrate mix and incubated at room temperature for 30 min while protected from light. The reaction was terminated by adding 50 µl of the stop solution and OD was measured at 490 nm. The cytotoxicity in the culture was expressed by the percentage of OD compared to the sample treated with the lysis buffer (9% Triton X-100).

Analysis for cytotoxicity

In order to evaluate the cytotoxicity induced by Ad infection in the co-culture, cells were harvested and stained with 1 µg/ml propidium iodide (PI) and analyzed by flow cytometry. PI positive cells in the samples were analyzed on FACScan (BD Immunocytometry Systems, Lincoln Park, NJ, USA) using FlowJo flow cytometry analysis software (Treestar, OR, USA). Also, the co-culture with MLE and EGFP-labeled
Detection of cytokine expression

Total RNA was extracted from cells using Trizol (Invitrogen, Burlington, ON, Canada). The concentration was measured and 1 µg of RNA was used for reverse transcription with the random primer using Superscript II (Invitrogen, Burlington, ON, Canada). The cDNA products were then PCR-amplified for 30 cycles with the gene specific primers and analyzed on agarose gels. The primer sequences are listed in Table 1. The quantitative analyses were conducted for IL-6 and IL-1α using real-time PCR as described previously (Kushwah et al., 2010). In addition, IL-6 and IL-1α were detected from cell culture supernatant obtained 24 h after infection according to the manufacturer’s instruction.

Nitric oxide (NO) measurement

The amount of nitric oxide produced in the culture was indirectly measured by assaying for the nitrite concentration in the medium using Griess Reagent System (Promega, Madison, WI, USA) according to the manufacturer’s instruction.

Detection of ROS producing cells

The ROS positive cells in the MLE-Raw co-culture were analyzed 24 h after Ad infection. The cells were harvested in FACS buffer (5 mM EDTA, 2% FBS in PBS) and stained with 2 µM 3 -(p-aminophenyl) fluorescein (APF; Invitrogen, Burlington, ON, Canada) for 20 min at room temperature (Setsukinai et al., 2003). Flow cytometry was performed on the stained cells on FACScan and ROS positive cells in the samples were analyzed using FlowJo flow cytometry analysis software. In order to visualize ROS
Table 2-1. The sequence of primers used in the RT-PCR experiment.

<table>
<thead>
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</tr>
<tr>
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</tr>
<tr>
<td>iNOS</td>
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<tr>
<td>GAPDH</td>
<td>5′-GTTGGCAAGTTGCCAGATGTTGCC</td>
<td>5′-GATGATGACCCGTGGCTCC</td>
</tr>
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</table>
positive cells in the culture, cells were loaded with 10 μM 2’,7’-dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen, Burlington, ON, Canada) for 20 min at 37 °C. The excess dye was washed and the cells were incubated at 37 °C for another 20 min and then observed under a fluorescence microscope.

**Western blot analysis**

Cells were harvested and cell extracts were prepared in the lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing phosphatase inhibitors as well as protease inhibitors (50 mM NaF, 10mM sodium pyrophosphate, 50 μM sodiumorthovanadate). The protein concentration was measured and 25 μg of cell extracts were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with the indicated antibody and visualized with an enhanced chemiluminescence detection system (Perkin Elmer, Waltham, MA, USA).

**Statistical Analysis**

Student’s t-test was used to assess statistical significance between means. Significance was set at p<0.05. All the data are presented as mean ± standard deviation.
2.4 Results

Ad infection of macrophage-epithelial cell co-culture resulted in macrophage activation

In order to study the interaction between macrophages and epithelial cells during the Ad-induced innate immune response, a co-culture system was established using two mouse cell lines. Raw is a widely used mouse macrophage cell line isolated from Abelson mouse leukemia virus induced tumors (Raschke et al., 1978). MLE is a mouse cell line obtained from lung tumors showing many characteristics of alveolar type II cells (Wikenheiser et al., 1993). In the co-culture environment, these two cells grew normally as they would in their mono-cultures. When the co-culture was 80-90% confluent, an Ad vector (HDAd-CMV-LacZ) was added to the medium at approximately 20 MOI. At this Ad dose over 90% of MLE and less than 5% of Raw cell transduction was achieved in their mono-cultures (Figure 2-1).

During the first 12 h there were no major changes in the Ad-infected co-culture compared to the non-infected co-culture. However, approximately 12 to 24 h post-infection we noticed substantial changes in the Ad-infected co-culture, among which the most evident was the dramatic change in pH in the cell culture, easily noticeable by the change of the medium color to yellow in phenol-red containing medium (Figure 2-2A). Therefore, we monitored the pH change every 6 h after Ad infection and found that the co-culture infected with Ad began to show a lower pH by 18 h of Ad infection and turned acidic between 24 to 30 h after infection (Figure 2-2B). Although there were minor fluctuations in the Ad-infected mono-cultures we did not see the drastic drop in pH as observed in the co-culture even 30 h after infection. Also, the non-infected co-cultures did not show any changes in pH during 30 h (Figure 2-2B), indicating that the decrease in pH was due to Ad infection. These changes in the macrophage and epithelial cell co-culture suggest that Ad infection induces important cellular changes resulting in macrophage activation. More importantly, the absence of similar changes in Ad-infected Raw mono-culture indicates
Figure 2-1. Ad transduction efficiency in MLE and Raw cells. MLE (A) and Raw (B) cells were cultured to 90% confluency and transduced with HDAd-CMV-LacZ at 20 MOI. After 24 h, β-galactosidase assay was performed to identify the cells expressing the transgene (stained blue). The images were taken at a low magnification (100X) and representative of 2 independent experiments.
Figure 2-2. Ad infection of MLE-Raw co-culture causes a decrease in medium pH. (A) MLE, Raw mono-culture and MLE-Raw co-culture were infected with HDAd-CMV-LacZ at 20 MOI. The decrease in pH was apparent in co-culture 30 h after Ad infection as indicated by the change of medium color. (B) The medium from each culture was obtained every 6 h up to 30 h after Ad infection and the pH was measured. In addition, the corresponding control cultures without Ad infection were maintained for 30 h and analyzed for medium pH changes (far right column). The results represent the mean ± standard deviation of 3 independent experiments.
that the interaction between macrophages and epithelial cells might be essential for the innate immune response against Ad.

**Ad infection of co-culture resulted in cytotoxicity**

Along with the lower pH, the Raw cells in the co-culture showed changes in cellular behavior as early as 12 h after Ad infection, which were absent in the non-infected controls (Figure 2-3). Raw cells were easily distinguishable from MLE cells under the microscope as they are round, relatively similar in size, and appear brighter (Figure 2-3B). Even in the co-culture condition, they maintain their normal shape and grow on the culture dish surface next to MLE cell colonies (Figure 2-3C). However, we observed changes in Raw cells in the co-culture as they became irregular in shape and size as early as 12 h and by 24 h we found that only a fraction of cells could be identified as Raw cell (Figure 2-3F). In addition, Raw cells in the co-culture started to form clumps 12 h after Ad infection. These cells increasingly became less adherent and detached from the surface. By 30 h of Ad infection the majority of the Raw cells were detached from the culture dish and most of the cells remaining were MLE cells. In contrast, there were no notable differences in Ad-infected MLE and Raw mono-cultures compared to non-infected controls (Figure 2-3D and E, respectively). We have used HDAd-CMV-LacZ vector in all experiments in this study, which contains only one transcribed gene, the bacterial β-galactosidase gene. When we infected the co-culture with HDAd devoid of any transgene we also observed the same changes in the cell culture described above (data not shown), indicating that the response is an innate immune response against Ad and independent of transgene expression.

Ad infection of the co-culture resulted in loss of adherence and increased detachment of Raw cells, indicative of cell death and cytotoxicity. The signs of cell death were more apparent at higher Ad doses. To quantify Ad-induced cell death in the co-culture and the mono-cultures, LDH assays were performed. As shown in Figure 2-4A, the amount of LDH released in the medium of the Ad-infected co-culture at 18 h was almost three times higher than that of the non-infected co-culture. On the other hand, there was no increase
Figure 2-3. Microscopic observation of Ad infected MLE-Raw co-culture. In general, MLE epithelial cells (A) grow in colonies and appear darker under the microscope while Raw cells (B) are round, smaller in size, and appear brighter. MLE cells (arrows) and Raw (arrowhead) maintained their own morphology in the co-culture during 30 h culture period (C). The MLE, Raw, and MLE-Raw co-culture were infected with HDAd-CMV-LacZ at 20 MOI and the cells were observed 30 h later under the microscope. Compared to the non-infected MLE and Raw culture (A and B, respectively) there were no major morphological changes 30 h after Ad infection in their corresponding cultures (E and F, respectively). Ad infection changed the cell morphology and cellular behavior in the co-culture (F). The Raw cells (arrowhead) showed irregular shapes, formed clumps, became less adherent. Some of the Raw cells started detaching from the culture dish after 12 h, resulting in reduced number of cells on the culture surface. The images were taken at 100× magnification and are representative of 5 independent experiments.
Figure 2-4. Ad infection of MLE-Raw co-culture induced cytotoxicity. (A) MLE, Raw mono-culture and MLE-Raw co-culture were infected with HDAδ-CMV-LacZ at 20 MOI and the media was collected 18 h after infection. The amount of LDH in the medium was measured and the fold increase of LDH release was determined by comparing to the corresponding non-infected culture. The results represent the mean ± standard deviation of 3 independent experiments. *, p < 0.05. (B) The cell death in the MLE-Raw co-culture 18 h after Ad infection (right) or without Ad infection (left) was analyzed by PI staining followed by flow cytometry analysis. The proportions of PI positive are indicated in percentages. The plot is representative of 5 independent experiments.
in LDH release in Ad-infected MLE and Raw mono-cultures, indicating the cytotoxicity was induced by the interaction between macrophages and epithelial cells.

We confirmed the LDH assay results by staining the cell cultures with PI. As expected, Ad infection did not increase the number of PI positive cells in the MLE and Raw mono-cultures. However, Ad infection of the co-culture increased PI positive cells as early as 12 h post-infection and a large number of cells were PI positive by 18 h showing a considerable difference to the non-infected co-culture. In order to quantify the increase in cell death we harvested the co-culture 18 h after Ad infection and counted PI positive cells by flow cytometry. There were almost 4 times more PI positive cells in the Ad-infected co-culture than in the non-infected culture further confirming Ad induced cytotoxicity in the co-culture (Figure 2-4B). We found that most of the cells remained on the culture surface were MLE cells when maintained longer than 24 h after infection. In order to verify that most of the dying cells were macrophages we repeated the co-culture experiment with EGFP-labeled Raw and performed staining with PI 24 h post-infection. As shown in Figure 2-5, most of the PI positive cells were Raw macrophages. In addition, we found that the dying Raw cells do not go through the apoptotic pathway since very few cells stained positively for phosphatidylserine (PS) and most of them were epithelial cells (Figure 2-6). However, the small proportion of apoptotic epithelial cells cannot account for all the dead cells we observed in Ad-infected co-culture.

From these results we concluded that Ad infection causes cytotoxicity in the macrophage and epithelial cell co-culture. The absence of the cytotoxicity in the Ad-infected mono-cultures highlights the importance of the interaction between macrophages and epithelial cells in the Ad-induced innate immune response. Although it is possible that the cytotoxicity might be partially due to the acidic environment in the later stage of the cell culture, the increase in PI positive cells was observed as early as 12 h after Ad infection, even before the pH in the medium turns acidic enough to affect cell viability. Therefore, the acidification of the Ad-infected co-culture is likely to be a consequence of macrophage activation and cytotoxicity.
Figure 2-5. Majority of dying cells in the Ad infected co-culture are Raw macrophages. MLE-EGFP-labelled Raw co-culture was stained with PI 24 h after infection and pictures were taken at 400× magnification at the same viewing area without (A) or with rhodamine and FITC filter and the fluorescent images were superimposed (B). The images are representative of 3 independent experiments.
Figure 2-6. Ad infected co-culture did not induce Raw cell apoptosis. (A) The co-culture of MLE and mCherry-labelled Raw cells (red) were infected with 20 MOI of HdAd-CMV-LacZ. (B) After 24 h the cells were stained with FITC-Annexin V to detect phosphotidylserine (PS) positive cells (green). Most of the PS positive cells were identified as epithelial cells (not red). The proportion of PS positive cells cannot account for the large dead cell population (PI positive) in the Ad infected co-culture observed in Fig. 2. (A) and (B) are from the same viewing area and the images were taken at a low magnification to capture infrequent PS positive cells (100X).
Ad infection of co-culture induced pro-inflammatory cytokines and chemokines

Changes in the cell culture and induction of cytotoxicity in the Ad-infected co-culture suggest macrophage activation and inflammatory responses. In order to study whether Ad infection of the co-culture induced inflammatory response we analyzed induction of several cytokines and chemokines known to be important in Ad-induced inflammation by semi-quantitative RT-PCR. The time points at the very early (2 h), early (6 h), and late (24 h) phases were chosen to study the inflammatory responses. As shown in Figure 2-7, there was no significant cytokine induction at 2 h post-infection in the co-culture or the mono-cultures. However, after 6 h of Ad infection IFN-β, MIP-2, IL-6, and KC were highly induced in the Ad-infected co-culture. These cytokines and chemokines are known to be important mediators in Ad-induced inflammation (Liu and Muruve, 2003). Although some of these cytokines were also induced in the Raw mono-culture, their levels of induction were even higher in the co-culture, indicating that there is a synergistic mechanism that requires both epithelial cells and macrophages to induce pro-inflammatory cytokines and chemokines. Especially, the expression of IL-6 and KC was highly induced in the co-culture suggesting their critical role in Ad-induced inflammatory responses. Similarly, IL-1α and IL-1β were also highly induced in the co-culture 24 h after Ad infection. IL-6 and IL-1α are commonly induced during early inflammatory response and their induction in the co-culture was further confirmed by real-time RT-PCR (Figure 2-8A, and B) and ELISA assay (Figure 2-8C and D). These results demonstrate that Ad infection induced macrophage activation and inflammatory responses in the macrophage-epithelial cell co-culture.

Ad infection of co-culture generated NO and ROS

In addition to the cytokines and chemokines, NO is considered as an important mediator of inflammation. Therefore, we examined the expression of inducible nitric oxide synthase (iNOS) after Ad infection using semi-quantitative RT-PCR. As shown in Figure 2-7, iNOS was induced in the co-culture at 6 h after Ad infection and the level of induction was higher in the co-culture compared to the Raw mono-culture. To confirm
<table>
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**Figure 2-7. Synergistic induction of pro-inflammatory cytokines, chemokines and iNOS in Ad infected co-culture.** (A) MLE and Raw mono-cultures as well as MLE-Raw co-culture were harvested 2, 6, and 24 h with or without Ad infection. The expression of various cytokines, chemokines as well as iNOS was compared by semi-quantitative RT-PCR using the total RNA isolated from the cells. The data are representative of 2 independent experiments.
**Figure 2-8. Synergistic induction of pro-inflammatory cytokines in Ad infected co-culture.** MLE and Raw mono-cultures as well as MLE-Raw co-culture were cultured for 24 h with or without Ad infection. (A) and (B), Induction of IL-6 and IL-1α were quantitatively analyzed by real-time RT-PCR. (C) and (D), IL-6 and IL-1α were measured from supernatant of cell culture media 24 h after Ad infection and compared to non-infected culture. The results represent mean ± standard deviation of 3 independent experiments. *, p < 0.05.
the RT-PCR result, we measured the concentration of nitrite in the culture medium as an indirect measurement of gaseous NO using Griess reagent. Although induction of iNOS was evident as early as 6 h, nitrite accumulation in the media was detected only after 12 h. Ad-infected co-culture generated as much as 15 µM of nitrite 24 h post-infection (Figure 2-9A). In contrast, Ad infection did not induce NO in the MLE mono-culture and considerably less in the Raw mono-culture compared to the co-culture. These data show that there is a synergistic induction of iNOS and subsequent generation of NO in the Ad-infected co-culture.

NO generated by activated macrophages can affect two important processes in inflammation; regulation of inflammatory response and cytotoxic effector function (Bogdan et al., 2000). NO can be converted to peroxynitrite, a potent cytotoxic radical, in the presence of ROS, which is also known to be produced during inflammation. Like NO, ROS has also been implicated in innate immune response as a microbicidal mechanism as well as a signaling molecule (Lambeth, 2004). In order to examine whether Ad infection of co-culture produced ROS generating cells in addition to NO, we stained the cells with APF and analyzed by flow cytometry. As shown in Figure 2-9B, we found approximately 15% of the cells were ROS positive 24 h after infection, whereas very few cells were ROS positive in the non-infected co-culture. We found similar results when we directly stained the cells in the culture using DCFDA and most of the ROS positive cells were identified as macrophages using mCherry-labeled Raw cells (Figure 2-10). On the other hand, there was no increase in the ROS positive population in the Ad-infected mono-cultures compared to their corresponding non-infected culture. These results further confirm that NO and ROS are generated during Ad-induced inflammation and the macrophage and epithelial cell interaction can synergistically enhance production of these inflammatory mediators.

**Inflammatory response in Ad-infected co-culture is regulated by NF-κB pathway and IRF3**
Figure 2-9. Ad infection of the co-culture resulted in induction of NO and ROS.
(A) In order to evaluate NO generation in the MLE, Raw, and MLE-Raw co-culture, the media was collected 24 h after Ad infection and subjected to Griess assay. The results represent mean ± standard deviation of 3 independent experiments. *, p < 0.05. (B) The ROS positive cells in the co-culture 24 h after Ad infection were analyzed by flow cytometry. The cells were harvested and loaded with 2 μM APF and incubated for 20 min at room temperature. The fluorescence of 50,000 cells was monitored and presented as APF fluorescence intensity versus the frequencies. The flow cytometry results are shown in histogram and the ROS positive population is indicated in percentages. The plot is a representative of 5 independent experiments.
Figure 2-10. Ad infection induced ROS in macrophages. (A) The co-culture of MLE and mCherry-labelled Raw cells (red) were infected with 20 MOI of HdAd-CMV-LacZ. (B) After 24 h the cells were stained for ROS generating cells using DCFDA. All of the ROS positive cells (green) were identified Raw macrophages (red). (A) and (B) are from the same viewing area and the images were taken at 400× magnification.
The induction of the pro-inflammatory cytokines and chemokines indicates activation of inflammatory signaling pathways. Two of the key signaling pathways that lead to expression of cytokines and chemokines in the early innate immune response are mediated by NF-κB and IRFs (Kawai and Akira, 2006). In particular, NF-κB and IRF-3 are known to be activated in early stages of Ad infection (Nociari et al., 2009; Nociari et al., 2007). Therefore, we investigated whether these inflammatory transcription factors are activated by Ad infection in the co-culture and compared their activation to those of the mono-cultures. As shown in Figure 2-11, by Western blot analysis, we detected activation of NF-κB, indirectly measured by phospho-IκB-α, as early as 4 h after Ad infection. The level of activation was higher in the co-culture compared to the Raw cell mono-culture even at 8 h after Ad infection. Similarly, IRF-3 was also activated in the co-culture by 4 h post-infection and the level of activation was higher in the co-culture compared to the Raw mono-culture.

The activation of NF-κB and IRF-3 pathways would lead to the induction of pro-inflammatory cytokines and chemokines in the Ad-infected co-culture shown in Figure 2-7 and 8. In order to study the importance of these inflammatory signaling pathways in macrophage activation we used an NF-κB specific inhibitor, CAPE and a tyrosine kinase inhibitor, genistein (Wu et al., 2008). Pre-treatment of the co-culture with these inhibitors prior to Ad infection not only inhibited IL-6 cytokine induction, (Figure 2-12A) but also significantly reduced NO generation (Figure 2-12B) and decreased cytotoxicity (Figure 2-12C), indicating their suppressive effects on Ad induced inflammatory responses. These results show that inhibition of NF-κB activation can reduce inflammatory responses against Ad in the co-culture.

**Ad-induced macrophage activation requires interaction with neighboring epithelial cells**

Since the macrophage activation occurred only in the co-culture but not in the Raw mono-culture, we asked whether these responses are mediated by soluble factors secreted by Ad-infected epithelial cells. We used Transwell system, where MLE and Raw cells
Figure 2-11. **Inflammatory signaling pathways play a key role in inflammatory response in the co-culture.** The activation of NF-κB and IRF-3 transcription factors was studied by Western blot analysis. The cell extracts from MLE, Raw, and MLE-Raw co-culture were harvested before or 4 and 8 h after Ad infection. The induction of NF-κB pathway was detected indirectly by anti-phospho-IκB-α antibody. IRF-3 activation was detected by anti-phospho-IRF3 antibody and compared to the total IRF3. The results are representative of 2 independent experiments.
Figure 2-12. Inflammatory signaling pathways play a key role in inflammatory response in the co-culture. The co-cultures were treated with CAPE or genistein at the indicated concentrations prior to Ad infection. Media were collected 24h after infection and IL-6 (A) and NO (B), and the increase in cytotoxicity (C) were measured. *, p < 0.05 against Ad infected co-culture without treatments (Ad).
were grown in separate compartments but allowed free movement of molecules in the medium. Ad infection of MLE and Raw cells in Transwell system did not generate NO (Figure 2-13) or caused changes observed in our co-culture where the two cell types were cultured in contact with each other. It suggests that soluble factors, such as cytokines and chemokines alone, may not be sufficient for Ad-induced macrophage activation but the intercellular interaction between macrophages and epithelial cells might be necessary.

To investigate the importance of the intercellular interaction, we prepared partial co-culture by first seeding MLE cells and then scraping off cells from half of the culture dish and then seeding Raw cells. This procedure resulted in a cell culture dish with half of the dish with a co-culture of MLE and Raw cells and the other half with Raw cell mono-culture (Figure 2-14A). If the macrophage activation is mediated by soluble factor we would expect Raw cell activation not only on the co-culture side but also on the Raw mono-culture side. However, we found that the Raw cell activation occurred only on the co-culture side. Eighteen hours after Ad infection there were higher levels of cell death, shown by PI staining (Figure 2-14B), along with more ROS positive cells (Figure 2-14C) on the co-culture side compared to the Raw mono-culture side. These results indicate that macrophage activation requires interaction with neighboring epithelial cells. A local intercellular interaction can be carried out by contact dependent cell-cell interaction or through paracrine mechanism. Although the data from our experiments do not address what mechanisms are involved in macrophage activation, our observations suggest a paracrine-like interaction since there are some activated macrophages close to the co-culture side.
Figure 2-13. Ad-induced macrophage activation requires interaction with neighboring epithelial cells. NO generation after Ad infection was measured and compared between the co-culture and Transwell culture. Due to the structure of Transwell, the experiment was carried out in 3 ml volume and NO concentration was measured 48 h after Ad infection. n.d., not detectable.
A

Raw mono-culture | MLE-Raw co-culture

B

C
Figure 2-14. Ad-induced macrophage activation requires interaction with neighboring epithelial cells. (A) A partial MLE-Raw co-culture was established such that one area with MLE-Raw co-culture and the other area with Raw cells only. The partial co-culture was infected with Ad and macrophage activation was studied by examining at the border area of the Raw mono-culture (left side) and the co-culture (right side) (200×). (B) The cell death was analyzed by PI staining. (C) The cells were loaded with 10 μM DCFDA and stained for 20 min to determine ROS positive cells. A, B, and C are from the same viewing area. The images are representative of 3 independent experiments.
2.5 Discussion

Despite major improvements in the development of Ad vectors, their clinical application has been hampered by safety issues. Since the unfortunate incident several years ago, where a patient succumbed to a systemic inflammatory response syndrome during an Ad gene therapy clinical trial (Raper et al., 2003), the innate immune response against Ad as well as other types of gene therapy vectors has been under close scrutiny. Recent studies have suggested that Ad infection can be sensed by cellular PPRs, such as NLRP3, DAI, and AIM2, which recognize intracellular DNA to induce inflammatory responses during viral infection (Muruve et al., 2008; Schroder et al., 2009; Takaoka et al., 2007). However, there is a substantial lack of knowledge on how activation of these PRRs elicits massive immunological response in the host.

In general the innate immune response has been studied by two approaches. First, KO animal models that are deficient in innate immune genes are used to look at their response to pathogens. Although this approach can predict which genes might be involved in the innate immune responses, it would be difficult to unravel the complex mechanisms leading to inflammatory responses. Alternatively, the innate immune response is studied in vitro by examining the molecular interactions and signal transduction pathways that lead to inflammatory responses at the cellular and molecular levels. This approach, however, lacks the context of the inflammatory environment in the tissue, where various cell types can communicate and interact with each others. In fact, most of the in vitro studies use one type of cell and ignore the cooperative interaction among different cell types within the tissue, which might be essential in initiating and amplifying the inflammatory signal in vivo.

In this study we investigated the interaction between macrophages and epithelial cells in Ad-induced inflammation. These cells are the two major cell types that reside on the airway surface to recognize and respond to pathogens and harmful substances and induce airway inflammation. In a recent study, it has been shown that direct cell-cell interactions
between these two cell types in the airway can modulate innate immune responses against pathogens (Takabayshi et al., 2006). Their interactions have also been shown to play an important role in inflammation caused by air pollution particles and ischemic injury (Fujii et al., 2002; Ishii et al., 2005; Sharma et al., 2007; Tao and Kobzik, 2002). These studies suggest that the interaction between macrophages and epithelial cells may be a critical component in inflammatory responses.

To study the epithelial cell interaction during Ad infection we utilized an in vitro co-culture system consisting of mouse epithelial cells and macrophages. We have shown that Ad infection of the co-culture induced macrophage activation and inflammatory responses resulting in cytotoxicity (Figure 2-4), pro-inflammatory cytokine and chemokine induction (Figure 2-7 and 8), NO and ROS generation (Figure 2-9) and activation of inflammatory transcription factors (Figure 2-11). These bona fide inflammatory responses culminated in drastic changes in the cell culture and a decrease in medium pH by 24 h of Ad infection (Figure 2-2). The fact that these inflammatory responses occurred only in the co-culture but were absent or much weaker in the macrophage mono-culture indicates that the macrophage and epithelial cell interaction is essential in Ad-induced inflammation. We have used HDAd-CMV-LacZ vector in all of the experiments in this study, which contains the bacterial β-galactosidase gene. When we infected the co-culture with an HDAd devoid of any transgene (C4HSU) we also observed the same responses including induction of NO (Figure 2-15), indicating that the Ad induced inflammatory response observed in the co-culture was independent of transgene expression.

Macrophages often need multiple stimuli for their activation and they tend to generate more robust inflammatory responses when they are exposed to more than one stimulatory signal (Duffield, 2003). The multiple stimuli requirement might be an important regulatory mechanism to prevent unwarranted spontaneous activation of macrophages, which can cause devastating consequences to the host. In our study, Ad infection of macrophage mono-cultures induced little response whereas the response was synergistically increased in the co-culture. This suggests that the macrophage and
Figure 2-15. **Infammatary responses Ad is independent of the transgene in the vector.**

Infection of MLE-Raw co-culture with a HDAd containing no transgene, C4HSU, induced NO at a similar level to HDAd-CMV-LacZ.
epithelial cell interaction might generate an extra signal that triggers inflammatory responses.

Infection by pathogens causes expression of cytokines and chemokines and many studies suggest that their expression can trigger macrophage activation. However, our data indicate that their expression alone may not be sufficient since macrophage and epithelial cell co-culture in the Transwell system failed to induce macrophage activation during Ad infection. Several other studies also reported similar results that inflammatory responses seen in co-culture models were diminished if epithelial cells and macrophages were cultured in Transwells (Rosseau et al., 2005; Tao and Kobzik, 2002). Furthermore, they showed that treatment of the co-culture with pro-inflammatory cytokines had no effect on inflammation. We also tested whether adding cytokines after Ad infection induces macrophage activation without epithelial cells but such treatments did not reproduce changes observed in the co-culture (data not shown). On the other hand, we observed low levels of cytokine induction and activation of the NF-κB pathway when epithelial cells and macrophages were mixed without Ad infection (Figure 2-7 and 2-11). Therefore, it is likely that induction of cytokines and activation of pro-inflammatory pathways might be pre-requisites to respond to Ad infection, but other stimuli generated by intercellular interaction are also involved in macrophage activation.

It has been proposed that the immune response requires a “danger signal” in addition to recognition of pathogens (Matzinger, 2002). In fact, there is accumulating evidence that activation of macrophages and inflammatory response are enhanced in the presence of endogenous danger signals, which are thought to be released by damaged or stressed cells (Zhang and Mosser, 2008). We have seen significantly higher cell death in the co-culture as early as 18h after Ad infection (Figure 2-4), suggesting that endogenous danger signals released by cell death could be involved in the inflammatory responses in the co-culture. The danger signals released from virus infected cells might be sensed by neighboring innate immune cells such as macrophages to trigger a cascade of inflammatory events observed in this study. Currently, we are examining the possibility of this hypothesis.
Ad-induced macrophage activation in the co-culture resulted in striking changes in the cell culture such as decrease in pH and increase in cytotoxicity (Figure 2-2 and 2-4). Although it may seem reasonable to assume that cytotoxicity in the culture may contribute to lower pH, it is not clear what causes the drastic decrease in pH in a short period of time. In the respiratory burst of neutrophils and macrophages, superoxide and subsequently other ROS are generated by the activated NADPH oxidase complex upon engulfing pathogens, which is believed to be one of the major microbicidal mechanisms (Lambeth, 2004). Other effects of ROS generation in the respiratory burst are apoptotic cell death and decrease in pH to compensate for the charge imbalance as a consequence of electron transfer (Cross and Segal, 2004). Although the activation of NADPH oxidase has been studied mostly in the context of phagocytosis-mediated innate immune response, it is now well accepted that viral infection also induce ROS generation via NADPH oxidase activation (Grandvaux et al., 2007). Therefore, it is possible that the cytotoxicity and decrease in pH in our Ad-infected co-culture might be the consequences of activation of NADPH oxidase and ROS production. However, there may be other mechanisms responsible for the cytotoxicity. For example, it has been shown that IL-1 plays a major role in Ad-induced toxicity in vivo (Shayakhmetov et al., 2005b). IL-1α and IL-1β were highly induced in the co-culture in the late phase of Ad infection, (Figure 2-7 and 2-8) and they might play a role in the cytotoxicity we observed in the co-culture.

We used CAPE and genistein to show that inhibition of inflammatory signaling pathways can reduce Ad-induced inflammation (Figure 2-12). CAPE is a specific inhibitor for NF-κB, and genistein is shown to have various anti-inflammatory as well as anti-oxidant effects including inhibition of NF-κB. These agents significantly reduced inflammatory responses in the co-culture demonstrating that the Ad-induced inflammatory response is dependent on activation of NF-κB. Previous studies have also reported that activation of NF-κB as well as IRF-3 and MAPKs is involved in Ad-induced inflammation (Liu and Muruve, 2003; Nociari et al., 2007). Therefore, we suggest that activation of the NF-κB pathway plays a critical role in macrophage activation during Ad-induced inflammation and can be a target to control Ad induced inflammation during gene delivery.
Our study provides evidence that the interactions between macrophages and epithelial cells may be essential in macrophage activation during Ad infection. The synergistic effects of the co-culture in the inflammatory response shown in this study including the cytotoxicity and production of inflammatory mediators may explain how a high dose Ad administration could cause acute inflammatory response \textit{in vivo}. The macrophage and epithelial cell interaction might also be important in induction of inflammatory responses during the wild type adenovirus infection. Given the significant effect of the synergism in our co-culture, it would be interesting to examine whether a similar synergism is involved in macrophage activation by the wild type Ad and other viruses. Future studies directed to identify the mechanism that mediates the interactions between macrophages and epithelial cells can provide strategies to reduce undesirable inflammation during Ad gene therapy or in the treatment of other inflammatory diseases.
Chapter 3

The role of ATP in regulating inflammatory responses during an acute viral infection

The contents of this chapter have been published in PloS One:

3.1 Rationale and summary

Acute viral infection causes damage to the host due to uncontrolled viral replication but even replication deficient viral vectors can induce systemic inflammatory responses. Indeed, overactive host innate immune responses to viral vectors have led to devastating consequences. Macrophages are important innate immune cells that recognize viruses and induce inflammatory responses at the early stage of infection. However, tissue resident macrophages are not easily activated by the mere presence of virus suggesting that their activation requires additional signals from other cells in the tissue in order to trigger inflammatory responses. Previously, we have shown that the cross-talk between epithelial cells and macrophages generates synergistic inflammatory responses during adenoviral vector infection.

Objective of Study: Here, we investigated whether ATP is involved in the activation of macrophages to induce inflammatory responses during an acute adenoviral infection.

Summary of Results: Using a macrophage-epithelial cell co-culture system we demonstrated that ATP signaling through the P2X7 receptor (P2X7R) is required for induction of inflammatory mediators. We also showed that ATP-P2X7R signaling regulates inflammasome activation as inhibition or deficiency of P2X7-R as well as caspase-1 significantly reduced IL-1β secretion. Furthermore, we found that intranasal administration of replication deficient adenoviral vectors in mice caused a high mortality in wild-type mice with symptoms of acute respiratory distress syndrome but the mice deficient in P2X7-R or caspase-1 showed increased survival. In addition, wild-type mice treated with apyrase or inhibitors of P2X7-R or caspase-1 showed higher rates of survival. The improved survival in the P2X7-R deficient mice correlated with diminished levels of IL-1β and IL-6 and reduced neutrophil infiltration in the early phase of infection.
Conclusion: These results indicate that ATP, released during viral infection, is an important inflammatory regulator that activates the inflammasome pathway and regulates inflammatory responses.

3.2 Study background

Acute viral infection poses serious health problems as seen in the recent outbreaks caused by the new strains of influenza virus and the SARS corona virus. Although rapid viral replication and its cytopathic effects can directly damage the infected tissue, the overwhelming host response to acute viral infection can lead to a fatal outcome due to systemic inflammation and multiple organ failure (Chen and Subbarao, 2007; Maines et al., 2008). For this reason, the innate immune response is referred to as a double-edged sword as it is essential for inducing immune responses against pathogens but its over-activation can lead to immunopathologic consequences (Fukuyama and Kawaoka, 2011; Rouse and Sehrawat, 2010).

Adenovirus is a double stranded DNA virus that can infect various organs in humans and often causes acute upper respiratory tract infection with relatively mild symptoms. Adenovirus has been engineered as replication deficient viral vectors for gene therapy purposes and shown to be one of the most effective gene delivery vehicles for the lung (Cao et al., 2004). Although adenoviral vectors (Ads) have been used in gene therapy with mild side effects, a fatality occurred during a clinical trial. The patient suffered from systemic inflammatory response syndrome with pathological features of acute respiratory distress syndrome (ARDS) after receiving a high dose Ad (Raper et al., 2003). The fact that administration of replication deficient Ads can induce severe inflammatory responses supports the notion that an overactive innate immune response is responsible for devastating consequences in the host during acute viral infection.
Studies on influenza virus demonstrated that NLRP3 inflammasome activation is a critical component of the innate immune response against acute viral infection (Allen et al., 2009; Ichinohe et al., 2009; Thomas et al., 2009). It has been shown that DNA viruses such as adenovirus can also activate NLRP3 and AIM-2 inflammasomes to induce secretion of IL-1β and IL-18 (Muruve et al., 2008; Rathinam et al., 2010). Although the pathogen recognition receptors (PRRs) such as NLRP3 are essential components of inflammasomes it has been recognized that additional stimuli are necessary for activation of the inflammasome pathway (Kanneganti, 2010; Martinon et al., 2009; Pang and Iwasaki, 2011). In studies examining the mechanism of inflammasome activation, ATP is often applied to induce secretion of IL-1β from macrophages following stimulation with pathogens or the relevant pathogen associated molecular patterns (PAMPs). ATP has been considered as an endogenous danger signal since cells maintain a high concentration of ATP but it is mostly absent outside of the cell (Di Virgilio, 2007). In addition, P2X7R, the cell surface receptor for ATP in macrophages and other immune cells, has an unusually low affinity for ATP, raising speculation that the release of high concentrations of ATP from stressed or dying cells might provide an important regulatory mechanism for induction of inflammatory responses (Bours et al., 2011). In most of the studies, ATP is exogenously added at milli-molar concentrations to stimulate P2X7R. Other studies utilized strong cytotoxic treatments or directly applied necrotic cells to show that release of ATP from dying cells can induce inflammatory responses (Ghiringhelli et al., 2009; Iyer et al., 2009). While these studies indicate that ATP is involved in inflammatory responses, the role of ATP in regulating innate immune response during viral infection is still not clear.

In this study, we investigated whether ATP plays a role in the induction of inflammatory responses during acute viral infection using replication deficient Ads. Replication deficient viral vectors can be a useful tool for studying the innate immune response...
against viral infection. Unlike wild-type virus models where the host is infected with a small number of viruses which then proliferate over time to reach the level of acute viral infection, a replication deficient viral vector can be administered at a pre-determined titer to emulate the condition of acute viral infection. Moreover, since there is no further viral replication that generates ongoing cytopathic effects, the infection models using replication deficient viruses allow us to examine the host induced innate immune responses. In this view, it should be noted that the mice with a deficiency in the innate immune system often do worse than wild-type mice when infected with wild-type viruses because the deficiency usually hinders induction of innate immune responses resulting in unchecked viral replication. However, the same deficient mice could be affected less by the host-damaging effects of the relevant innate immune mechanism when infected with non-replicating viruses.

Although studies have reported that administration of replication deficient Ad induced inflammatory responses their mechanisms have not been well characterized. However, studies clearly demonstrated that macrophages are a major player in Ad induced inflammatory responses as they uptake a large proportion of administered Ad and produce pro-inflammatory cytokines (Kuzmin et al., 1997; Muruve, 2004; Zsengeller et al., 2000). Using an *in vitro* model we have previously shown that Ad infection of macrophage and epithelial cell co-cultures produced substantially stronger inflammatory responses and increased cytotoxicity compared to infecting macrophages alone, suggesting synergistic interactions between these two cell types in regulating innate immune responses (Lee et al., 2010). The study also showed that macrophage activation requires interaction with neighboring epithelial cells. Since macrophage activation often requires multiple stimuli (Zhang and Mosser, 2008), we postulated that the synergic responses are mediated by ATP released from Ad-infected cells, which allows macrophage activation and triggers inflammatory responses. In this study, we found that
ATP signaling via P2X7-R plays a key role in the regulation of inflammatory responses during acute viral infection including inflammasome activation. Furthermore, we show that ATP-mediated signaling is an important mechanism that regulates induction of systemic inflammation \textit{in vivo}.
3.3 Materials and methods

Ethics statement

All animal use procedures were conducted according to the guideline set by the Canadian Council on Animal Care. The Animal Care Committee at the Hospital for Sick Children approved all protocols developed for this work.

Reagents

LPS, oATP, apyrase were purchased from Sigma (St. Louis, MO, USA), z-YVAD-fmk from BioVision (Mountain View, CA, USA), and A-438079 from Tocris (Ellisville, MO, USA).

Adenovirus vectors.

The Ads used in this study were prepared as described previously (Lee et al., 2010; Shayakhmetov and Lieber, 2000). The helper-dependent Ad was used in the in vitro experiments and the E1-deleted first generation Ad was used in the in vivo studies in order to achieve the high viral dosages required for the experiment.

Cell culture and in vitro infection

The cell lines and the primary macrophage cells were cultured in DMEM (Invitrogen, Burlington, ON, Canada) supplemented with 10% FBS (Invitrogen) and cultured at 37 °C.
in 5% CO₂. The macrophage and epithelial cell co-cultures were established and infected with Ad as previously described (Lee et al., 2010). Briefly, MLE and macrophage (Raw, SF, J774, ATPR, or peritoneal macrophages) co-culture was prepared on 6 well plates by first seeding MLE cells and the macrophages on the following day, each at 25% confluency. On the third day the co-culture was about 80~90% confluent with MLE and macrophages at 50:50 ratio. The mono-cultures of MLE or macrophages were also grown to 80~90 % confluency before Ad infection. The cell culture was replaced with 1 ml of fresh medium and Ad was directly inoculated to the medium at approximately 20 multiplicity of infection (MOI). The mouse primary peritoneal macrophages were collected by peritoneal lavage as previously described (Muruve et al., 2008) except lavage was performed without the thioglycollate induction.

**Measuring inflammatory mediators and cytotoxicity.**

Analyses of inflammatory mediators and cytotoxicity were performed as previously described (Lee et al., 2010). Briefly, IL-6 and KC were measured from the culture medium using available ELISA kits (R&D Systems, Minneapolis, MN, USA) and NO was measured by Griess assay (Promega, Madison, WI, USA). The ROS positive cells were analyzed by flow cytometry using 3-(p-aminophenyl) fluorescein (APF; Invitrogen, Burlington, ON, Canada). The cytotoxicity in the culture was assessed by using a LDH assay kit (Promega, Madison, WI, USA). Commercially available ELISA kits were used to measure IL-1β (BD Biosciences, Mississauga, ON, Canada) and IL-18 (MBL, Nagoya, Japan). IL-1β was detected by Western blot analysis following immuno-precipitation with an antibody against mouse IL-1β (R&D Systems, Minneapolis, MN, USA).
In vivo Ad infection.

C57Bl/6 mice (Charles River, St. Constant, QC, Canada), caspase-1-KO (gift from Dr. Alberto Martin), and P2X7-KO mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for in vivo experiments. Mice 6~10 weeks of age were briefly anesthetized by isoflurane inhalation and 50 µl of Ad solution containing $1 \times 10^{11}$ of viral particles was administered through nares into the lungs. The inhibitors were administered by intraperitoneal injection for the first two days of infection. BALF was obtained as described elsewhere (Gasse et al., 2009) and used for cytokine measurement and differential cell count. Mice with more than 20% weight loss were euthanized.

Histology

The mouse lung sections were prepared from the whole lung samples fixed in 4% buffered formaldehyde. The sections were stained with hematoxylin and eosin and the severity of pathological features were assessed according to the criteria described in the Supplementary Material (Table 3-1).

Differential cell count

The cell fraction from the BALF were prepared on a slide by cytospin and differential cell counts were performed in a blinded manner, with a total of 300 cells counted per sample.

Statistical analyses
Table 3-1. The criteria for assessing mouse lung pathology score.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Pathology score</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Overall extent of inflammation</td>
<td>none</td>
</tr>
<tr>
<td>Airway inflammation/injury</td>
<td>none</td>
</tr>
<tr>
<td>Alveolar inflammation/injury</td>
<td>none</td>
</tr>
</tbody>
</table>
Student’s t-test was used to assess statistical significance between means. Survival curves were compared by using the log rank test. Significance (*) was set at p<0.05. The error bar represents mean ± standard deviation (SD) of three independent experiments.
3.4 Results

**Oxidized-ATP (oATP) inhibited inflammatory responses in the Ad-infected macrophage and epithelial cell co-culture**

Previously, we found that Ad infection of the co-culture consisting of a mouse lung epithelial cell line, MLE-15 (MLE), and a mouse macrophage cell line, Raw 264.7 (Raw), resulted in induction of inflammatory mediators including pro-inflammatory cytokines, nitric oxide (NO), and reactive oxygen species (ROS) (Lee et al., 2010). These inflammatory mediators were absent or much lower when Raw cell mono-culture was infected with Ad. In order to test whether ATP is involved in the synergistic response we first measured the concentration of ATP in the media after Ad infection (Figure 3-1). Although there was a variation in the magnitude, we consistently observed higher extracellular ATP in the Ad-infected co-culture 12~18 h after infection, which coincides to the time when there is a significant increase in the cytotoxicity measured by LDH in the medium (Lee et al., 2010).

To further investigate the correlation between the increase in extracellular ATP and the inflammatory responses, we utilized oATP (Murgia et al., 1993) to inhibit ATP-mediated macrophage activation. Infection of the co-culture with approximately 20 multiplicity of infection (MOI) of Ad induced a significant amount of NO within 24 h. However, adding 50~100 µM of oATP completely abolished NO generation in the Ad-infected co-culture (Figure 3-2A). We also found that the treatment with 100 µM of oATP reduced the number of ROS positive cells to a basal level (Figure 3-2B). Furthermore, oATP treatment significantly decreased induction of key inflammatory cytokines such as IL-6 and KC (Figure 3-2C and D). These inhibitory effects of oATP on induction of various inflammatory mediators suggest that ATP plays a role as an intercellular signal molecule...
Figure 3-1. Ad infection of MLE-Raw co-culture increased extracellular ATP. The MLE or Raw cell mono-culture as well as MLE-Raw co-culture were infected with 20 MOI of Ad and the samples of media were obtained every 2 h to measure the relative content of extracellular ATP using a luciferase based assay kit (Perkin Elmer, Woodbridge, ON, Canada). The assay was performed according to the manufacturer’s recommended procedure and the luminescent emission was measured using EnVision Plate Reader (Perkin Elmer, Woodbridge, ON, Canada). Shown is a representative plot of three independent experiments.
Figure 3-2. Treatment with oATP inhibited inflammatory responses in Ad infected MLE-Raw co-culture. The MLE-Raw co-culture was infected with Ad for 24 h in the presence of oATP at the designated concentrations. (A), The amount of NO produced in the culture was measured from the medium using Griess reagent. Data are expressed as mean ± SD. (B), The MLE-Raw co-culture was infected with Ad and ROS positive cells from were counted by flow cytometry after treating 3-(p-aminophenyl) fluorescein (APF) without gating for specific cell types. Shown is a representative FACS histogram of three independent experiments with the percentage of ROS positive population. (C and D) Mouse IL-6 and KC, respectively, were measured from the medium using commercially available ELISA kits. Data are expressed as mean ± SD.
in the macrophage and epithelial cell co-culture during Ad infection. In addition, oATP treatment reduced the cell death caused by Ad infection in the co-culture (Figure 3-3).

**Deficiency in P2X7R inhibited inflammatory responses in Ad-infected macrophage and epithelial cell co-culture**

Despite the common usage of oATP to inhibit ATP-mediated inflammatory responses, there is a debate about whether its effect is caused by inhibition of cell surface ATP receptors or by inhibition of other signaling pathways (Beigi et al., 2003). Macrophages express several ATP receptors on the cell surface but P2X7R is known to play the major role in regulating inflammatory responses (Ferrari et al., 2006). In order to confirm our findings from the experiments with oATP we performed co-culture experiments with a P2X7R deficient Raw cell line (SF) (Pfeiffer et al., 2007). Unlike the co-culture with the wild-type Raw cells, Ad infection of the MLE-SF co-culture produced only basal level of NO (Figure 3-4A). Even at the five time higher Ad dose we did not detect any significant NO induction in the co-culture of P2X7R deficient Raw cells whereas LPS treatment induced a large amount of NO indicating that these cells are capable of NO generation (Figure 3-4B). In addition, Ad infection of the MLE-SF co-culture did not generate ROS positive cells (Figure 3-4C) and produced less IL-6 compared to its wild-type counterpart (Figure 3-4D), confirming the results obtained from the experiments using oATP. These data indicate that P2X7R activation by extracellular ATP is an important component of the inflammatory responses in macrophages during Ad infection.

**MLE-Raw co-culture failed to secrete IL-1β after Ad infection**
Figure 3-3. Treatment with oATP reduced cytotoxicity in Ad infected MLE-Raw co-culture. The MLE-Raw co-culture was infected with Ad for 18 h and stained with propidium iodine (PI). The cells without Ad infection (A and B) showed limited cell death and oATP (100 μM) treatment did not cause cytotoxicity. Ad infection at 20 MOI resulted in significant cell death in the culture (C) but the treatment with oATP reduced cytotoxicity (D).
Figure 3-4. Ad infection of the co-culture with P2X7R deficient Raw cells generated significantly less inflammatory mediators. The MLE and a P2X7R deficient Raw cell (SF) co-culture was established as MLE-Raw co-culture. (A) MLE-Raw and MLE-SF co-cultures were infected with the designated MOI of Ad. The amount of NO produced in the culture medium was measured by Griess assay. Data are expressed as mean ± SD. (B) To verify that SF cells has no inherent defect in NO generation MLE-SF co-culture was treated with LPS (2.5 µg/ml) and NO generation was compared with Ad infected MLE-SF as well as MLE-Raw co-cultures. Data are expressed as mean ± SD. (C) The ROS positive cells in the Ad infected MLE-SF co-culture were counted by flow cytometry analysis after treating cells with APF. Shown is a representative FACS histogram of three independent experiments with the percentage of ROS positive population. (D) Mouse IL-6 was measured from the media of mono-cultures (MLE, Raw, and SF) and co-cultures (MLE-Raw and MLE-SF) 24 h after Ad infection using a commercially available ELISA kit. Data are expressed as mean ± SD.
While our study was in progress Pelegrin et al. reported that Raw cells do not express ASC (apoptotic speck protein containing a caspase recruitment domain), one of the key molecules of the inflammasome complex, and, therefore, they are deficient in IL-1β and IL-18 processing and secretion (Pelegrin et al., 2008). Indeed, we detected no IL-1β in the media of the Ad-infected MLE-Raw co-culture although there was induction of IL-1β gene expression (Lee et al., 2010) and pro-IL-1β was detected from the cell extract (Figure 3-5). Therefore, we utilized another mouse macrophage cell line, J774.A1 (J774), in the co-culture system to investigate whether ATP is involved in the inflammasome activation and IL-1β secretion during Ad infection.

**Ad infection of MLE-J774 co-culture induced secretion of IL-1β and IL-18**

Ad infection of the MLE-J774 co-culture showed a similar response to the MLE-Raw co-culture including an increase in cytotoxicity (Figure 3-6A) and induction of pro-inflammatory cytokines (Figure 3-6B and C). In addition, MLE-J774 co-cultures secreted a significant amount of IL-1β in the media within 24 h after Ad infection, which was absent in the J774 mono-culture (Figure 3-7A). We also detected a significant amount of IL-18 in the Ad-infected co-culture (Figure 3-7B) suggesting that Ad infection of MLE-J774 co-cultures induced activation of the inflammasome. There was a significant IL-1β secretion when infected with more than 20 MOI indicating that it requires above a threshold level of infection (Figure 3-7C). As shown by Western blot analysis the majority of IL-1β in the media was in the mature form and detected after 18 h (Figure 3-8), corresponding to the time when drastic changes were observed in the cell culture. We found that the amount of IL-1β was maximized when the co-culture was at 50:50 ratio of two cell types (Figure 3-9A). This result along with the fact that infection of J774 cells
Figure 3-5. Ad infection of MLE-Raw co-culture induced IL-1β but fail to process it to mature form. The Raw cell mono-culture and MLE-Raw co-culture were infected with Ad (20 MOI) for 18 h and cells were harvested for Western blot analysis. Proteins were resolved in 15% SDS-PAGE, and IL-1β was detected by an anti-mouse IL-1β antibody (R&D Systems, Minneapolis, MN, USA), which detects both pro-IL-1β (p-IL-1β) and mature IL-1β (m-IL-1β).
Figure 3-6. Ad infection of MLE-J774 co-culture increased cytotoxicity and induced pro-inflammatory cytokines. The MLE-J774 co-culture was infected with the designated doses (MOI) of Ad. (A) The cytotoxicity was measured by LDH assay using the culture media 18 h after infection. The increase in cytotoxicity was presented as the relative fold increase compared to the non-infected culture. (B and C) The induction of pro-inflammatory cytokines, IL-6 (B) and KC (C), were measured by ELISA using the media obtained from MLE-J774 co-culture 24 h after infection with different doses of Ad. Data are expressed as mean ± SD.
Figure 3-7. Ad infection of macrophage and epithelial cell co-culture induced secretion of IL-1β and IL-18. (A and B) MLE and J774 mono-culture as well as MLE-J774 co-culture were infected with Ad. IL-1β (A) and IL-18 (B) secretion was measured by ELISA assay using the media 24 h after infection. Data are expressed as mean ± SD. (C) MLE-J774 co-culture was infected with 0, 2, 20, or 100 MOI of Ad and IL-1β in the medium was measured 24 h after infection. Data are expressed as mean ± SD.
Figure 3-8. Ad infection of macrophage-epithelial cell co-culture resulted in secretion of mature IL-1β. J774 monoculture or MLE-J774 co-culture was infected with Ad and medium was collected at designated time for Western blot analysis following immunoprecipitation.
alone did not induce IL-1β secretion indicates that inflammasome activation requires additional signals generated from the interaction between neighboring macrophages and epithelial cells. Interestingly, co-cultures consisting of mouse macrophage cells and human airway epithelial cells (A549) also induce IL-1β secretion, supporting the idea that inflammasome activation might be regulated by a biologically universal molecule such as ATP (Figure 3-9B).

Inhibition of P2X7R and caspase-1 reduced IL-1β secretion in Ad-infected co-culture

To test whether ATP is involved in IL-1β secretion we treated the MLE-J774 co-culture with oATP. As shown in Figure 3-10A, 200-400 µM of oATP significantly reduced IL-1β secretion. IL-1β secretion was also inhibited by z-YVAD-fmk indicating that this process is caspase-1 dependent and involves inflammasome activation (Figure 3-10B). To address the role of P2X7R in inflammasome activation and IL-1β secretion during Ad infection, we used a J774 cell line deficient in P2X7R (ATPR-B2 (ATPR)) (Beyer and Steinberg, 1991). Ad infection of the MLE-ATPR co-culture produced significantly less IL-1β compared to the MLE-J774 co-culture (Figure 3-10C). Furthermore, we observed similar results when the MLE-J774 co-culture was treated with a P2X7R specific inhibitor (McGaraughty et al., 2007), A-438079 (Figure 3-10D), suggesting that P2X7R activation by ATP is an important process in inflammasome activation and IL-1β secretion during Ad infection.
Figure 3-9. Secretion of IL-1β is dependent on macrophage-epithelial cell interaction and might be mediated by a biologically universal molecule. (A) The co-cultures of different ratio of MLE and J774 were established and IL-1β was measured in the medium 24 h after Ad infection. Data are expressed as mean ± SD. (B) A human lung epithelial cell line, A549, instead of MLE was used to establish the co-culture with J774 macrophages and infected with Ad. IL-1β was measured 24 h after infection. Data are expressed as mean ± SD.
Figure 3-10. Inhibition of P2X7R and caspase-1 reduced IL-1β secretion in the Ad infected co-culture. (A and B) MLE-J774 co-culture was infected with Ad in the presence of oATP (0–400 μM) or z-YVAD-fmk (0–50 μM) and IL-1β secretion was measured in the medium. Data are expressed as mean ± SD. (C) The co-culture of MLE and a P2X7R deficient J774 cell line (ATPR) was establish and IL-1β in the medium was measured 24 h after Ad infection. Data are expressed as mean ± SD. (D) MLE-J774 co-culture was treated with a P2X7R specific inhibitor, A438079 (0–200 μM) at the designated concentration before Ad infection and IL-1β was measured from the medium 24 h after infection. Data are expressed as mean ± SD.
Co-cultures consisted of primary macrophages with deficiency in P2X7R and caspase-1 secreted less IL-1β after Ad infection

We confirmed our results obtained with the J774 macrophage cell line by replacing it with primary peritoneal macrophages obtained from mice. When infected with Ad, the co-culture containing peritoneal macrophages from wild-type mice showed IL-1β secretion comparable to the co-culture with J774 cells. IL-1β secretion in the co-culture with peritoneal macrophage was significantly reduced when treated with P2X7R inhibitors (Figure 3-11A) in a similar manner as with the MLE-J774 co-culture (Figure 3-10). Furthermore, the co-cultures of MLE and peritoneal macrophages from P2X7R or caspase-1-knock out (KO) mice secreted significantly less IL-1β after Ad infection compared to the co-culture prepared with peritoneal macrophages obtained from wild-type mice (Figure 3-11B and C), demonstrating that Ad infection induced IL-1β secretion via inflammasome activation in mouse primary macrophages. We also performed the same experiment with peritoneal macrophages from the NLRP3-KO mice since NLRP3 has been shown to be involved in Ad induced inflammation (Muruve et al., 2008). Although there was some reduction in IL-1β secretion in the NLRP3-KO macrophage co-culture the difference was less pronounced and limited to earlier time points (Figure 3-11D).

Intranasal (i.n.) Ad infection in mice caused systemic inflammation and fatality but deficiency or inhibition of P2X7R or caspase-1 enhanced survival

In order to study the role of ATP during acute Ad infection in vivo we established a mouse i.n. Ad infection model. Although Ad administration has been known to induce inflammatory responses, i.n. administration of Ad at dosages up to $5 \times 10^{10}$ viral particles
Figure 3-11. Co-cultures with primary macrophages from P2X7R and caspase-1 knockout mice secreted less IL-1β. (A) The co-culture was established with MLE and peritoneal macrophages from wild-type (C56BL/6) mice and infected with Ad with or without oATP (200 μM) or A438079 (100 μM). IL-1β was measured from the medium 24 h after infection. Data are expressed as mean ± SD. (B-D), Peritoneal macrophages were collected from and P2X7R (B), caspase-1 (C), or NLRP3 (D) knockout mice and the co-cultures were established with MLE. IL-1β was measured from the medium 24 h (18 h and 24 h for NLRP3-KO) after Ad infection and compared to the wild-type counterpart. Data are expressed as mean ± SD.
Figure 3-12. Intranasal administration of Ad caused ARDS-like symptoms and fatality. Ad was intranasally administered to wild type mice (C57/Bl6) with matching age and gender under brief isoflurane inhalation anesthesia. The mice were monitored and the body weight was measured each day. The survival curves was generated based on the humane end point of 20% weight loss. Mice were infected with Ad at either of the two dosages, 5x10^{10} or 1x10^{11} vp/mouse. The mice that received 5x10^{10} vp (n=5) showed no apparent symptoms and sustained only slight weight loss but recovered within a few days. On the other hand, all of the mice that received 1x10^{11} vp (n=5) showed ARDS like symptoms and continuously lost their body weight to reach the experimental humane end point. In the subsequent in vivo experiments Ad dosage of 1x10^{11} vp/mouse was used. Data are expressed as mean ± SD.
(vp)/mouse was well tolerated, showing only minor symptoms (Figure 3-12). On the other hand, when dosages higher than \(1 \times 10^{11}\) vp/mouse were given mice showed visible symptoms such as lethargy, dyspnea, ruffled fur, and significant and continuous weight loss followed by mortality within a few days (Figure 3-12). These pathological features of systemic inflammation were very similar to ARDS described in other mouse models for acute pulmonary viral infection (Chen and Subbarao, 2007; Maines et al., 2008). In order to test whether inhibiting ATP-mediated inflammatory responses can alleviate the severity of symptoms caused by acute Ad infection, we compared the weight loss and the survival rate between the wild-type and P2X7R-KO mice. As shown in Figure 3-13A, three days after infection most of the wild-type mice had considerable weight loss and showed symptoms of severe respiratory distress. However, the weight loss in P2X7R-KO mice was delayed and less severe (Figure 3-13A). More importantly, more than 30% of P2X7R-KO mice survived the infection (Figure 3-13B) and most of the surviving mice recovered the normal body weight and exhibited normal behavior by day 7. We found similar results from caspase-1-KO mice, which also exhibited less severe weight losses (Figure 3-14A) and a higher survival rate (Figure 3-14B) compared to the wild-type mice.

**Inhibition of ATP-P2X7R signaling enhanced survival of wild-type mice after Ad infection**

To further support the hypothesis that the inhibition of ATP-P2X7R-mediated inflammatory responses can alleviate acute inflammation and reduce fatality, we treated wild-type mice with A437980 (Taylor et al., 2009), z-YVAD-fmk (Gasse et al., 2009), or apyrase (Wilhelm et al., 2010), an ATP hydrolyzing enzyme. These inhibitors were intraperitoneally administered twice, once at the time of Ad infection and once at 24 h
Figure 3-13. Mice deficient in P2X7R showed reduced symptoms after intranasal Ad administration and increased survival. Ad (1x10^{11} vp/mice) was intranasally administered to the wild-type (n=12) and the P2X7R-KO mice (n=13) with matching age and gender. The mice were monitored and the body weight was measured each day. (A) The individual body weight on day 3, when all the subject mice were still alive, was presented as the relative retained body weight. The bold line represents the average body weight. (B) The survival curves were generated based on the humane end point of 20 % weight loss and the comparison was made by the log rank test. *, p < 0.05.
Figure 3-14. Mice deficient in caspase-1 showed reduced symptoms after intranasal Ad administration and increased survival. Ad (1x10^{11} vp/mice) was intranasally administered to the wild-type (n=6) and the P2X7R-KO mice (n=6) with matching age and gender. The mice were monitored and the body weight was measured each day. (A) The individual body weight on day 3, when all the subject mice were still alive, was presented as the relative retained body weight. The bold line represents the average body weight. (B) The survival curves were generated based on the humane end point of 20% weight loss and the comparison was made by the log rank test. *, p < 0.05.
after infection, in order to limit the effect of the inhibition to the initial stage of the innate immune response. As shown in Figure 3-15, the treatment with the inhibitors only during the first two days of infection significantly improved the survival in a similar manner as for the P2X7R and caspase-1-KO mice. These results from the inhibitor study along with the KO mouse studies demonstrate that ATP-P2X7R-mediated signaling and subsequent activation of the inflammasome pathway is critical for induction of systemic inflammation during acute viral infection in vivo.

**Enhanced survival in P2X7R-KO mice is due to reduced inflammatory responses**

Despite the apparent differences in the overall response to Ad infection the histopathologic features of the lungs in P2X7R-KO mice were similar to those of the wild-type mice (Figure 3-16 and 3-17A), suggesting that their survival advantage might be related to reduced host immune responses rather than the cytopathic damage caused by viral infection. We also found that there is no difference between wild-type and P2X7R-KO mice in the viral titers in the bronchoalveolar lavage fluid (BALF) 24 h after infection (data not shown) suggesting that the different phenotype was not due to the difference in viral clearance. It is well known from studies on acute viral infection as well as sepsis that overwhelming cytokine production and excessive neutrophil infiltration are the main immunopathological features linked to systemic inflammation and ARDS (La Gruta et al., 2007; Rittirsch et al., 2008). Therefore, we measured the levels of pro-inflammatory cytokines, IL-1β and IL-6, in the lung 24 h after Ad infection. Both IL-1β and IL-6 in the BALF were significantly lower in the P2X7R-KO mice compared to the wild-type mice (Figure 3-17B). In addition, we found fewer neutrophils and more macrophages in the BALF of the P2X7R-KO mice compared to the wild-type mice at 24 h after infection (Figure 3-17C) indicating that neutrophil infiltration is delayed or attenuated. These attenuated responses in the P2X7R-KO mice compared to the wild-type
Figure 3-15. Inhibition of ATP-P2X7R pathway early in Ad infection enhanced survival in vivo. Ad was intranasally administered to wild type mice (C57/Bl6) with matching age and gender. A438079 (300 μmol/kg), a P2X7R specific inhibitor, z-YVAD (10 mg/kg), a caspase-1 inhibitor, or apyrase (4 units/mouse), a ATP hydrolyzing enzyme, was administered intraperitoneally twice, at the time of Ad infection and 24 h after infection (n=15/group). The survival curves were generated based on the humane end point of 20 % weight loss and the comparison was made by the log rank test. *, p < 0.05.
Figure 3-16. The survival advantage of P2X7R-KO mice is not due to better lung histopathology. The wild-type (C57/Bl6) and the P2X7R-KO mice were infected with Ad of $1 \times 10^{11}$ vp/mouse by i.n. administration. The mice were sacrificed on day 2 ($n=3$), day 4 ($n=4$) or day 6 ($n=3$) after administration and the lungs were obtained for histological analysis. There were no major differences between the wild-type and the P2X7R-KO mice. Representative microscopic sections are shown. See also Figure 3-17A for the lung histology score analysis.
Figure 3-17. P2X7R-KO mice generated less inflammatory cytokines and neutrophil infiltration in the early stage of Ad infection. The wild-type (C57/Bl6) and the P2X7R-KO mice were infected with Ad (1x10^{11} vp/mouse) by i.n. administration. (A) The lung pathological scores were used to compare difference between the wild-type and the P2X7R-KO mice according to the criteria described in the Materials and Methods. Data are expressed as mean ± SD. (B and C) Twenty four hours after Ad administration BALF was collected from the wild-type (n=10) and the P2X7R-KO mice (n=8). (B) IL-1β and IL-6 in the BALF were analyzed by ELISA. (C) Differential cell counts were performed with the cell fraction of BALF. Data are expressed as mean ± SD.
C57/Bl6 mice underscore the significant role of P2X7R, especially when taking into consideration that the C57/Bl6 strain has been shown to be less responsive to its agonists such as ATP due to a point mutation in the cytoplasmic TNFR1 domain of P2X7R (Adriouch et al., 2002). Taken together these results suggest that ATP signaling through P2X7R regulates induction of pro-inflammatory cytokine and neutrophil infiltration, which consequently lead to the host-damaging systemic inflammation during acute viral infection.
3.5 Discussion

In this study we investigated the inflammatory responses against acute viral infection using replication deficient Ad to examine the effects of host-mediated responses. Specifically, we focused on the role of ATP in induction of inflammatory responses. Using macrophage and epithelial cell co-culture systems, we showed that ATP signaling through P2X₇R is essential for induction of inflammatory responses including activation of the inflammasome during acute viral infection. Our co-culture system was an effective model for examining the role of ATP in inflammatory responses during viral infection without the use of exogenous ATP. Furthermore, our in vivo study demonstrates that the ATP-P2X₇R signaling plays an important role in the development of systemic inflammation during acute viral infection.

Although a number of studies have examined the role of viral PAMPs and their relevant PRRs in inflammation, relatively little attention has been given to the role of endogenous danger signals in viral infection. This is partly due to the paucity of models that can be used to study the effect of danger signals. Infection with a wild-type virus results in proliferation of the virus and cell lysis that generate not only more PAMPs but also various danger signal molecules, making it difficult to dissect the effects of one from another. The replication deficient viral vector provides a unique model allowing us to generate the condition of acute viral infection without ongoing viral proliferation. In this model, cytopathic effects should be limited to the early stage of infection and the consequent inflammatory responses would be largely mediated by the innate immune mechanisms. Therefore, in the replication deficient viral infection model, the innate mechanisms triggered by endogenous danger signal should be more specifically revealed.
Although it was suggested more than a decade ago that ATP can modulate various immune responses, there is an increasing interest in the function of extracellular ATP with the recent discovery of the inflammasome pathway (Mariathasan et al., 2004). Stimulation of innate immune cells like macrophages with PAMPs followed by treatment of ATP has been commonly used to trigger inflammasome activation and IL-1β secretion in \textit{in vitro} experiments. However, the role of ATP in inflammation and its physiological and pathological implications are only beginning to emerge. Using a bleomycin-induced lung injury model Riteau et al. have shown that ATP released from the injured cells is responsible for release of IL-1β and pulmonary inflammation (Riteau et al., 2010). Idzko and co-workers have examined the role of ATP in various inflammatory conditions from lung injury to asthma and graft-versus-host disease (Idzko et al., 2007; Muller et al., 2011; Weber et al., 2010; Wilhelm et al., 2010). Using experimental animal models their studies showed that inhibition or deficiency of P2X7-R resulted in reduced severity in inflammation. Although these studies were conducted in models that cause tissue damage or immunogenic responses in the absence of pathogenic infection, they provide important evidence that ATP can act as a potent inflammatory stimulator.

In our \textit{in vivo} infection model, a high dose of replication deficient Ad is administered by intranasal route emulating the condition of acute viral infection. The infection resulted in ARDS-like symptoms and fatality demonstrating that a high titer of virus even without viral replication can trigger systemic inflammation in mice. Although it is not clear what causes systemic inflammation and ARDS, over production of cytokines has been proposed as a factor contributing to the severity of the disease in viral infection (La Gruta et al., 2007). Our data support this idea as the decrease in pro-inflammatory cytokines, such as IL-1β and IL-6, in the P2X7-R-KO mice correlated with reduced severity in inflammatory symptoms and higher survival rates (Figure 3-17B). IL-1β has been considered one of the key cytokines involved in the pathology caused by acute
inflammation (Dinarello, 2011). In particular, studies on influenza virus demonstrated that IL-1β is responsible for the acute lung pathology (Schmitz et al., 2005), and the inflammasome pathway that produces IL-1β has been shown to be essential in the inflammatory response against influenza infection (Allen et al., 2009; Ichinohe et al., 2009; Thomas et al., 2009). Therefore, the immunopathology seen in our acute viral infection model is likely to be related to activation of the inflammasome pathway and excessive IL-1β production.

In our in vivo study, we found it remarkable that only a two-fold difference in the viral titer can lead to such pronounced difference in the overall host responses (Figure 3-12). This result suggests that there is a threshold titer required for the induction of acute inflammation. We also observed a similar threshold effect in the in vitro co-culture studies where inflammasome activation and induction of the inflammatory mediators required a minimum of 20 MOI of Ad (Figure 3-4A, 3-6, and 3-7C). Since the induction of the inflammatory mediators was dependent on P2X7R, it is likely that Ad infection at the threshold titer would cause ATP release from the infected cells. Although how virus infected cells release ATP is not clear, most often ATP release is a consequence of cell damage or cell death (Di Virgilio et al., 2001). Recently, a luciferase-based detection method was used in mice to demonstrate that extracellular ATP can accumulate at the site of inflammation to a concentration high enough to activate P2X7R (Weber et al., 2010; Wilhelm et al., 2010). In our co-culture experiments, we detected a substantial increase in cytotoxicity, even before any significant induction of inflammatory mediators (Figure 3-6A). Since such cytotoxicity would potentially increase the extracellular ATP, it is conceivable that ATP released from cells dying from Ad infection would trigger the ATP-P2X7R-mediated inflammatory responses. However, it is difficult to identify the source of ATP at the cellular level because of the instantaneous and transient nature of ATP release.
Our results from the *in vitro* study suggest that ATP is likely to be initially provided by epithelial cells as the infection of macrophages alone fails to induce inflammasome activation (Figure 3-7A, B and 3-9A). ATP is known to be released from stressed or injured epithelial cells (Riteau et al., 2010), and infection with high dosages of Ad can induce some cell death (Teramoto et al., 1995). The mechanism of macrophage activation by neighboring virus infected epithelial cells through ATP-P2X7R activation is in line with our previous observation that the synergistic inflammatory responses were absent when the two cells were infected in separate compartments of a Transwell (Lee et al., 2010). Since P2X7R requires unusually high concentrations of ATP, which can be readily degraded by ectoenzymes in the extracellular space, it is believed that activation of P2X7R can only occur when ATP is secreted by dying cells very close to the macrophages. This mode of macrophage activation is further supported by the fact that IL-1β secretion was maximized when macrophages and epithelial cells were in 50:50 mixtures (Figure 3-9A). This co-culture condition should provide an ideal condition where ATP released from an Ad-infected epithelial cell can be readily sensed by the macrophages in the vicinity.

Nevertheless, we found that the majority of the dying cells in the Ad-infected co-culture were macrophages (Lee et al., 2010). Stimulation of P2X7R by ATP induces inflammasome activation and release of IL-1β but an extensive exposure to ATP results in activation of pannexin-1, membrane permeabilization, and eventual cell death, which would release more ATP and activate more macrophages (Bours et al., 2006). Therefore, we propose a model (Figure 3-18) that ATP released from virus infected epithelial cells activates neighboring macrophages in the infected tissue, culminating in inflammasome activation and IL-1β secretion, which can further aggravate the local tissue inflammation by induction of other inflammatory mediators such as IL-6. Moreover, redundant and prolong exposure to ATP caused by persistent infection would lead to macrophage cell
Figure 3-18. **Model of ATP regulating inflammatory response during viral infection.** A viral infection is sensed by macrophages via various pathogen recognition receptors (PRRs) on the plasma membrane and in the cytoplasm. Sensing of viral infection activates NFκ-B and other inflammatory signaling pathways to induce pro-inflammatory cytokines (Signal 1). As one of the key pro-inflammatory cytokines, IL-1β is produced in the inactive form of pro-IL-1β. Acute viral infection can cause release of a high concentration of ATP by the infected epithelial cells. The released ATP is sensed by P2X7R on the cell membrane and induces channel activation and potassium ion efflux in macrophages (Signal 2). These changes allow inflammasome formation and activation of caspase-1, which cleaves pro-IL-1β to active IL-1β. IL-1β is then secreted from the cell to induce various inflammatory responses. An extensive activation of P2X7R causes pennexin-1 activation, membrane permeablization, and eventual macrophage cell death, which leads to release of more ATP and other danger signals. These self-amplifying events would lead to escalation of inflammatory responses and may result in systemic inflammation.
death. This will in turn generate more ATP for activation of macrophages and other recruited immune cells in the infected tissue, which may cause systemic inflammation through this self-amplifying mechanism.

In the experiments with ASC deficient macrophages (Raw cell line), we found that various inflammatory mediators were induced by ATP-P2X7R-mediated signaling despite the absence of inflammasome activation. In particular, we found that NO (Figure 3-4A) and ROS (Figure 3-4C) generation was completely dependent on P2X7R as previously reported (Pfeiffer et al., 2007). These results indicate that ATP-P2X7R signaling may regulate various cellular responses in addition to the inflammasome activation. Since the mechanism downstream of ATP-P2X7R leading to the inflammasome activation is not clearly understood, it would be of interest to explore how ATP triggers induction of these important inflammatory mediators in cells lacking inflammasome components. In this regard, it is noteworthy that Ad infection induced ROS in Raw cells (Figure 3-2C) whereas the inhibition or deficiency of P2X7R did not produce ROS (Figure 3-2B and 3-4C). Currently, there is a controversy about whether inflammasome activation is mediated by ATP or ROS (Pang and Iwasaki, 2011). Although it requires further analyses our data indicate that ATP-P2X7R activation might be upstream of ROS generation.

While production of NO and ROS was completely dependent on P2X7R in the MLE-Raw co-culture (Figure 3-2 and 3-4), IL-1β secretion and inflammasome activation in the MLE-J774 co-culture was partially inhibited by the deficiency of P2X7R or caspase-1 (Figure 3-10 and 3-11). Moreover, P2X7R or caspase-1 deficiency protects 40% of mice while others succumbed to ARDS in our in vivo study (Figure 3-13 and 3-14). Similar results were obtained from previous studies that examined the role of P2X7R in systemic inflammation in a graft-versus-host disease model (Wilhelm et al., 2010). The reason for the partial inhibition might be due to the redundancies and complex network of
interrelated pathways in the innate immune system (Bours et al., 2011). For instance, other purinergic receptors like P2Y2R are known to provide ATP mediated inflammation in recruiting neutrophils (Cicko et al., 2010). In addition, other cytokines may be able to compensate for reduced IL-1β secretion. In particular, IL-1α, which binds to the same receptor and induces the same inflammatory responses as IL-1β, does not require post-translational modification through inflammasome pathway for its biological function (Dinarello, 2011). Considering these redundancies and compensating mechanisms, the significant difference in the survival rate underscores the importance of ATP-P2X7R-mediated inflammatory responses.

Taken together, our findings support a model that a high concentration of ATP released from virus infected cells during an acute viral infection functions as a danger signal, which activates the inflammatory responses including the inflammasome pathway and may serve as a link between the local infection and systemic inflammation (Figure 3-18). The results of this study indicates that controlling excessive inflammation by inhibiting ATP-P2X7R-mediated signaling pathway could provide a possible therapeutic approach for diseases caused by acute inflammation. Although such a therapeutic approach could provide beneficial effects in many conditions in the absence of infective agents, it should be noted that disruption of an innate immune mechanism can also compromise pathogen clearance and increase fatality as seen in infections with wild-type viruses (Kanneganti, 2010; Pang and Iwasaki, 2011). Therefore, the therapy should consider measures to control both pathogen and ATP induced inflammatory responses.
Chapter 4
Summary and Future Directions

The viral vectors developed for gene therapy other than for certain cancer therapy purposes are replication deficient viruses and they were once considered relatively safe to be used in human. However, since the tragedy during a gene therapy clinical trial in 1999, where a patient who had been treated with a FG-Ad developed and succumbed to a systemic inflammatory response syndrome, the gene therapy field was prompted to make more efforts to understand the immune responses triggered by viral vectors. Although the adaptive immune responses against viral vectors as well as wild type viruses have been extensively studied, induction of innate immune responses by viruses and viral vectors has not been well characterized. The pathologic inflammatory responses induced by administration of replication deficient viral vectors indicate that viral particles even without viral replication can trigger strong innate immune responses. How non-replicating viral vectors can cause such massive inflammatory responses remains to be determined, but it is likely that multiple pathways and mechanisms are involved in generating the host-damaging consequences.

This study was conducted based on the hypothesis that the interaction between macrophages and epithelial cells plays an important role in eliciting innate immune responses against viral vectors. Epithelial tissues such as the digestive and respiratory tracts have a unique environment, as the cells in the epithelial lining are constantly exposed to various harmful substances and microbes. Consequently, the concept of an immune response based on recognition of self and non-self cannot fully explain the level of tolerance observed in the epithelial environment. Tissue resident innate immune cells such as macrophages are equipped with various pathogen sensing mechanisms, but they
do not elicit inflammatory responses at every encounter with PAMP containing microbes. In fact, their excessive activation in the epithelial environment may only cause unnecessary and costly inflammatory responses and lead to host-damaging outcomes. Therefore, it is reasonable to assume that epithelial cells, which usually encounter invading pathogens first, could be actively involved in innate immune responses by providing additional signals that may regulate the function of innate immune cells such as tissue resident macrophages.

In this study, we provide evidence that the interaction between epithelial cells and macrophages generates essential signals that activate macrophages and induce inflammatory responses during viral infection. In Chapter 2, we found that macrophages behave remarkably differently in response to Ad infection in the presence of epithelial cells compared to when they are infected by themselves. Induction of inflammatory mediators including pro-inflammatory cytokines, NO, and ROS was significantly enhanced when macrophages were infected with Ad in the presence of epithelial cells. In addition to the inflammatory responses, Ad infection of macrophage and epithelial co-culture resulted in substantial cytotoxicity. These results demonstrate the importance of the synergistic interactions between macrophages and epithelial cells during an Ad infection and suggest that epithelial cells play a critical part in inflammatory responses by regulating macrophage functions.

Although \textit{in vitro} cell culture systems were used to demonstrate the interaction between macrophages and epithelial cells, the synergistic interaction is likely to be relevant in inflammatory responses \textit{in vivo}. Acute viral infection in the lung caused by viruses such as influenza virus can lead to overwhelming inflammation characterized by extensive tissue damage in the epithelium and massive induction of pro-inflammatory cytokines, referred to as a “cytokine storm” (La Gruta et al., 2007). It is believed that rapid viral
replication causes cytotoxicity and inflicts tissue damage during an acute influenza infection. However, the excessive production of cytokines that follows after viral proliferation is also known to cause tissue damage in the infected organ and leads to systemic inflammation. Currently, it is not clear how an acute viral infection causes over-production of cytokines which may be responsible for the morbidity and mortality. In light of our finding that macrophage and epithelial cell interaction considerably increases the production of pro-inflammatory cytokines during Ad infection, more studies are needed to examine whether the interaction between macrophages and epithelial cells plays a role in the over-production of pro-inflammatory cytokines and generation of deleterious inflammatory responses in the infections caused by clinically important viruses such as influenza virus.

In addition to our study, the synergy between macrophages and epithelial cells has been reported in other inflammation models such as particle inhalation and ischemia induced inflammation (Fujii et al., 2002; Ishii et al., 2005; Sharma et al., 2007; Tao and Kobzik, 2002). Therefore, the interaction might provide a fundamental mechanism that regulates pathways related to an acute inflammation. In order to understand the mechanism, the essential questions that need to be addressed are 1) what signals mediate the interaction and 2) how do the signals regulate the inflammatory responses? Although pro-inflammatory cytokines such as IFN-γ are best known for activating macrophages during inflammation, our attempts to stimulate Ad-infected macrophages with various cytokines failed to enhance inflammatory responses to the level we observe in the co-culture. In addition, the synergistic inflammatory responses occurred only when macrophages and epithelial cells were cultured in mixtures whereas culturing them in separate compartments in Transwells abolished the synergism. These results suggest that the interaction is not entirely mediated by cytokines and chemokines but dependent on a novel intercellular signaling mechanism among the cells in the microenvironment.
It is well known that macrophage activation often requires multiple stimuli. Activation of macrophages to the M1 state requires sequential stimulation with IFN-γ and LPS. Similarly, macrophages need to be primed with LPS before applying specific PAMPs in order to achieve significant inflammatory responses in *in vitro*. This mode of macrophage activation is relevant to the two signal model that is commonly observed in the immune system (Fontana and Vance, 2011). For example, T cell activation requires signals from the T cell receptor and a co-stimulatory molecule. The two signal requirement is considered as a safety mechanism to prevent unwarranted activation of the immune system, which can cause more harm than good to the host. According to the two signal model, the immune system detects infection not only through direct pathogen sensing but also through the contextual signals associated with the infection. The contextual signal is thought to be mediated by various molecules known as danger signals.

The results described in Chapter 2 suggest that the activation of macrophages by Ad infection also fits into the two signal model; the first signal is generated by the innate immune sensing mechanism and the second signal is generated from the interaction between macrophages and epithelial cells. As discussed in Chapter 1, the first signal is generated by PRRs upon recognition of Ad, which activates inflammatory signaling pathways such as NF-κB pathway, resulting in induction of pro-inflammatory genes. Several cytosolic PRRs have been identified to respond to Ad and other DNA viruses through the recognition of viral DNA. In particular, NLRP3 and AIM2 have been shown to play important roles in inducing innate immune responses against DNA viruses through the activation of corresponding inflammasomes. However, activation of the inflammasome and secretion of IL-1β and IL-18 often require additional stimuli such as a high concentration of extracellular ATP. Extracellular ATP has been known to function as a danger signal and it activates P2X7R to form channels on the plasma membrane, which allows K+ efflux. It has been suggested that the change in the intracellular ionic
environment caused by ATP signaling through P2X7R is considered to be a key 
mechanism that activates inflammasomes in macrophages (Rathinam et al., 2012). This 
two signal mechanism of inflammasome activation is compatible with the observations 
from our co-culture studies and provided a rationale to test whether ATP is the danger 
signal generated from the interaction between macrophages and epithelial cells during an 
Ad infection.

Based on the rationale, we investigated whether ATP plays a role in the inflammatory 
responses during an Ad infection. In Chapter 3, using the macrophage and epithelial cell 
co-culture system we have demonstrated that the inhibition of ATP-P2X7R signaling can 
significantly reduce inflammasome activation as well as induction of various 
inflammatory mediators during an Ad infection. We also found that the ATP-P2X7R 
pathway is important in inducing acute inflammation *in vivo* as mice deficient in P2X7R 
showed reduced mortality and diminished inflammation after Ad infection. These 
experimental results support that Ad infection causes secretion of ATP, which then 
regulates activation of the inflammasome and induction of inflammatory responses.

Our results in Chapter 3 provide a basis for further investigation on the mechanisms of 
the ATP-mediated inflammation at the cellular level. Although it has been long suggested 
that ATP has pro-inflammatory properties, demonstrating the function of ATP in 
inflammatory conditions has been difficult because of its unique mode of action. At the 
cellular level, activation of P2X7R requires ATP at a concentration that can only be 
achieved when a cell spontaneously releases its cellular content. In *in vitro* experiments, 
P2X7R activation is often attained by artificially adding mili-molar concentrations of 
ATP into the cell culture. However, such treatments may cause undesired effects, as an 
extensive exposure of macrophages to ATP is known to cause membrane permeablization 
and cell death. In this sense, our co-culture infection model provides a useful
experimental model to examine the mechanism of the ATP-induced P2X_7R activation in a physiologically relevant condition. In our study, the co-culture infection model has been effectively used to show the importance of intercellular interaction between macrophages and epithelial cells in proximity (Figure 2-14). This result concurs with the mode of ATP-mediated responses, which predicts that only the macrophages located in the same microenvironment of the infected epithelial cell would be affected. In the future, this experimental approach can be applied to study ATP-mediated P2X_7R activation in macrophages at the microscopic level. In particular, by examining the responses in co-cultures with a different distribution of the two cell types and how it affects the inflammatory response in macrophages we can gain mechanistic insights of P2X_7R activation. What we learn from such a study would help us to understand the process of inflammation.

An important question to be addressed regarding the mechanism of the ATP-mediated inflammatory response in the co-culture is which cell provides ATP out of the two cell types? We have determined that the majority of the dying cells in the Ad-infected co-culture were macrophages. However, there is limited cell death in Ad-infected macrophage mono-cultures suggesting that the initial signal of ATP should come from Ad-infected epithelial cells. Although it may be possible that Ad-infected epithelial cells may stimulate macrophages through other pathways, it is believed that release of ATP at a concentration required for the activation of P2X_7R can only occur during a spontaneous cell death. Based on these observations, we proposed a model for the inflammatory response during acute viral infection (Figure 3-18). It suggests that ATP is initially released from the virus-infected epithelial cells, which activates macrophages in the vicinity and induces inflammatory responses including inflammasome activation and IL-1β secretion. However, an extensive exposure to ATP eventually leads to macrophage cell death, which in turn produces more ATP to intensify the inflammation in the area.
Although similar mechanisms have been suggested in other studies, the intercellular interaction has not been clearly demonstrated because of the lack of an appropriate study model. Most of the studies relied on stimulating macrophages in the cell culture by adding a large amount of ATP which would result in spontaneous P2X7R activation but quickly lead to cell death. The Ad infection co-culture model does not require addition of ATP and the analysis of the cellular changes in the culture can reveal inflammatory mechanisms mediated exclusively by the intercellular interaction between the macrophages and epithelial cells. In order to demonstrate the sequence of events that has been proposed in our model the future studies should examine the cellular changes during the process and the changes in the local ATP concentration. These studies would enhance our understanding of how virus-induced inflammation is mediated by dynamic intercellular interactions and elucidate the roles of ATP in regulating the inflammatory responses.

Our study provides evidence that activation of the inflammasome pathway and induction of IL-1β may be critical in the development of acute inflammation during a viral infection. We have shown that ATP is an essential factor that mediates the inflammasome pathway during an acute Ad infection. Despite the rapid accumulation of knowledge on inflammasomes in recent years, the mechanism of inflammasome activation has remained unclear. Because the earlier studies were focused on identifying PAMP sensing molecules, the role of danger signals in inflammasome activation has been mostly overlooked. Recently, the controversy over the mechanism of NLRP3 inflammasome activation has attracted more interest in the role of danger signals. Currently, ATP-P2X7R-mediated K⁺ efflux, generation of ROS, and phagosomal destabilization have been suggested as possible NLRP3 activating mechanisms. These different conclusions were likely drawn due to different experimental systems and it is also possible these volatile cellular conditions happen simultaneously in the cell. As discussed in Chapter 3,
our data indirectly show that ATP-P2X7R signaling might affect ROS production in macrophages during an acute viral infection. Further studies to test the hypothesis that ATP can mediate ROS generation via potassium efflux should help us to resolve the inflammasome activation mechanism.

The end result of inflammasome activation is secretion of active IL-1β and IL-18. As a major pro-inflammatory cytokine, IL-1β promotes the functions of innate immune cells by inducing production of other cytokines. Therefore, release of IL-1β significantly amplifies the inflammatory responses at the local and systemic level. While IL-1β should be beneficial to the host by stimulating the immune system against infections, its over production can cause serious pathological conditions. The mechanism of over-active innate immune responses leading to pathological conditions such as ARDS has not been well-characterized, but it is believed that IL-1β may play an important role. In acute inflammation caused by a viral infection, IL-1 has been shown to be responsible for the immunopathology (Schmitz et al., 2005; Thomas et al., 2009).

Our *in vivo* study results are in line with this view. Although our study utilized replication deficient viral vectors, administration of Ad particles at high dosages caused mortality with symptoms of ARDS in mice. More importantly, disruption of the inflammasome activation pathway by inhibiting the ATP-P2X7R signaling significantly enhanced survival. These results indicate that ATP-mediated inflammasome activation and IL-1β secretion are key mechanisms that lead to immunopathology of acute inflammation during a viral infection. Although this study was mainly focused on the inflammatory responses in an acute viral infection, the results revealed the importance of the ATP signaling, which has broader implications in various other immunopathological conditions. In fact, there is evidence that ATP mediates inflammatory responses in chronic inflammation, such as asthma and chronic obstructive pulmonary disease,
hypersensitivity, graft versus host disease, and sterile inflammation. In the future, investigations should be carried out to explore the role of ATP in the induction of inflammation in other immunopathological conditions.

Although this study is focused on macrophages, studies addressing how ATP affects other types of cells may help us to understand the role of ATP during inflammatory conditions. Most of the immune cells that play a critical role in eliciting inflammatory responses, including neutrophils, DCs, monocytes, and NK cells, are known to express P2X7R at varying degrees (Wiley et al., 2011). Thus, it would be interesting to explore whether ATP exerts the same response in these cells as in macrophages. P2X7R is also present in non-immune cells such as epithelial cells, endothelial cells, fibroblasts, and cells of the nervous system (Burnstock and Knight, 2004). While these cells do not express P2X7R at a significant level under normal conditions, P2X7R expression can be up-regulated during inflammation allowing these cells to contribute to ATP mediated responses. Taken together, future studies on how ATP elicits different responses in various cells and how ATP modulates the interactions between them can broaden our understanding of the complex and dynamic phenomenon of inflammation.
References


