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Interaction of respiratory bacteria and viral ligands with phagocytes and alveolar epithelium: inflammatory response and cell death

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Abbreviations

AEC: Alveolar epithelial cell

AECB: acute exacerbations of chronic bronchitis

AM: Alveolar macrophage

A. phagocytophilum: Anaplasma phagocytophilum

APS: Ammonium persulfate

Bcl-2: B cell lymphoma 2

BEC: Bronchial epithelial cell

BH3: Bcl-2 homology 3

BSA: Bovine serum albumin

Cfu: Colony forming unit

COPD: chronic obstructive pulmonary disease

dsRNA: double-stranded RNA

ECL: Enhanced chemiluminescene

EDTA: Ethylenediamine tetraacetic acid

EGFR: Epidermal growth factor receptor

ELISA: Enzyme-linked immunosorbent assay

EBV: Epstein-Barr virus

ERK: Extracellular signal-regulated kinase

FCS: Fetal calf serum

Fig: Figure

GM-CSF: Granulocyte-macrophage colony-stimulating factor

G-CSF: Granulocyte colony-stimulating factor

h: hour

***H. influenzae*:** Haemophilus influenzae

H_2O_2: Hydrogen peroxide

H_2SO_4: Sulphuric acid

HBEpC: Human Bronchial Epithelial Cells

HCl: Hydrochloric acid

HRP: Horseradish peroxidase

IAV: Influenza A virus

ICAM: Intercellular adhesion molecule

IFN: Interferon

IKK: IkB kinase

IL: Interleukin

IRAK: IL-1 receptor-associated kinase

JNK: c-jun N-terminal kinase

KCl: Potassium dihydrogen phosphate

KH_2PO_4: Potassium dihydrogen phosphate

KO: Knockout

L: Liter

LOS: Lipooligosaccharide

LPS: Lipopolysaccharide

M: Molar

MAPK: Mitogen-activated protein kinase

McF: MacFarland

Mcl -1: Myeloid cell leukemia

$MgCl_2$: Magnesium Chloride

min: Minute

mL: Mililiter

MM: multiple myeloma

MMP: Matrix metalloproteinase

MMP: Mitochondrial membrane permeabilization

MNC: Mononuclear cells

MOMP: Mitochondrial outer membrane permeabilization

mRNA: messenger Riboneucleic acid

MyD88: Myeloid differentiation marker 88

Na_2HPO_4: Sodium hydrogen phosphate

NaCl: Sodium chlorid

$NaHCO_3$: Sodium bicarbonate

NaOH: Sodium hydroxide

NEAA: Nonessential amino acid

NF-κB: Nuclear factor-κB

ng: Nanogram

Hi: Haemophilus influenzae

OMP: Outer membrane proteins

PAMP: Pathogen-associated molecular patterns

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

pg: Picogram

PI3-K: Phosphatidylinositol 3-kinase

PKR: Protein kinase R

PMN: Polymorphonuclear leukocyte

poly(I:C): polyinosinic acid : cytidylic acid

PRR: Pattern recognition receptor

PVA: Polyvinyl alcohol

rpm: revolutions per minute

RSV: Respiratory syncytial virus

RV: Rhinoviruses

s: second

SAEC: Small airway epithelial cells

SAPKs: Stress-activated protein kinases

SARS-CoV: Severe acute respiratory syndrome coronavirus

SDS: Sodium dodecyl sulfate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SEM: Standard error of mean

ssRNA: single-stranded RNA

S. pneumoniae: *Streptococcus pneumoniae*

***S. aureus*:** *Staphylococcus aureus*

TBS: Tris buffered saline

TEMED: Tetramethylethylenediamined

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor-α

TRAF: Tumor necrosis factor receptor-associated factor-α

Tris-Cl: Tris-chlorine

T-TBS: Tween-tris buffered saline

ug: Microgram

uM: Micromolar

v/v: volume per volume

v: volt

w/v: weight per volume

WT: Wild-type

y: year

Introduction

Although most respiratory infections are associated with a single bacterial pathogen, a growing body of evidence suggests that a significant proportion of patients diagnosed with this disease have mixed infections of bacteria and virus (Thorburn et al.,2006). Moreover, inappropriate antibiotic treatment contributes to the worldwide emergence of antibiotic-resistant strains and leads to increased incidence of polymicrobial infections.

Substantial evidence implicates common respiratory viral infections in the pathogenesis of asthma and chronic obstructive pulmonary disease(COPD). Respiratory viral infections are a major trigger for acute exacerbations of both asthma and COPD (Proud and Chow.,2006). Importantly, viral infection may impact on exacerbation severity indirectly by increasing bacterial load in addition to the direct effects of viral infection itself(Wilkinson et al.,2006). The clinical severity and inflammatory responses in COPD exacerbations can be modulated by the nature of the infecting organism, bacterial and viral pathogens interact to cause additional rises in inflammatory markers and greater exacerbation severity (Wilkinson et al.,2006). Papi et al performed a prospective controlled study concluding that the presence of coinfection with both viruses and bacteria was found in 25% of exacerbations in COPD patients and that these patients had more severe functional impairment and longer hospitalization (Papi et al.,2006). Changes were more severe when evidence for both bacterial and viral infection was present. These exacerbations were associated with higher bacterial loads which may suggest a synergistic interactive effect of viral infection which allows greater proliferation of airway bacteria.

1.1 Host defense against infection

1.1.1 Respiratory epithelial cells and infection

In the host innate immune system, the surface epithelial cells are situated at host/environment boundaries and thus act as the first line of host defense against

pathogenic bacteria and viruses. Bronchial epithelial cells (BEC) are equipped with several means of defense against invading pathogens (Boyton et al.,2002). BECs build up a tight physical barrier and are embedded in a mucus layer that hinders pathogens from penetrating the body. Another method of defense used by BECs is their ability to use mucociliary clearance to entrap and remove microorganisms. The bronchial epithelium also produces antimicrobial substances, including lysozyme, lactoferrin, collectins, and antimicrobial peptides, which provide a further protective cover. Despite these functions, epithelial cells can recognize invading pathogens by directly interacting with pathogen-associated molecular patterns(PAMPs) on a variety of pathogens via Toll-like receptors (TLRs) expressed on host cells, which leads to the inducible secretion of further mediators, including chemokines to orchestrate a local inflammatory response (Iwamura and Nakayama et al.,2008). Experiments demonstrate that BECs express functional TLR1–6 and TLR9 and thus make use of a common principle of professional innate immune cells. It regulates its sensitivity to recognize microbes by managing receptor expression levels (Mayer et al.,2007).

1.1.2 Proinflammatory cytokines and infection

Cytokine production is an important effector function of host cells. Intensity of proinflammatory cytokine production correlates with the severity of symptoms in infectious diseases. In particular, IL-8, IL-6 and TNF-α are key proinflammatory cytokines, which are produced early in infection（Khair et al.,1996). TNF-α is a potent inflammatory cytokine implicated in the pathogenesis of asthma and COPD. Significantly higher levels of IL-8, TNF-α and neutrophil elastase in sputum were seen in exacerbations associated with Hi when compared with nonbacterial exacerbations in the same patients (Sethi et al.,2000). One study provides further evidence for a role of TNF-α in severe asthma, anti-TNF treatment may be a new treatment in subjects with severe asthma (Howarth et al.,2005). Bresser et al measured sputum levels of TNF-α and IL-8 in chronic bronchitis patients who were chronically infected with Hi. Sputum levels of these inflammatory mediators were significantly higher in the Hi-infected group compared with noninfected patients (Bresser et al.,2000). IL-6 has also been implicated in the pathogenesis of asthma

and COPD exacerbations. Another study showed that COPD patients who have frequent exacerbations have increased airway cytokine levels when stable which rise further during an exacerbation, especially in the case of IL-6. Measurement of the cytokines IL-6 and IL-8 in sputum can determine which COPD patients are susceptible to frequent exacerbations (Bhowmik et al.,2000). Stankiewicz et al found that the concentrations of IL-8, IL-6 and TNF-α were considerably higher in bronchial asthma patients than in COPD patients (Stankiewicz et al.2002).

1.1.2 Toll-like receptors (TLRs) and infection

The innate immune system is crucial to our day-to-day survival and defense against infection. To date, 10 human TLRs have been described, they recognize distinct microbial components and are involved in the inflammatory and immune responses (Akira et al.,2006). TLRs are broadly expressed by many cells, including epithelial cells, mast cells, macrophages, fibroblasts, and airway smooth muscle. While there is variation between cell types in the panel of TLRs expressed in vivo, it is likely that there is cooperative signaling between cells of the immune system and tissue cells to enable an effective immune response to pathogens (Morris et al.,2006).

TLRs can be broadly classified as having antibacterial or antiviral responses. TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6 principally serve antibacterial roles, through the detection of Gram-negative Lipopolysaccharide (LPS) (TLR-4) and Gram-positive lipoproteins (TLR-2, acting as a heterodimer with TLR-1 or TLR-6). TLR-5 recognizes flagellin. TLR-3, TLR-7 and TLR-8 respond RNA (double stranded for TLR-3, single stranded for TLR-7 and TLR-8) and have been implicated in antiviral responses (Alexopoulou et al., 2001; Heil et al.,2004). TLR-9 is principally antibacterial, although it has clearly some ability to recognize viral DNA and may play a role in a broader range of bacterial and viral infections (Wagner et al.,2004). In response to ligand binding, TLR signaling mainly through Myeloid differentiation marker 88 (MyD88) leads to activation of nuclear factor (NF)-κB and Mitogen-activated protein kinases (MAPK), resulting in inflammatory cytokine release and recruitment of cells required for host defense (Akira

and Takeda., 2004).

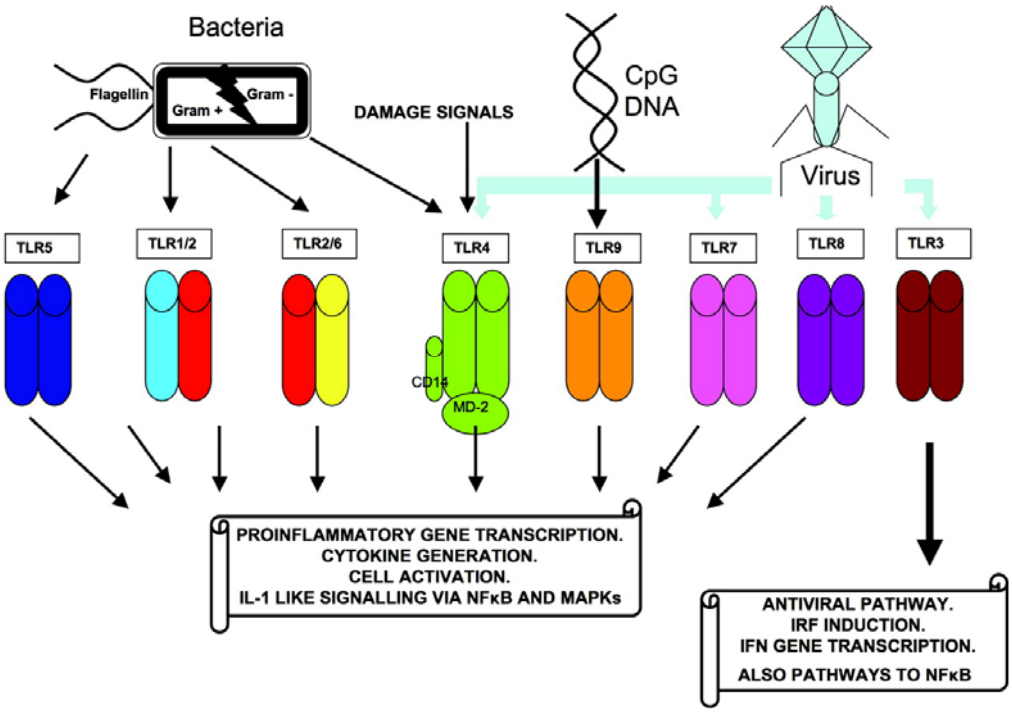


Fig.1: TLRs respond to a broad range of agonists. TLRs can be broadly classified into those that exhibit antibacterial and antiviral responses. TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6 principally (although not exclusively) serve antibacterial roles through the detection of Gram-negative and -positive bacteria. TLR-3, TLR-7, and TLR-8 respond to RNA and have been implicated in antiviral responses. TLR-9 distinguishes between human and nonhuman DNA through recognition of bacterial CpG DNA. Agonist binding and activation of TLRs result in a complex series of protein-protein interactions ultimately leading to cellular activation responses, the activation of transcription factors such as NF-κB and MAPKs, and the production of type I IFNs (in the case of antiviral responses) (Chaudhuri et al.,2007).

1.1.3 TLRs and Nontypeable Haemophilus influenzae (Hi)

Viral and bacterial pathogens cause inflammation via TLR signaling. Different bacteria use different sets of TLRs and each bacterium can activate several TLRs. Some observations suggest that several TLRs may cooperate in activating an inflammatory response in a synergistic manner (Mogensen et al.,2006).

Hi is a nonencapsulated gram-negative pleomorphic rod-shaped bacterium which colonizes the upper respiratory tract of the majority of individuals. It is the most commonly recovered bacterium during acute exacerbations of chronic bronchitis (AECB) (Sethi et al.,

2002). TLR2 is involved in the recognition of a wide array of bacterial products, including peptidoglycan, lipopeptides, zymosan, and bacterial lipoproteins, whereas TLR4 is activated by LPS. LPS has been clearly documented to play a central role in the pathogenesis of gram-negative infections. Additional evidence in knockout (KO) and mutant mice has shown that the MyD88-dependent pathway of TLR4 is important for an effective innate immune response to respiratory tract infection caused by Hi (Wieland et al.,2005). Recent studies demonstrated that TLR2 also plays a key role in activating host immune and inflammatory response against the Hi. There is significant evidence that other components of Hi, including porins, can induce cytokine production mediated by the TLR2/MyD88 pathway (Galdiero et al.,2004). Some results indicate that some changes in the lipooligosaccharide(LOS) subunit of Hi can favor signaling through non-TLR4 receptors, such as TLR2. These results also indicate a close interaction between TLR4 and TLR2 that tightly regulates the expression of both receptors (Lorenz et al.,2005). Distinct and overlapping sets of TLRs are used to mount the inflammatory response.

1.1.4 TLRs and virus

The invasion of viruses is initially sensed by the host innate immune system, triggering rapid antiviral responses that involve the release of proinflammatory cytokines, and leading to the subsequent activation of adaptive immune responses. Diverse components of infecting viruses can induce the signalling pathways that regulate the cellular antiviral gene program. Among them, double-stranded RNA (dsRNA) has been viewed as the most important component. It is a common signature linked to the viral replication cycle and lysis of virus-infected cells is hypothesized to release dsRNA. A major transducer of cell signalling generated by dsRNA is the TLR3 (Alexopoulou.,2001). Several studies established that TLR3 does have an important function in response to a common human viral infection of its natural host cell. Rhinoviruses (RV) can induce TLR3 messenger Ribonucleic acid (mRNA) and surface protein expression. TLR3 plays an important role in innate immune responses against RV infection (Hewson et al.,2005). Another study demonstrated the importance of dsRNA in the induction of pro-inflammatory cytokines by RV in BECs. Their data suggest a role for both TLR3 and Protein kinase R (PKR) in

dsRNA induced pro-inflammatory cytokine induction and possible overlap between TLR3 and PKR pathways (Edwards et al.,2007). TLR3 also mediates inflammatory cytokine and chemokine production in Respiratory syncytial virus(RSV)-infected epithelial cells（Rudd et al.,2005). A study showed that TLR3 plays a key role in the expression of proinflammatory cytokines in epithelial cells infected by Influenza A virus (IAV) (Le Goffic et al.,2007). TLR3 is constitutively expressed in distinct human alveolar and bronchial epithelial cells and plays a key role in the immune response of respiratory epithelial cells to both dsRNA and IAV (Guillot et al.,2005). However, other studies showed for the first time that TLR3-IAV interaction critically contributes to the debilitating effects of a detrimental host inflammatory response (Le Goffic et al.,2006). The functional role of TLR3 *in vivo* still remains unclear.

In contrast, how additional virus component receptors such as TLR7 or TLR8 are regulated remains largely unknown. TLR7 and TLR8 have been identified as receptors for single-stranded RNA (ssRNA) and the imidazoquin-like molecule resiquimod R-848 (Jurk et al.,2002; Diebold et al.,2004). Martin et al indicate that Epstein-Barr virus(EBV) initially uses TLR7 signaling to enhance B-cell proliferation (Martin et al.,2007). Another study demonstrated that the eosinophil uses TLR-dependent pathways, in particular TLR-7, to promote host defense against RSV (Phipps et al.,2007). Moreover, Chuang and Ulevitch detected expression of TLR7 and TLR8 in lung tissue, implying a potential role for TLR7/8 in host antiviral response to respiratory pathogens (Chuang and Ulevitch.,2000).

1.2 Interaction between Bacteria and virus

1.2.1 Virus - Bacteria

Respiratory viruses frequently promote bacterial opportunistic infections, through complex mechanisms that still deserve to be investigated in detail. RV infection promotes infections with facultative pathogens in the lower airways such as *Staphylococcus aureus*, possible mechanisms involving the virus induced release of cytokines (Interleukin(IL)-6 and IL-8),

and the overexpression of intercellular adhesion molecule (ICAM)-1 (Passariello et al.,2006). Recent data indicate that Interferon (IFN)-β, which is produced by virally infected respiratory epithelial cells, converts normally innocuous Hi outer membrane proteins (OMP) into potent inflammatory stimulants for the production of inflammatory mediators by alveolar macrophages (Punturieri et al.,2006). A mouse model of bacterial superinfection in which a mild, self-limiting influenza virus infection is followed by mild, superinfection with *Streptococcus pneumoniae (S. pneumoniae*) was used to demonstrate that *S. pneumoniae* superinfection results in rapid dissemination of the bacterium from the respiratory tract and systemic spread to all major organs, resulting in fatal septicemia (Speshock et al.,2007). Granulocyte colony-stimulating factor (G-CSF) is a major contributor to synergistic exacerbation of disease leading to fatal septicemia (Speshock et al.,2007). Another study indicated that both suppression of neutrophil function and neutrophil-independent mechanisms such as enhanced cytokine production are responsible for increased susceptibility to a secondary *S. pneumoniae* infection after an influenza infection (McNamee and Harmsen et al., 2006).

Recently it was shown that sustained desensitization of lung sentinel cells to TLR lasts for several months after resolution of influenza or RSV infection. The consequence of attenuated TLR responsiveness may be susceptibility to secondary bacterial pneumonia (Didierlaurent et al.,2008). To determine the role of TLR2 in the host response to postinfluenza pneumococcal pneumonia TLR2 KO and wild-type (WT) mice were infected intranasally with influenza A virus. Fourteen days later they were administered with *S. pneumoniae* intranasally. The results indicate that TLR2 does not contribute to host defense during murine postinfluenza pneumococcal pneumonia (Dessing et al.,2008). Taken together viral infection may alter the immunologic environment by a number of mechanisms that allow either proliferation of colonizing airway bacteria or a new pathogen to infect the lower airways.

1.2.2 Bacteria-Virus

Despite the relatively well-known role of viral infections in promoting bacterial infections,

it is still not clear whether bacterial infection also promotes viral infection in polymicrobial infections. Newcomb et al conclude that RV infection and Tumor necrosis factor-α (TNF-α) stimulation induce cooperative increases in epithelial cell chemokine expression (Newcomb et al.,2007). TNF-α stimulation increases expression of ICAM-1 in airway epithelial cells. ICAM-1 serves as a receptor for the major subtype RV (90%) (Greve et al.,1989). Hi is an important respiratory pathogen. It initiates infection by adhering to the airway epithelium. Adherence of Hi to epithelial cells increases ICAM-1 expression by respiratory epithelial cells (Avadhanula et al.,2006). A recent study provided evidence that Hi enhances host antiviral responses via TLR2-dependent up-regulation of TLR7 expression in human airway epithelial cells in vitro and mouse lung tissue in vivo. Moreover, Hi induces TLR7 expression via a MyD88- IL-1 receptor-associated kinase (IRAK)- Tumor necrosis factor receptor-associated factor-α(TRAF)6- IκB kinase(IKK)-NF-κB-dependent mechanism (Sakai et al.,2007). Another study found that infection of airway epithelial cells with Hi increases expression of the RV receptors ICAM-1 and TLR3, leading to increased RV binding and exaggerated RV-induced chemokine responses (Sajjan et al.,2006). Together, these data provide a mechanism for increased susceptibility to RV infection in COPD patients who are also infected with Hi.

Bacterial and viral infections cause various cellular responses, including the expression of inflammatory mediators, cytokines and apoptosis. There is growing evidence that apoptosis plays an important role in modulating the pathogenesis of a variety of infectious diseases.

1.3 Infection and Apoptosis

Apoptosis, or programmed cell death, is essential for normal development and homeostasis. Insufficient apoptosis may contribute to the pathogenesis of malignancy and acute and chronic inflammation. According to the classical scheme, apoptosis can be induced by two main pathways. The extrinsic pathway is initiated by binding of death ligands, such as Fas or TNF, to their corresponding receptors (Sharma et al.,2000). Upon complex formation

between ligand and receptor, the signal for the induction of apoptosis is transmitted to the cell interior. In the intrinsic pathway, apoptosis is triggered by internal signals leading to permeabilization of the outer mitochondrial membrane, which causes the release of cytochrome c into the cytoplasm (Lee and Kleiboeker..2007). However, there is crosstalk between the mitochondrial and the death receptor-mediated pathways.

1.3.1 Bacterial infection and apoptosis

Bacterial infection is a potent stimulus for apoptosis. Apoptosis can be regulated by bacteria and their products, some bacteria have evolved mechanisms that can perturb these processes (DeLeo,2004). For example, *Anaplasma phagocytophilum* can evade phagocytic killing and delay neutrophil apoptosis, allowing the bacterium to replicate within the neutrophil and be dispersed throughout the body (Ge et al.,2005). Other bacteria such as *Pseudomonas aeroginosa* produce pyocyanin to suppress the acute inflammatory response by pathogen-driven acceleration of neutrophil apoptosis (Allen et al.,2005). In contrast some bacteria such as *Chlamydia pneumoniae* can multiply in neutrophil granulocytes and delay their spontaneous apoptosis (van Zandbergen et al.,2004). Hi are commonly found as commensal organisms in the upper respiratory tract of adults. Colonizing Hi produces significant airway inflammation, which is increased during exacerbations (Chin et al.,2005). Inflammatory activation by cytokines, adhesion, and transmigration can all delay neutrophil apoptosis and extend their lifespan to allow them to perform their function in inflammation (Edwards et al.,2003). Thus, neutrophils recruited into tissues exhibit prolonged survival (Droemann, Chest 2000) and generally undergo apoptosis when their function is complete. Naylor et al show that rather than killing the bacteria, the neutrophils themselves rapidly undergo cell death and release their granule contents into the extracellular environment after Hi infection which may result in the further infiltration of neutrophils into the lungs, thereby amplifying the inflammatory response (Naylor et al.,2007).

Identifying the mechanisms by which bacteria such as these regulate host-cell apoptosis and necrosis will shed new insights into the molecular processes that control these events.

This may help to a better understanding of disease pathology and could lead to new anti-microbial treatment strategies.

1.3.2 Viral infection and apoptosis

Host cell apoptosis is a commonly employed immune defense mechanism against viral infection, and many viruses potently induce or suppress cell death during infection. Incubation of granulocytes with RSV leads to a delay in the constitutive apoptotic program. The molecular mechanism is dependent on both phosphatidylinositol 3-kinase (PI3-K) and NF-κB activation (Lindemans et al.,2006). A recent study showed that, following RV infection, several signs of apoptosis could be detected in bronchial epithelium cells contributing to the destabilization of the cell and facilitating viral progeny release (Deszcz et al.,2005). Both apoptosis and necrosis have been observed in cells infected by severe acute respiratory syndrome coronavirus (SARS-CoV) and other coronaviruses, suggesting that the regulation of cell death is important for virus replication and/or pathogenesis (Tan et al.,2007).

Mechanisms of induction and/or suppression of apoptosis during virus infection have been discussed in detail in several recent review articles (Iannello et al.,2006; McLean et al.,2008). Viruses encode various proteins to modulate apoptosis to their own advantage (such as p53, E1B-19K, BHRF1 et al). They inhibit premature apoptosis of the virus-infected cells (before replication of the virus has occurred). After completion of the viral replication, viruses may promote apoptosis to disseminate progeny virus without causing inflammatory responses. Viral antiapoptotic strategies also help the virus evade CTL and NK cell-mediated killing of the virus-infected cells (Iannello et al.,2006).

1.3.3 Bcl-2 family and apoptosis

B cell lymphoma 2 (Bcl-2) family members are critical regulators of apoptosis in many cell types. This family can be divided into two groups, antiapoptotic proteins (such as Bcl-2, Bcl-XL, Mcl-1, and A1/Bfl-1) and proapoptotic proteins (such as Bax, Bad, Bak, Bik, and Bid) (van Delft and Huang.,2006; Gustafsson and Gottlieb et al.,2007). The relative

abundance of these proteins is thought to control the commitment of a cell to apoptosis or survival.

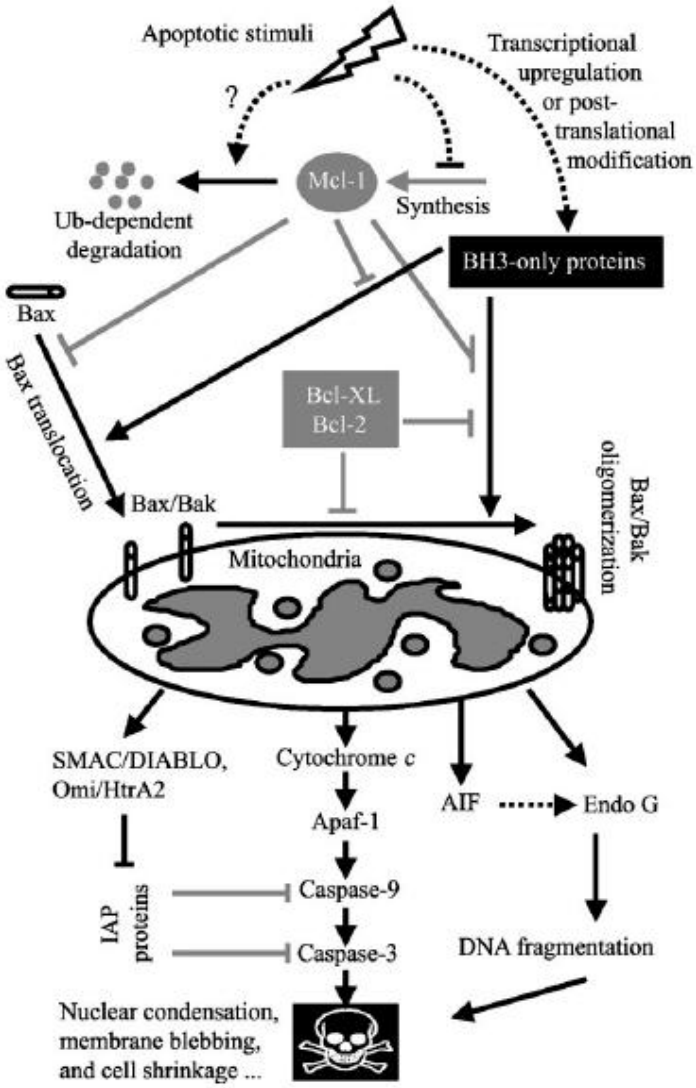


Fig.2: Regulation of the mitochondrial apoptosis pathway by the Bcl-2 family members (modified according to Jiang and Wang, 2005).

The antiapoptotic Bcl-2 family plays critical roles in regulating apoptosis in immune cells (Marsden and Strasser,2003; Zhang et al.,2005). They exert their effect primarily by maintaining mitochondrial homeostasis and inhibiting mitochondrial outer membrane permeabilization (MOMP) (Wong and Puthalakath.,2008). Among the proapoptotic members, Bak and Bax have been categorized as the last gateway of cytochrome c release, and their homooligomerization on the mitochondrial membrane is essential for release.

Homology domain 3 (BH3)-only proteins function primarily as antagonists to neutralize all protective antiapoptotic proteins to allow Bak/Bax to induce mitochondrial disruption (Uren et al.,2007; Willis et al.,2007). Myeloid cell leukemia (Mcl)-1 is a quick- turnover protein, and it can be degraded by the ubiquitination- proteasome pathway. Loss of Mcl-1 is an apical event in the apoptotic cascade. When cells are treated with various proapoptotic signals, Mcl-1 protein levels decrease dramatically, due to a blockage of its synthesis as well as acceleration of its degradation. Mcl-1 disappearance is a prerequisite for downstream apoptotic events, such as Bcl-XL inactivation, Bim dephosphorylation, Bax translocation, and Bax/Bak oligomerization. Once activated, Bax and Bak mediate permeability of the mitochondrial outer membrane, releasing proapoptotic factors, particularly cytochrome c, that provoke caspase activation and subsequent cell death (Green et al.,2005).

1.3.4 Mcl-1 and apoptosis

The molecular basis of cell resistance to apoptosis includes expression of antiapoptotic Bcl-2 family members. Mcl-1 is an unusual antiapoptotic member of the Bcl-2 family, with some unique properties that distinguish it from other family members. It is a much larger protein than other family members (40/42 kDa), and its N-terminal region contains PEST sequences and other motifs that target it for proteolysis by the proteasome (Akgul et al.,2000). It has a very short half-life, which is due to the fact that it has a large N-terminal domain which plays a major regulatory role in the function of Mcl-1 (Germain and Duronio.,2007). Motifs within this N-terminal domain are targets for posttranslational modifications and give the molecule the unique property to be acutely up- or down-regulated within minutes when the cell has received a particular stimulus. Mcl-1 can be tightly regulated both transcriptionally and through proteasomal degradation (Michels et al.,2005). Indeed, the half-life of the mature protein is estimated to be between 2 and 3 h, depending upon the cell type and culture conditions (Moulding et al.,2001). Mcl-1 is thus a rapidly induced survival protein that is subject to rapid turnover. It contributes to the control of mitochondrial integrity, which is critical for maintaining cell viability. These properties make it ideally suited to dynamic regulation of host-cell survival and could

allow the rapid changes in cell viability required during infection.

There is now much evidence demonstrating the central role of Mcl-1 in regulating neutrophil and other cells survival. Mcl-1 plays an essential role in neutrophil survival. Several studies have demonstrated that various stimuli that promote or inhibit neutrophil apoptosis can modulate Mcl-1 expression in these cells (Derouet et al., 2004; Derouet et al.,2006). Lymphocytes lacking Mcl-1 expression undergo apoptosis and exhibit defective differentiation (Opferman et al.,2003). In addition, Mcl-1 may also be essential for macrophage survival. Inhibition of Mcl-1 expression by antisense oligonucleotides in human monocyte-differentiated macrophages resulted in apoptosis (Liu et al.,2001). Marriott HM et al indicate that levels of Mcl-1 expression contribute to the loss of macrophage viability and bacterial clearance after pneumococcal infection (Marriott et al.,2005). These results suggest that Mcl-1 may play essential roles in the survival of a wide range of host cells.

1.4 MAP kinases

MAPK pathways provide a mechanism to regulate cell proliferation, differentiation, motility, survival and apoptosis. In mammalian cells, there are at least three major subfamilies that have been identified: c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), extracellular signal-related kinases (p44/42 MAPK; ERK1/2), and p38 MAPK. The ERK cascade appears to mediate signals promoting cell proliferation, differentiation, or survival. The p38 MAPK and JNK cascades appear to be involved in the cell responses to stresses (Roux and Blenis.,2004). Activation of JNK and p38 can also promote apoptosis in many systems (Xia et al.,1995). The exact function of MAPKs on cellular survival and apoptosis are complex. Therefore, the dynamic balance between growth factor- activated ERK and stress-activated JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis.

1.4.1 p38MAPK and apoptosis

p38 MAPK, a MAPK family member, is phosphorylated and activated by cellular stress and inflammatory stimuli, and its physiologic role seems to involve the regulation of important cellular responses, such as apoptosis and inflammation (Aoshiba et al.,1999).

p38 MAPK can promote either cellular survival or apoptosis (Roux and Blenis et al., 2004). For example, p38 MAPK contributes to neutrophil survival, pharmacological inhibition of p38MAPK activity augmented both spontaneous and Fas-mediated apoptosis (Alvarado-Kristensson et al.,2002). Similarly, another study showed that p38 MAPK plays an important role in the delayed apoptosis of *Anaplasma phagocytophilum* - infected neutrophils (Choi et al.,2005). Zhang et al showed that MAPK and NF-κB pathways function together to up-regulate apoptosis inhibitor gene expression in macrophages in response to *Yersinia* infection. Inhibition of p38 and JNK activity, in addition to inhibition of NF-κB activation, is important for rapid *Yersinia*-induced macrophage apoptosis (Zhang et al.,2005). Recent data showed that glucocorticoid- mediated enhancement of Mcl-1 levels and survival were significantly suppressed by pharmacologic inhibition of p38 MAPK or PI3-K. These data indicate that PI3-K and p38 MAPK are protein kinases that regulate the antiapoptotic effect of glucocorticoids on human neutrophils (Saffar et al.,2008). In contrast, pneumococci strongly induced apoptosis of human endothelial cells, p38 MAPK and JNK were activated in pneumococci-infected cells and inhibitors of both kinases strongly reduced pneumococci-induced caspase activation and apoptosis (N'Guessan et al.,2005). *Mycobacterium tuberculosis* signals via TLR2, leading to the activation of the p38 MAPK pathway, which in turn can induce neutrophil activation and apoptosis (Alemán et al., 2005). Thus, it appears that cell type and stimulus have a powerful influence on the role of p38 MAPK on cell life or cell death.

1.4.2 p44/42MAPK or extracellular signal-related kinase (ERK) and apoptosis

The p44/42 MAPK in addition to p38 MAPK and JNK is one of three parallel serine/threonine kinase modules that regulate diverse cellular processes, including cell proliferation, differentiation, and apoptosis (Fan and Chambers.,2001). Activated ERK is

well-known for its role in cytoprotection and cell survival. Leptin promotes survival of blood monocytes by preventing the triggering of the apoptosis process. This effect is mediated by the p44/42 MAPK pathway (Najib and Sánchez-Margalet., 2002). RSV activates epidermal growth factor receptor (EGFR) in lung epithelial cells. Activation of EGFR results in increased ERK activity, contributing to both the inflammatory response (IL-8 release) and prolonging the survival of RSV-infected cells (Monick et al.,2005). ERK triggers survival signaling through multiple mechanisms, including transcriptional regulation and phosphorylation of pro- or antiapoptotic proteins (Ballif and Blenis.,2001). Activated ERK leads to downregulation of the proapoptotic BH3-only proteins Bim and Bad. Inhibiting ERK activation attenuated the ability of the bacteria to induce cytoprotection (Howie et al.,2008). Phosphorylation of ERK and Akt also increases the levels of the antiapoptotic protein Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells (Petlickovski et al.,2005). The apoptotic pathway itself is complex and may prove to be a target for future drug therapies. These findings will hopefully reveal novel therapeutic targets and approaches that can improve the survival of the critically ill patient with severe infections (Wesche-Soldato et al.,2007).

1.5 Aims of the current study

Over the past two decades, tremendous efforts have been made towards understanding the host response to bacteria and viruses. Most studies, however, have focused on investigating bacteria-induced antibacterial response or virus-induced antiviral response. Given that under *in vivo* conditions such as polymicrobial infections bronchial epithelial surfaces are often exposed to multiple pathogens including bacteria and viruses, the interactions between bacteria and viruses are still unclear and the mechanisms that underlie the heterogeneity of exacerbations are poorly understood. We used the synthetic TLR3 ligand polyinosinic acid : cytidylic acid (poly(I:C)) and the TLR7/8 ligand CL097 to model stimulation by virus infection. Moreover, we addressed the interaction between Hi and virus ligands in inflammatory response and apoptosis/necrosis of host cells.

2. Materials and Methods

2.1 Matetials

2.1.1 Chemicals and Kits

All chemicals and reagents were used in analytic degree of purity.

Acetic acid ……. Merck, Darmstadt,Germany

Acrylamide(30%)/Bisacrylamid(0.8%)……. ….Bio-Rad, Munich,Germany

Agarose ……..…..Invitrogen, Karlsruhe, Germany

Amino Acids ……..…..……PAA, Pasching, Austria

Ammonium persulfate(APS)……..…..……Sigma, Steinheim,Germany

Amphotericin-B ……..…..…… .PAA, Pasching, Austria

Bovine serum albumin(BSA)……...…...Sigma, Steinheim,Germany

Bromophenol blue ……...…...Bio-Rad, Muenichen,Germany

Bronchial/Tracheal epithelial cell

Serum-free growth medium…….…..…..ECACC, Salisbury, UK

Faramount mounting medium…..…….……...Dako, Hamburg, Germany

Hank's BSS …….…..…..…….PAA, Pasching, Austria

HOPE-solution ……..……..……DCS, Hamburg, Germany

Hydrochloric acid(HCl) …….……..……..…..Merck, Darmstadt, Germany

Hydrogen peroxide(H_2O_2) …….……..……...R&D, Minneapolis, USA

Isopropanol…….…..……...Merck, Darmstadt, Germany

L-Glutamin……..……...PAA, Pasching, Austria

Lymphocyte separation medium……..……..PAA, Pasching, Austria

May-Grünwald solution……..Merck, Darmstadt, Germany

Methanol …….…..…….Merck, Darmstadt, Germany

Milk powder(non-fat) ……..…….Frema, Lueneburg, Germany

Mounting fluid……..…… Dakocytomation,Glostrup,Demmark

Nonessential amino acid(NEAA) ……...……..PAA, Pasching, Austria

Paraformaldehyd……..……..Merck, Darmstadt, Germany

Polyvinyl Alcohol(PVA) ……..……..Merck, Darmstadt, Germany

Potassium chloride(KCl) ……..……..Merck, Darmstadt, Germany

Potassium dihydrogen phosphate(KH_2PO_4) ….……Merck, Darmstadt, Germany

RPMI 1640 ……..……...Biochrom, Berlin, Germany

Sodium bicarbonate($NaHCO_3$) ……..…….Merck, Darmstadt, Germany

Sodium chlorid (NaCl)……...Merck, Darmstadt, Germany

Sodium dodecyl sulphate(SDS)……..…Bio-Rad, Muenichen,Germany

Sodium hydrogen phosphate(Na_2HPO_4) ……...Merck, Darmstadt, Germany

Sodium hydroxide(NaOH) ……Merck, Darmstadt, Germany

Substrate Reagent Pack……...R&D, Minneapolis, USA

Sulphuric acid(H_2SO_4) ……..….Merck, Darmstadt, Germany

SYTO-16 ……Invitrogen, Oregon, USA

Tetramethybenzidine……...R&D, Minneapolis, USA

Tetramethylethylenediamined(TEMED)……..…..Sigma, Steinheim,Germany

Tris-aminomethan……..….Bio-Rad, Muenichen,Germany

Tris-chlorine(Tris-Cl) ……..…...Sigma, Steinheim, Germany

Trypan blue ……..…… Sigma, Steinheim, Germany

Trypsin inhibitor……..…..Sigma, Steinheim, Germany

Trypsin/ Ethylenediamine tetraacetic acid (EDTA)……..PAA, Pasching, Austria

Tween-20……..…..Merck, Darmstadt, Germany

2.1.2 Solutions and buffers

1 x PBS for ELISA**:** 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.2-7.4

1 x PBS for FACS**:** 100ml 10 x DPBS in 1L dH_2O

1 x T-TBS**:** 100ml 10 x TBS, 1 ml Tween-20 in 1L dH_2O

10 x TBS**:** 80g NaCl, 24.2g Tris-aminomethan in 1L dH_2O (PH 7.4)

10%APS**:** 0.1g APS in 1mL dH_2O

10%SDS**:** 0.1g SDS in 1mL dH_2O

5 x SDS-PAGE running buffer**:** 0.125 M Tris base, 0.96 M glycine, 0.5% SDS

Blocking buffer for ELISA**:** 0.1% BSA, 0.05% Tween-20 in 1 x TBS (20mM Trizma-aminomethan, 150 mM NaCl), pH 7.2-7.4, 0.2 uM filtered (for IL-8)**;** 1% BSA in 1 x PBS, 0.2 uM filtered (for IL-6 and TNF-α)

Blocking buffer for immunoblot**:** 5g non-fat dried milk in 100ml T-TBS

Blotting buffer**:** 25mM Tris-aminomethan, 193 mM glycine, 20% methanol

Protein lysis buffer**:** 4% w/v SDS, 10mM dithiothreitol, 20 % v/v glycerol, 0.125 M Tris-Cl(PH7.8), 0.4% bromophenol blue

Running gel buffer**:** 1.5 M Tris-Cl (PH 8.8)

Stacking gel buffer**:** 0.5M Tris-Cl (PH 6.8)

Stop solution**:** 1M H_2SO_4

Substrate solution**:** 1:1 mixture of Color Reagent A (Stabilized Peroxide Solution) and Color Reagent B (Stabilized Chromogen Solution)

Wash buffer**:** 0.05% Tween-20 in 1 x PBS, 0.2 uM filtered

2.1.3 Bacterial strain and cell line

Haemophilus influenzae (Hi), originally a clinical isolate from the blood of a patient with community acquired pneumonia, was used in this study. Hi was grown on chocolate agar plates at 37°C with 5% (vol/vol) CO_2 and taken from fresh overnight culture plates of chocolate-agar and suspended in sterile NaCl-Solution.

Alveolar epithelial cell (AEC) line A549 is obtained from European Collection of Cell Cultures.

Human bronchial epithelial (HBEp) cells is obtained from European Collection of Cell Cultures.

2.1.4 Antibodies, Ligand, inhibitors and their characteristics

Anti-phospho-p38MAPK (rabbit monoclonal antibody) is from Cell Signaling Technology, Beverly, USA and used at 1:1000 dilution.

Anti-phospho-p44/42 MAPK (rabbit monoclonal antibody) is from Cell Signaling Technology, Beverly, USA and used at 1:1000 dilution.

Anti-β-actin (rabbit monoclonal antibody) is from Cell Signaling Technology, Beverly, USA and used at 1:1000 dilution.

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody is from Cell Signaling Technology, Beverly, USA and used at 1:4000 dilution.

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody is from Cell Signaling Technology, Beverly, USA and used at 1:4000 dilution.

Imidazoquinoline Compound (CL097, TLR7/8 ligand) is from Invivogen, San Diego, USA and used at 1ug/ml.

Polyinosinic-Polycytidylic acid (Poly(I:C), TLR3 ligand) is from SIGMA, Missouri, USA and used at 25ug/ml.

Prestained protein marker is from New England biolabs, Beverly MA, USA, used at 7ul.

Purified mouse anti-human Mcl-1 monoclonal antibody is from BD Biosciences Pharmingen, Heidelberg, Germany and used at 1:500 dilution.

SB203580(p38 MAPK inhibitor) is from Calbiochem, CA, USA and used at 20 uM.

U0126 (p44/42 MAPK inhibitor) is from Calbiochem, CA, USA and used at 10 uM.

2.1.5 Instruments and eauipments

12-well cell culture plate ……..Greiner, Frickenhausen,Germany

6-well cell culture plate ……Greiner, Frickenhausen, Germany

Biological safety cabinets ……..Nuaire, Sarstedt, Germany

Cell counter AC-8 ……Assistant,Frankfurt,Germany

Cell culture flask ……...Greiner,Frickenhausen,Germany

Cell house 200 ……Heto,Allerod,Danmark

Centrifuge Rotina 35 ……..Hettich,Tuttlingen,Germany

Combitip plus ……Eppendorf,Hamburg,Germany

Cytocentrifuge Cytospin II ……Shandon,Frankfurt,Germany

Densimat…….....Bio-Merieux, Marcy-l'Etoile, France

ELISA plate ……Nalge-Nunc,Hereford,UK

Eppendorf pipette ……...Eppendorf, Hamburg,Germany

Hot Plate Magnetic Stirrer ……..IKAMAG, Staufen, Germany

Inverted phase contrast microscope CK-2……Olympus, Tokyo, Japan

Light microscope ……Carl Zeiss,Frankfurt,Germany

Microplate Reader Model 680 ……Bio-Rad,Munich,Germany

Mini-Proteam II Electrophoresis Cell …….Bio-Rad,Munich,Germany

Nitrocellulose membrane ……Sartorius, Goettingen,Germany

PH-meter …….WTW, Weilheim, Germany

Plate shaker……..Heidolph,Schwabach,Germany

Polymax wave platform shaker…….Heidolph,Schwabach,Germany

Polystyrene Round-Botton Tube …….BD Falcon, Erembodegem, Belgium

Superfrost^{+} microscope slides …….Menzel-Gläser,Braunschweig,Germany

Syringe ……Becton Dickson,Heidelberg,Germany

Syrings filter …….....Nalge,Rochester,USA

Thermostat 5320 …….Eppendorf,Hamburg,Germany

Upright fluorescence microscope Axioskop2……..Zeiss, Oberkochen, Germany

Weighter AE200 …….....Mettler-Toledo,Giessen,Germany

2.2 Subjects and Mathods

2.2.1 Cell preparation

2.2.1.1 Isolation of human monocyte enriched peripheral blood mononuclear cells (PBMC)

Mononuclear cells (MNC) were isolated from buffy coat blood obtained from healthy adult volunteers from the blood donor service of the University Medical Center (Luebeck, The Germany). Briefly, buffy coat was diluted 1/6 with sterile PBS，then layered on Ficoll Isopaque (LSM 1077, Lymphocyte Separation Medium, PAA Laboratories, Austria) and centrifuged for 20 min at 1600rpm without brake. The interphase consisting mainly of

lymphocytes and monocytes were collected. Human monocytes were selected from MNC by adherence. MNC were allowed to adhere in 175-cm^2 tissue culture flasks (Cellstar®, greiner bio-one, Germany) and cultured at 37℃ in a 5% CO2 air incubator in a humidified atmosphere for 3h. Nonadherent cells were removed, then adherent cells were washed twice with prewarmed medium (RPMI 1640, Biochrom, Berlin, Germany, 37°C). Adherent cells were removed by gentle agitation and light scraping. Cells were resuspended in RPMI 1640 medium supplemented with 5% FCS (FBS; Bioclear) and 2 mmol/L L-glutamine (Gibco BRL) and counted using a Fuchs-Rosenthal hemocytometer slide. All procedures were conducted at room temperature. In all cases, the preparations contained ≥70% monocytes, as determined by Giemsa staining of cytocentrifuged (Shandon, Pittsburgh, PA) samples. The viability of cells was greater than 95% immediately after purification , as determined by trypan blue exclusion. Cells were seeded in 12-well plates (Cellstar®, Greiner bio-one, Germany) at an initial density of 1×10^6 cells/ml and used for all experiments.

2..2.1.2 A549 cells

The type Ⅱ human epithelial cell line A549 was maintained at 5% CO_2 and 37°C in 175cm^2 polystyrene flasks(Greiner, Frickenhausen, Germany) with RPMI 1640 medium (PAA Laboratories, Austria), containing 5% fetal calf serum (Biochrome AG, Germany), 1% L-Glutamine (PAA Laboratories, Austria), 1000μg Amphotericin B (PAA Laboratories, Austria), 1ml Gentamicin (Sigma®, Germany), 1% Non Essential Amino Acids (PAA Laboratories, Austria). Cells were spread every 3-4 days . Antibiotic-free A549 cells were prepared the day before the stimulation experiment (1×10^6 cells/well in 1ml culture medium containing RPMI1640 and 10%fetal calf serum).

2.2.1.3 Human Bronchial Epithelial (HBEp) Cells

Human Bronchial Epithelial(HBEp) Cells were grown in Basal Medium adding with Growth Supplements(ECACC company). HBEp Cells were maintained following the manufacturer's recommendations. Growth medium was replaced every 3 days. Epithelial cells were seeded in 12-well plate at an initial density of 1×10^6 cells/ml and were left to

adhere overnight. All cells were maintained at 37°C in an atmosphere of 5% CO_2 (at 37°C in a humidified 5% CO_2 water-jacketed incubator). The number of nonviable cells, as assayed by trypan blue staining, was <5% of the total cell number for all time points.

2.2.2 Culture of bacteria

Hi was grown on chocolate agar plates at 37°C with 5% Volume per volume (v/v) CO_2 to generate a microaerophilic environment. Hi was taken from fresh overnight culture plates of chocolate-agar and suspended in sterile NaCl-Solution to a photometric density of 4 MacFarland (McF) resembling 10^9 colony-forming units (cfu)/ml. This solution is diluted 1:10 or 1:100 with the medium used for cell stimulation.

2.2.3 Infection of host cells

A549 cell cultures were shifted to 24 h before infection with Hi. Cells were infected with Hi at $10^{6,}$ 10^7, 10^8 cfu/ml or sham-infected media controls separately. After 3, 8 and 24h incubation at 37°C, supernatants were collected, centrifuged, and used for chemokine analysis. An optimal response to Hi was observed at 10^6 cfu/ml (Fig. 4), these conditions were used for subsequent experiments.

Cells were maintained as described above. All cell cultures were shifted to antibiotic-free medium 24 h before infection with Hi. 1 X 10^6 cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in a volume of 1 ml culture medium RPMI 1640 medium with or without the TLR7 ligand CL097 (1ug/ml), TLR3 ligand poly(I:C) (25ug/ml), and Hi (10^6 cfu/ml). After 3, 8 and 24h supernatants were collected from 12-well-plates for Elisa. MNCs (adjusted to 1 x 10^6 cells/ml) were resuspended in antibiotic-free medium (RPMI 1640 medium containing 5% FCS and 2 mmol/L L-glutamine) and incubated with 1ug/ml CL097, Hi(10^6 cfu/ml), or CL097/Hi combination. After every 3 hours from 0–24 hours and at the other indicated time points cells were collected for Western Blot. In selected experiments, MNC were preincubated with with 10µM UO126 and or 20uM SB203580 for 1h before being stimulated with Hi and CL097. At each time point, cells and supernatants were harvested for Western Blot or Elisa.

2.2.4 Cytokine assays

Culture supernatants were collected after 3,8 and 24 h as described above and centrifugated to remove cellular debris. Supernatants were stored as aliquots at -20°C until use. The supernatants were assayed for proinflammatory cytokine production by enzyme-linked immunosorbent assay (ELISA). IL-6, IL-8 and TNF-a were measured using DuoSet® Elisa (R&D systems, Wiesbaden- Nordenstadt, Germany), according to the manufacturers' instructions. Aliquots were measured in duplicates of at least three experiments.

2.2.5 Western blot analysis for Mcl-1

After removing medium from the wells, cells were collected in lysisbuffer (3,94g Tris-HCl pH 7,8 (125mM), 140ml Aqua dest, 20% Glycerol, 4% SDS, 10% 1M DTT, brome-phenol-blue; ad 200ml). Samples were then frozen at −20℃ until use. Protein extracts were separated by SDS-PAGE using the 12% acrylamide gel. Heat treatment at 95°C for 5 minutes(min) before loading on the gel. 7µl Marker and 30µl probes was loaded per lane. After electrophoresis proteins were transferred to Protran® Nitrocellulose Transfer Membrane (Schleicher & Schuell, Germany) using the BioRad mini protean II transfer apparatus (Bio-Rad, Hemel Hempstead, UK). Membranes were then blocked with a buffer containing 5% dried skimmed milk, Tris-buffered saline (TBS) (150 mmol/L NaCl; 10 mmol/L Tris-HCl, pH 8.0), and 0.1% Tween 20 for 1 hour at room temperature. After three brief washes in TBS (pH 8.0) and 0.1% Tween 20 (wash buffer), membranes were incubated with primary antibodies mouse anti-human Mcl-1 (1:500) (BD Pharmingen) diluted in 5% dried skimmed milk, TBS (pH 8.0), and 0.1% Tween 20 (Block buffer) overnight at 4°C on a shaker. Membranes were then washed 2 times for a few seconds and 3 × 15 min in wash buffer before incubation with HRP-linked secondary antibody (1:4.000) (HRP-linked anti-rabbit, Cell Signalling) diluted in Block buffer for 1 hour at room temperature. After washes, bound antibodies were detected using Amersham's ECL detection reagents（GE Healthcare,UK） and preflashed film. Care was taken not to overexpose film to allow comparison of expression of proteins between samples.

2.2.6 Western blot analysis for p-p44/42 MAPK and p-p38 MAPK

SDS-PAGE and electrotransfer to Nitrocellulose Transfer Membrane were performed as described above. Primary antibodies used were: anti-phospho p44/42 MAP Kinases (1:1.000), anti-phospho p38 MAPK(1:1.000), anti-β-Actin Akt. The second antibody was HRP-linked anti-rabbit (1:4.000, Cell Signalling). Bound antibodies were detected using the ECL system.

2.2.7 Live/dead staining

MNCs were seeded and treated with Hi and CL097 as described above. After the stimulation at the indicated time points, the cells were washed twice with prewarmed PBS (37°C), and then incubated with 1µl SYTO-16 (Molecular Probes, Leiden, The Netherlands) and 1 µl ethidium bromide (Sigma,Steinheim,Germany) in 200ul PBS for 60 min at room temperature. Later, cells were adjusted to 5 x 10^5 cells/ml, and were cytocentrifuged using a Shandon Cytospin 3 (Runcorn, Cheshire, UK). Slides were observed on a fluorescence microscope to detect SYTO-16 and EB staining. SYTO -16 is a green fluorescent cell-permeable dye that stains DNA, and EB is a red fluorescent dye that also stains DNA but is impermeable to intact cell membranes. Live cells stain green, and apoptotic cells, which have compromised membrane integrity, stain with both dyes, with the red dye being predominant visible by microscopy. Viable and dead cells were examined under fluorescence microscopy, (a minimum of 200 cells/slide was counted). The results for the Live/dead microscopy assay were displayed as percentage of dead cells.

2.2.8 Statistical analysis

Data are expressed as mean ± standard error of the mean(SEM). One-way analysis of variance (ANOVA) with Posthoc tests by LSD was used for statistical analysis of the differences between groups. Statistical significance was set at $p \leq 0.05$. Calculations were carried out with SPSS for windows software program 13.0.

3. Results

3.1 Hi independently increased chemokine production in A549 cells in a time-dependent manner

We assessed chemokine production by AEC-II cells (A549 cells) after Hi infection. A549 cells were infected with Hi (10^6 cfu/ml), supernatants were harvested at 3, 8 and 24 h, the amount of Il-8 and IL-6 released into the supernatants was assessed by ELISA(Fig.3). In the case of A549 cells, there was a steady increase of IL-8 in the supernatants starting at 3h, reaching statistical significance at 8h ($P < 0.05$) and continuing thereafter until 24h. The results indicate that Hi can independently increase IL-8 and IL-6 production in A549 cells in a time-dependent manner.

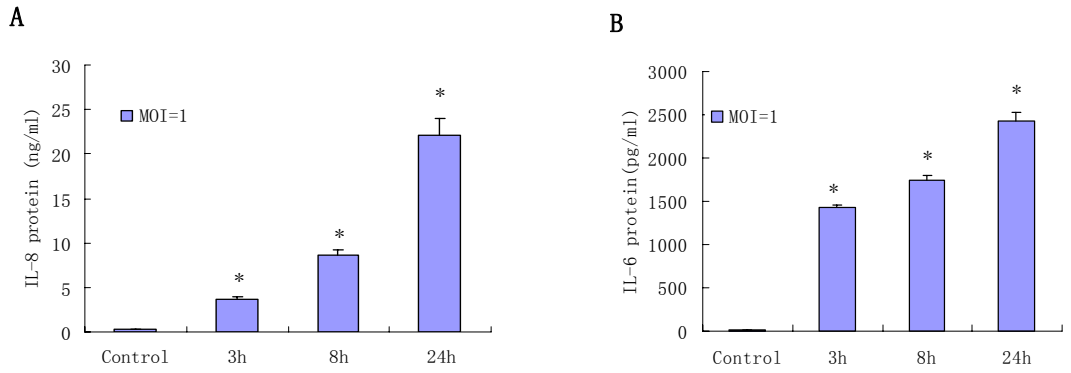


Fig.3: Time course of IL-8 and IL-6 protein release from A549 cells following exposure to Hi. A549 cells were infected with Hi (10^6 cfu/ml). 3, 8, 24 h after infection, cell supernatants were collected and examined for the C-X-C chemokines IL-8 (A, n=4) and IL-6 (B, n=4). Data are expressed as mean ± SEM for 4 experiments (*P < 0.05, ANOVA).

3.2 Hi induces IL-8 and IL-6 protein release by A549 cells not in a dose-dependent manner

To determine the optimal dose of bacteria that can induce cytokine production in A549 cells, epithelial cell cultures were infected with Hi at 10^6 or 10^7 and 10^8 cfu/ml and incubated for 3, 8, or 24 h (Fig. 4). The data showed that IL-6 protein release was found to be maximal when cultures were incubated with Hi at 10^6 cfu/ml. Exposure of A549 cells to Hi at various concentrations had similar effect on IL-8 protein release at each times point. These data demonstrate robust levels of IL-8 and IL-6 release by A549 cells after exposure

to low concentrations of Hi. Based on the results of these initial studies, we used Hi at 10^6 cfu/ml and an incubation time of 24 h for further studies.

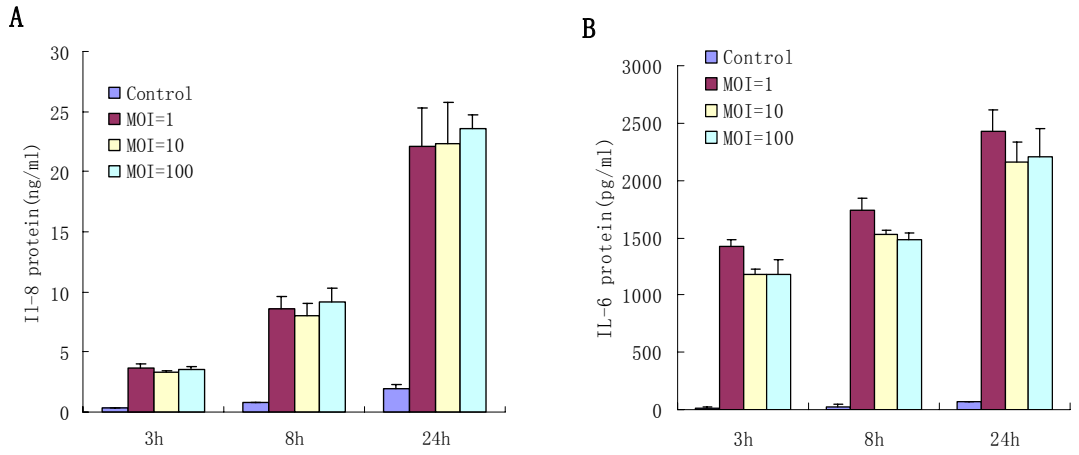


Fig. 4: Hi-induced IL-8(A, n=4) and IL-6(B, n=4) release by A549 cells was not dose-dependent. A549 cells were stimulated with control vehicle or various concentrations of Hi (10^6, 10^7, 10^8 cfu/ml) for 3,8 and 24h. Data are expressed as mean ± SEM from four independent experiments in A549 cells.

3.3 Hi and TLR3 agonist have cooperative effects on cytokine expression in A549 and HBEp cells

As a next step we wanted to assess if viral TLR agonists have an additive effect to bacterium on the inflammatory response in A549 cells. Cells were infected with Hi (10^6 cfu/ml) and costimulated with or without the TLR3 agonist poly(I:C) (25ug/ml) and TLR7 agonist CL097(1ug/ml). 8 and 24h after infection, cell supernatants were collected and examined for the C-X-C chemokine IL-8. The results indicate that Hi and poly(I:C) each independently increased IL-8 production, Hi and TLR3 agonist have cooperative effects on chemokine expression. In contrast no stimulation was observed after incubation with the TLR-7 agonist (Fig. 5A).

Since Hi infections have a predilection for the bronchial mucosa we extended our studies to Human Bronchial Epithelial (HBEp) Cells. Supernatants from the basolateral surface were collected and measured for IL-8 release after 8h and 24h. Again, Hi infection increased poly(I:C)-induced IL-8 protein levels relative to that found after poly(I:C) or Hi alone (Fig.5B). These data demonstrate that the additive effects observed in the A549 cells

occur also in a more physiological cell culture model.

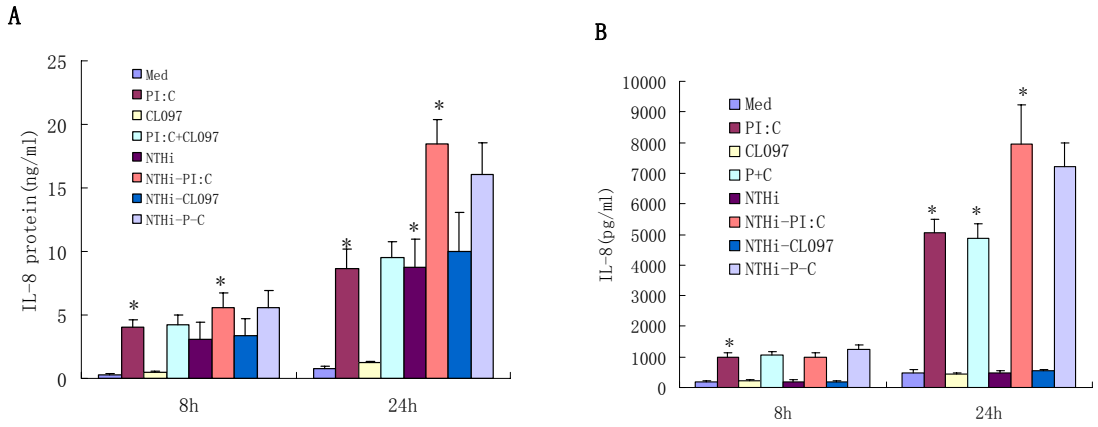


Fig. 5: Hi and poly(I:C) have cooperative effects on IL-8 and IL-6 production in A549 cells and HBEp Cells. A: IL-8 release from A549 cells increased significantly after combined stimulation with Hi/poly(I:C) when compared to Hi/CL097 or Hi monoinfection in 24h (Hi/poly(I:C) 18.48±1.93ng/ml vs. Hi/CL097 10.04±3.00ng/ml, Hi 8.70±2.22ng/ml, p< 0.05, n=4). B: Similar results were obtained using HBEp cells regarding IL-8 production (n=3). Data are expressed as mean ± SEM (*P < 0.05, ANOVA).

3.4 Combined effect of Hi and TLR7 agonist on TNF-α production by MNC

In MNC, an increased inflammatory response was associated with TLR7 agonist coincubation. Costimulation of Hi and Toll-like receptor 7 agonist shows an additive effect on TNF-α production in MNC (Fig 6). In contrast, TLR3 agonist has no additive effect on TNF-α production in MNC.

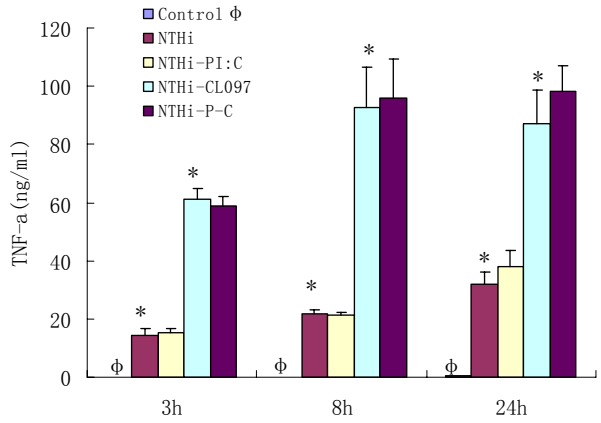
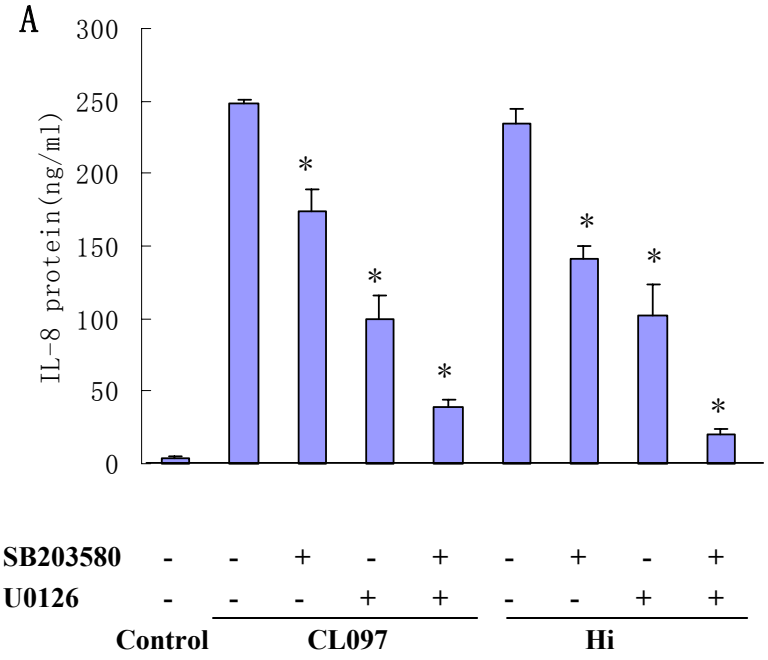


Fig.6: Combined effect of Hi and Toll-like receptor 7 agonist on TNF-α production by mononuclear cells (MNC). The concentrations of TNF-α increased significantly after stimulation with Hi/poly(I:C) together when compared to Hi/CL097 or Hi monoinfection (Hi/CL097 87.24±11.61ng/ml vs. Hi/poly(I:C) 37.88±5.73ng/ml, Hi 31.85±4.29ng/ml, p< 0.05, n=3). Data are

expressed as mean ± SEM for 3 experiments (*P < 0.05, ANOVA). φ, Control levels of TNF-α release by MNCs were at or below the minimum level of detection (15.6 pg/mL) of the TNF-α ELISA assay.

3.5 Proinflammatory cytokine secretion is dependent on p38 MAPK and p44/42 MAPK activation

To determine whether activation of p38MAPK and p44/42MAPK was required for the chemokine production of MNCs, we used two inhibitors of MAP kinases, SB203580 (inhibitor of p38 MAPK, 20uM) and U0126 (inhibitor of p44/42 MAPK, 10uM) to inhibit IL-8 and TNF-α expression induced by Hi or CL097 (Fig.7). The addition of any inhibitor attenuated Hi- or CL097-induced IL-8 expression significantly. The presence of both inhibitors strongly inhibited IL-8 production. In contrast, we found that treatment of MNCs with SB203580 inhibited TNF-α expression only after Hi infection, but not after treatment with CL097. Interestingly inhibition of p38 MAPK activity was able to augment the U0126-mediated TNF-α inhibition in CL097-treated MNCs. SB203580 reduced TNF-α expression 24 h after Hi infection by more than 95% and U0126 almost 75% in MNCs, respectively. The presence of both inhibitors resulted in complete blockade of TNF-α production in Hi-treated cells. Together, these data suggest that MAPKs are required for Hi- and CL097-mediated augmentation of cytokine production and that the relative importance of different MAP kinases is stimulus dependent.



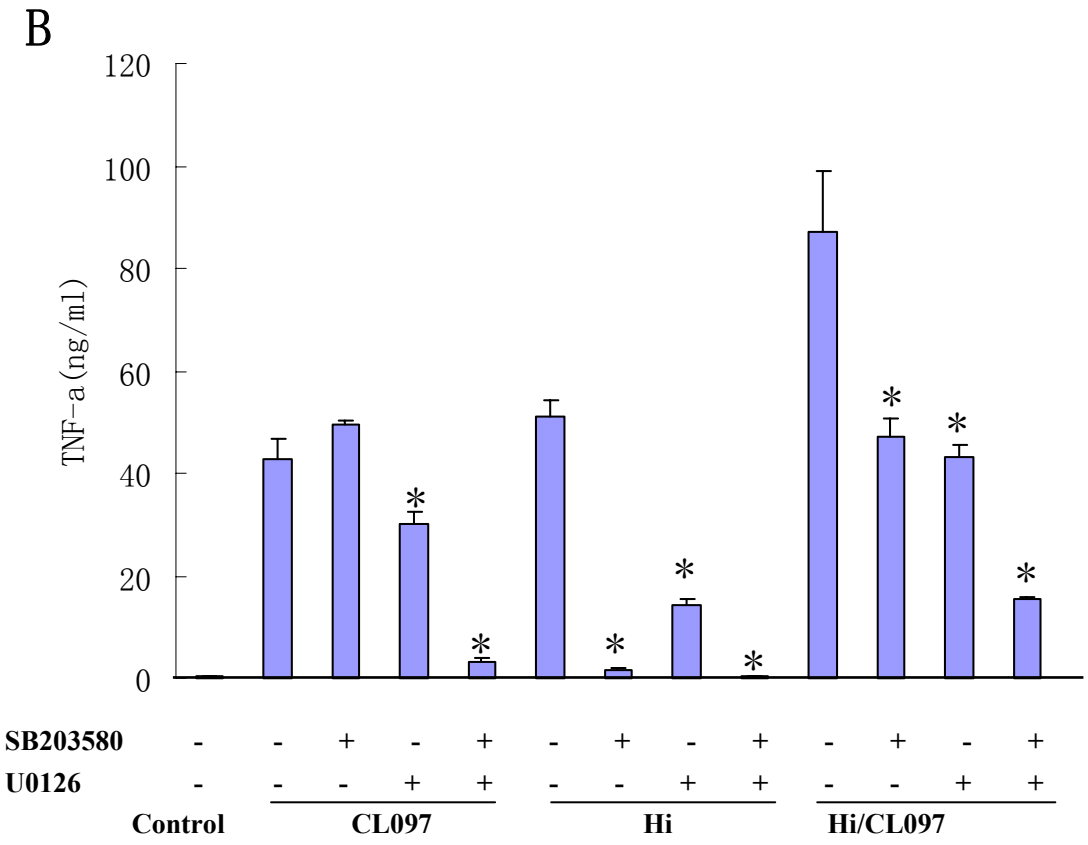


Fig. 7: Effect of ERK and p38 MAPK inhibitors on chemokine production of MNCs. MNCs were pretreated with inhibitors of p38 MAPK (SB 203580, 20 µM, 1 h), ERK1/2 (U0126, 10 µM, 1 h) to block MAP kinases. Afterwards, cells were incubated with Hi (10^6 cfu/ml) and or CL097 (1ug/ml). 24 h after incubation, supernatants were harvested for IL-8 (A, n=3) and TNF-α(B, n=3) analysis. Data presented are mean ± SEM of three separate experiments. Asterisks indicate significant difference of *p < 0.05.

3.6 p38 MAPK is activated in MNCs in response to Hi and CL097

To determine whether p38 MAPK kinase are activated in MNCs in response to Hi and CL097, we examined p38 MAPK activation in MNCs after Hi and CL097 stimulation by immunoblotting. The results show that this protein is present in MNCs, but in the presence of Hi and CL097, the phosphorylation of p38 MAPK was significantly increased**.** The data also show that phosphorylation of p38 MAPK in MNCs peaked at 30 – 60 min after CL097 and /or Hi stimulation (Fig. 8). The levels of activated p38 MAPK were maintained for about 2 h but then declined to basal levels by 3 h following CL097 or Hi/CL097 exposure.

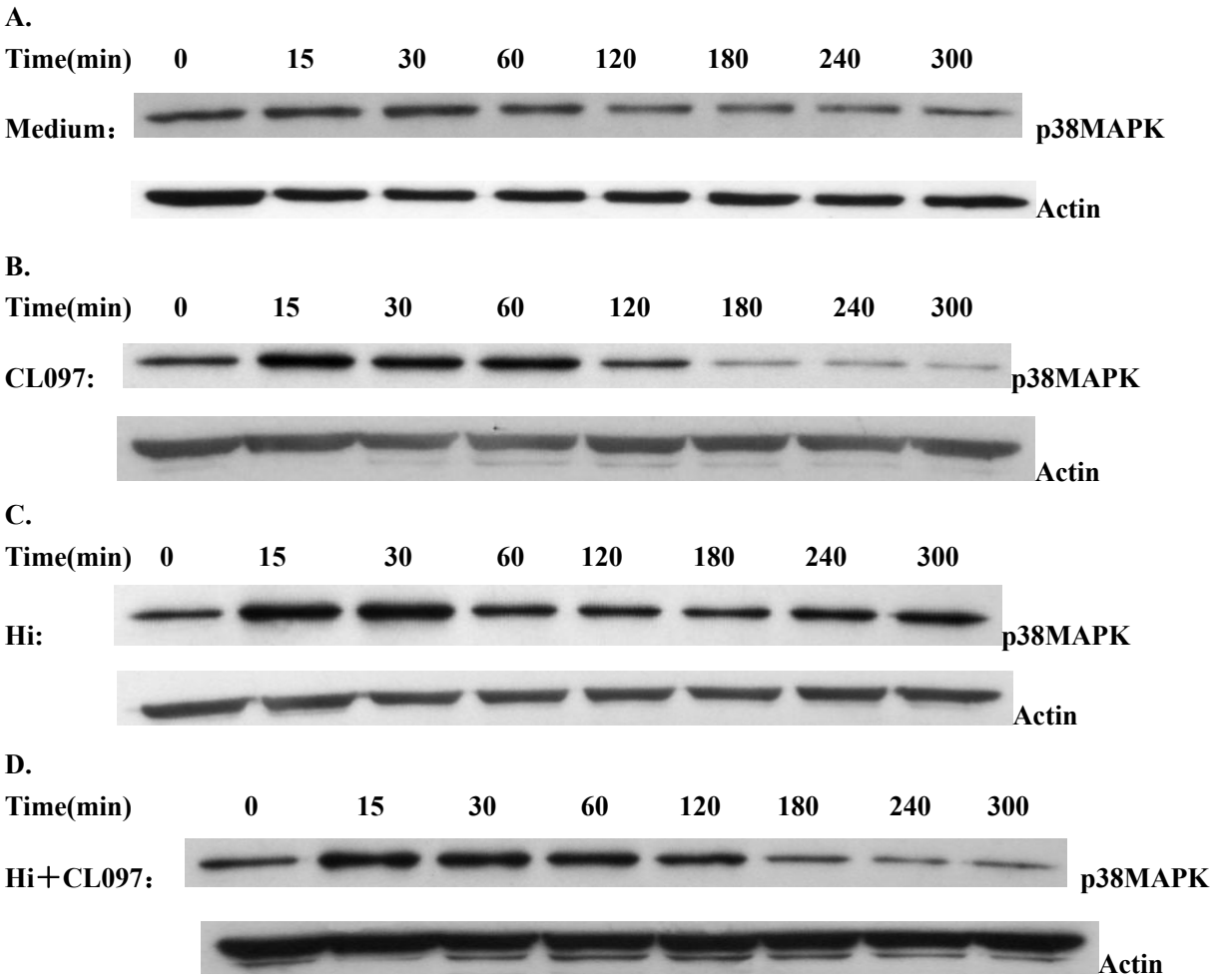


Fig. 8: Western blot analysis of activation and phosphorylation of p38 MAPK in human MNCs in response to Hi and CL097. A total of 1 x 10^6 cells were incubated for different periods of time (0, 15, 30, 60,120,180,240 and 300min) with or without Hi(10^6 cfu/ml) and CL097 (1ug/ml), lysed, blotted on a nitrocellulose membrane, and probed with anti-phospho-p38 MAPK antibody. (A) Time course of p38 MAPK activation in control, (B) in response to CL097, (C) in response to Hi, (D) in response to both. The data are representative of the data from two independent experiments.

3.7 p44/42 MAPK is activated in MNCs in response to Hi and CL097

Representative Western blots of p44/42 MAPK are shown in Fig.9 and indicate that p44/42 MAPK protein levels are detectable and maintain stability for 30 min, then decline slowly (Fig.9A). In contrast, significant p44/42 MAPK phosphorylations in CL097-treated, Hi-infected or Hi/CL097-costimulated MNCs are observed after 15min incubation compared to what occurred with untreated cells. The protein level decreased but increased again and remained elevated throughout the time course of the experiment in Hi-infected cells. (Fig.9C). However, CL097 alone induced rapid, transient increases in the activation

status of p44/42 MAPK, which peaked by 15 min and declined to basal levels by 2 h. This phenomenon was also observed in Hi-infected MNCs exposed to CL097.

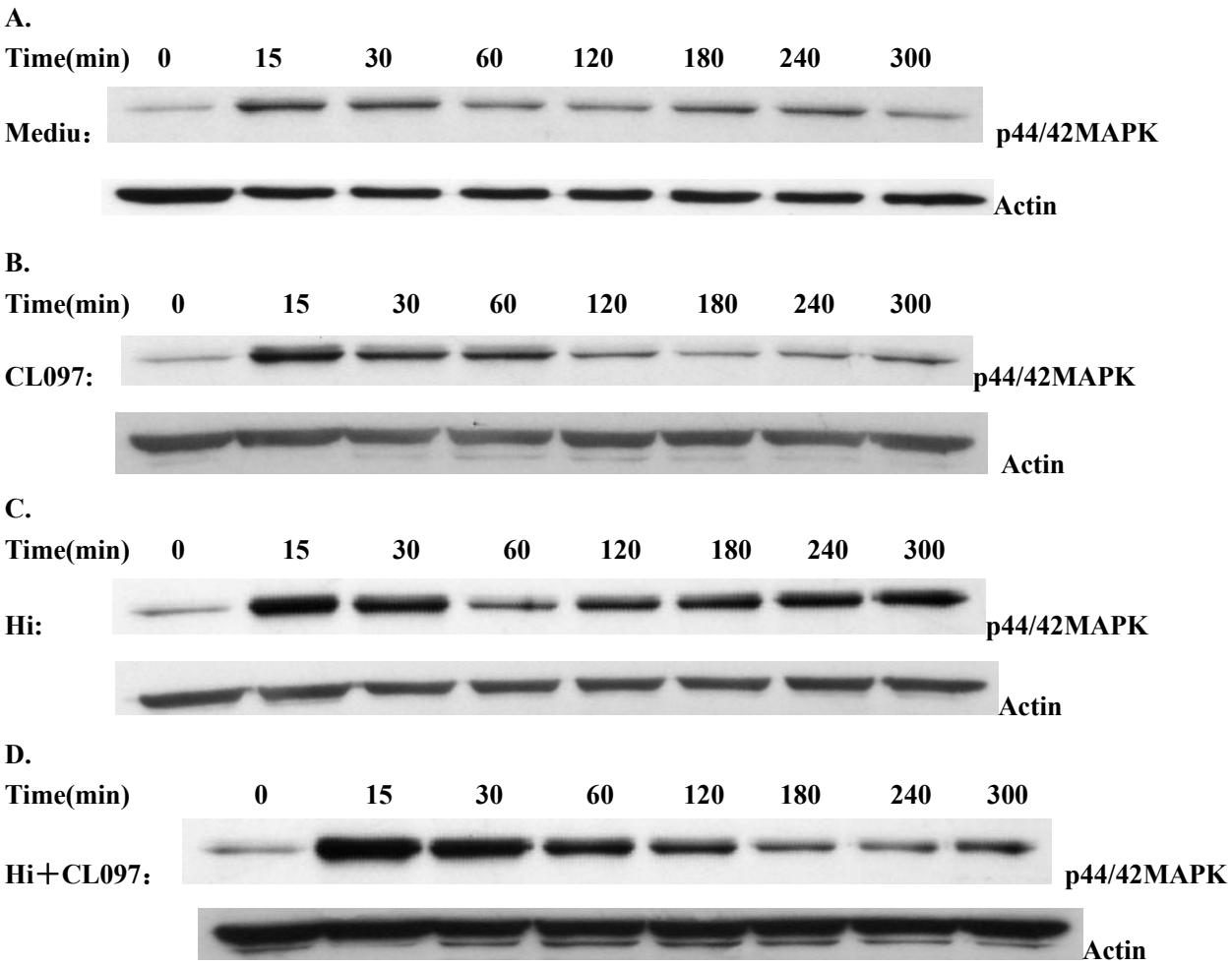


Fig.9: Effects of Hi and CL097 on activation of p44/42-MAPK. MNCs were incubated in the (A) absence (control) and (B) presence of Hi(10^6 cfu/ml), (C) CL097(1ug/ml) and (D) Hi/CL097 coincubation. At time intervals up to 5h, samples were removed for analysis of activated p44/42-MAPK by Western blotting. Typical result of two separate experiments is shown.

3.8 Increased Mcl-1 protein expression in MNC after Hi infection and Hi/CL097 coincubation

Mcl-1 is critical to both initial maintenance of cell viability and subsequent induction of apoptosis. A biphasic response in Mcl-1 protein expression is observed every 3 hours from 0–21 hours after isolating MNC freshly (Fig.10A). To investigate whether changes in Mcl-1 protein expression occurred in MNC in response to infection with Hi with or without TLR7 agonist(CL097), we serially measured Mcl-1 expression in MNC every 3

hours from 0–21 hours after stimulation. After initial stimulation, levels of Mcl-1 protein showed no obvious changes upon CL097 incubation(Fig.10B). Mcl-1 levels were markedly upregulated starting 9 h after coincubation in Hi-infected and Hi/CL097 -treated MNC when compared with controls and CL097 incubation (Fig.10E).

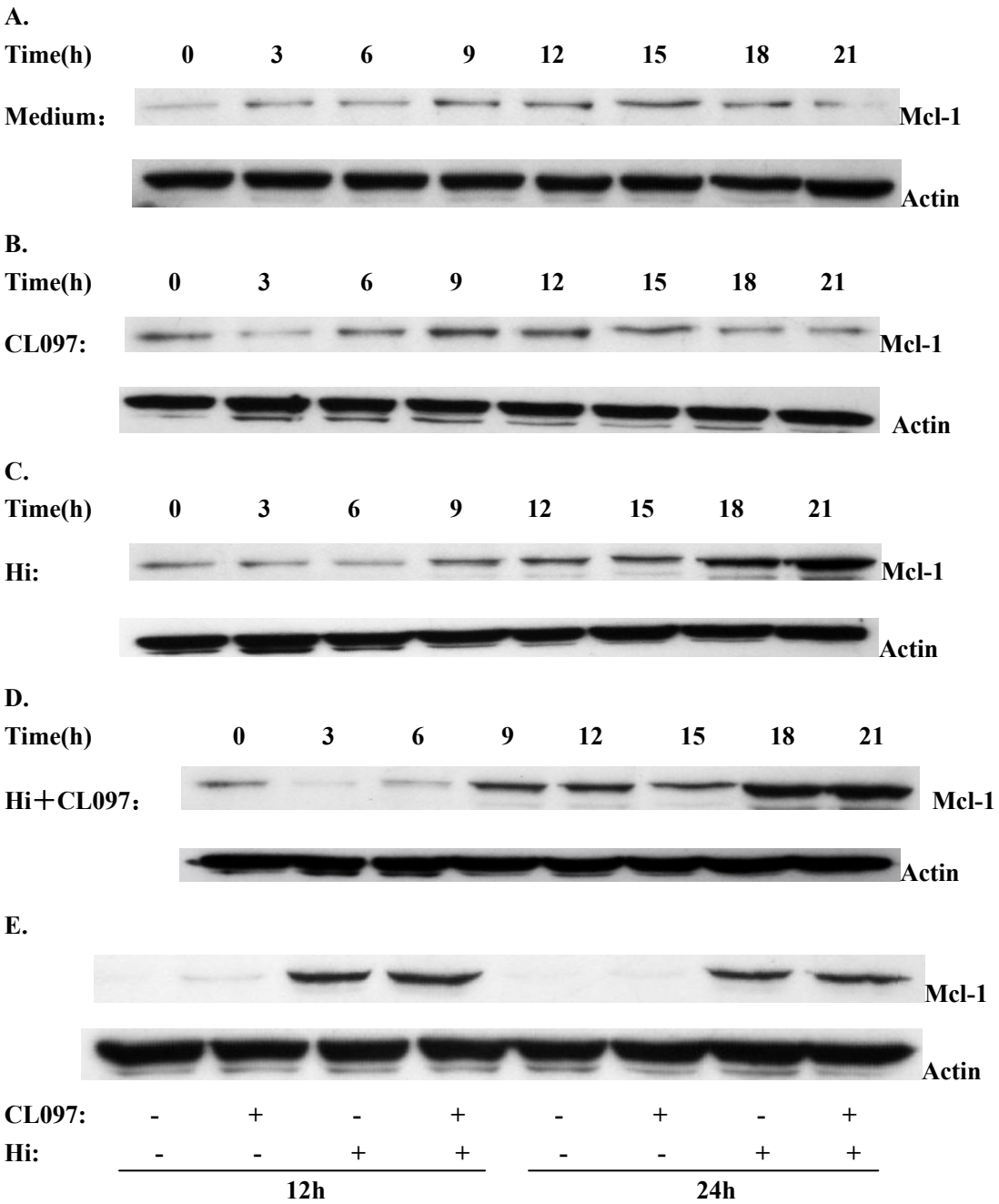


Fig.10: Hi infection and TLR7 agonist(CL097) coincubation in MNC results in increased expression of a proapoptotic Mcl-1 isoform. Data are representative of 2 independent experiments. (A)Western blot of total protein from MNC of control at the indicated time points, probed with anti–Mcl-1 and anti-actin antibodies showing the appearance of the band of approximately 40 kDa. Mcl-1 protein levels demonstrate biphasic variation in MNC. (B) MNCs were incubated in

the presence CL097, Expression of Mcl-1 and actin proteins were determined by Western blotting at the indicated time points. (C) Western blot of Mcl-1 and actin total protein from MNC at the indicated time points after infection with Hi. Hi infection of MNC results in increased expression of a proapoptotic Mcl-1 isoform. (D) Hi infection and CL097 coincubation in MNC results in increased expression of a proapoptotic Mcl-1 isoform. (E) Expression of Mcl-1 in MNC after 12 hours and 24 hours incubation under the conditions indicated were determined by Western blotting as described in Materials and Methods.

3.9 p38 MAPK and p44/42 MAPK regulate the levels of Mcl-1 in MNC

Previous work has shown that Mcl-1 has a short half-life within cells. p38 MAPK and p44/42 MAPK pathways may lead to or delay apoptosis via regulation of the cellular levels of Mcl-1. We therefore tested whether inhibition of MAPK activity had any effect on the cellular levels of Mcl-1 in MNCs. Hi- or NTH-/CL097-treated MNCs were cultured in the presence of p38 MAPK or p44/42MAPK inhibitors. Mcl-1 protein was detected by Western blot. In the absence of Hi and Hi/CL097, we found a low signal intensity for Mcl-1, which substantially increased in the presence of Hi and Hi/CL097. However, Hi-infected MNC incubated for 24 h in the presence of the p38 MAPK inhibitor SB203580 or p44/42 MAPK inhibitor U0126 had markedly decreased levels of Mcl-1. Expression of Mcl-1 was diminished even further in the presence of both inhibitors, indicating a rapid turnover of the protein when biosynthesis is inhibited. The addition of any inhibitors in CL097- and Hi-cotreated MNC attenuated the cellular levels of this protein. Remarkably, in the presence of both inhibitors expression of Mcl-1 was almost undetectable(Fig.11). These experiments confirm the hypothesis that Mcl-1 induction in MNCs in response to Hi or Hi/CL097 is dependent on the p38 MAPK and p44/42 MAPK pathway.

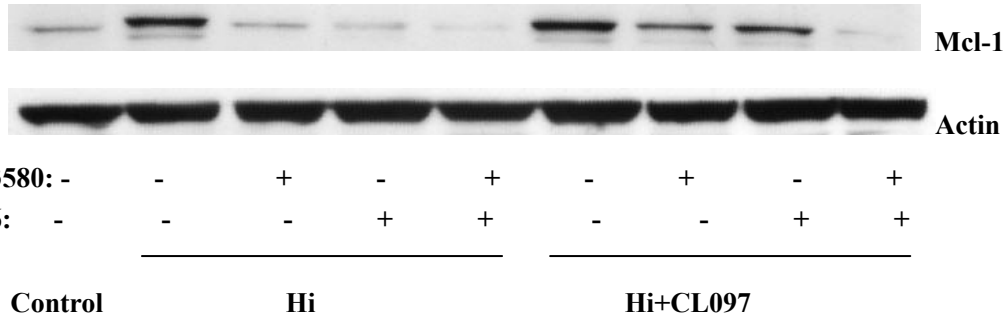


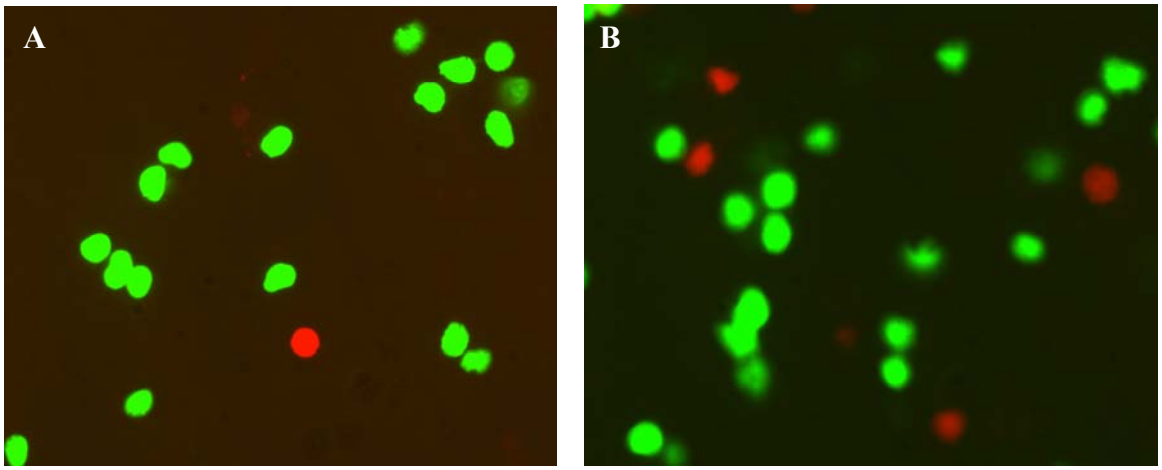
Fig.11: Effects of MAPK Inhibition on Mcl-1 Levels. Levels of Mcl-1 in MNCs extracts were measured by Western blotting after 24 h of incubation in the absence or presence of SB203580 (20

μM), U0126 (10 uM) in Hi- and Hi/CL097-treated MNCs. A representative Western blot of Mcl-1 is shown (2 independent experiments). Levels of actin are also shown.

3.10 Viable Hi are found intracellularly in MNC and induce cell death in a dose-dependent manner

Freshly isolated MNC were coincubated *in vitro* with Hi. A two-color immunofluorescent staining (live/dead staining) was used to investigate MNC viability. After staining with SYTO-16 (green) and ethidium bromide (red), viable cells and bacteria show green staining, whereas dead cells with compromised membranes stain red. After infection with Hi at 10^6, 10^7 and 10^8 cfu/ml in each case. Cytocentrifuge preparations were stained with live/dead staining and viewed by fluorescence microscopy to evaluate the apoptosis. After 24 h of culture, cell death was evident in cells infected at 10^7 and 10^8 cfu/ml (Fig.12C, D). As shown in Fig.12, Hi induced significant cell death induction at 10^7 and 10^8 cfu/ml at 24 h but not at either untreated-cells or 10^6 cfu/ml. Hi-induced death in MNCs was continue evaluated by fluorescent viability staining.

Fig.13 shows that bacteria were taken up by MNC after 24 h of coincubation. After coincubation with Hi, some MNC and the ingested bacteria still remain viable. However, instead of killing the bacteria, most MNC themselves are killed by Hi.



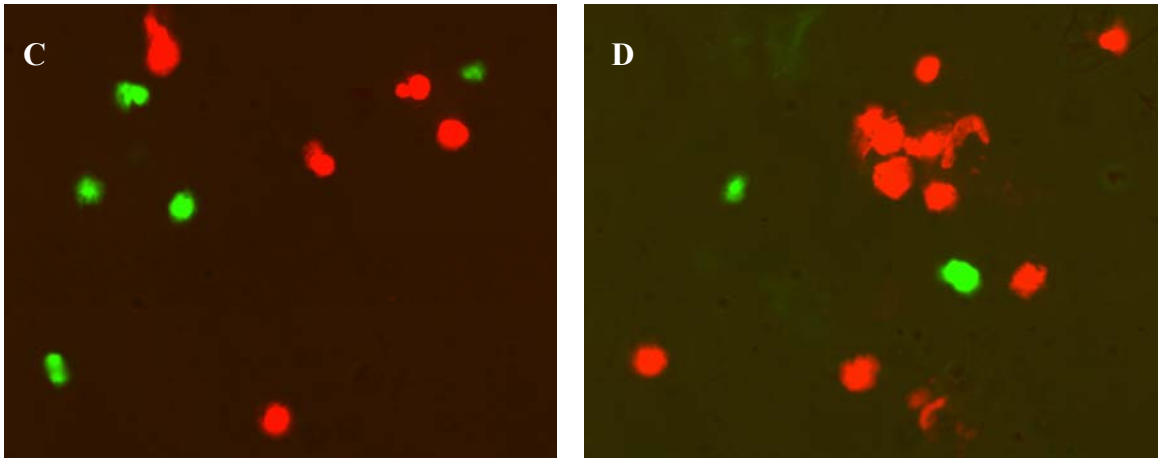


Fig. 12: Hi-induced cell death in MNCs. MNCs cells were infected with *Hi* at 10^6, 10^7 and 10^8 cfu/ml for 24 h. Cells were stained for viability using SYTO-16 – ethidium bromide under which viable cells appear green while apoptotic/necrotic cells appear red (magnification, x 400). A. unstimulated cells, B–D. Hi 10^6, 10^7 and 10^8 cfu/ml. Representative micrographs of three independent experiments are shown. For these studies, the percentage of dead cells was determined by evaluating ≥ 300 cells/condition in three independent experiments (data not shown).

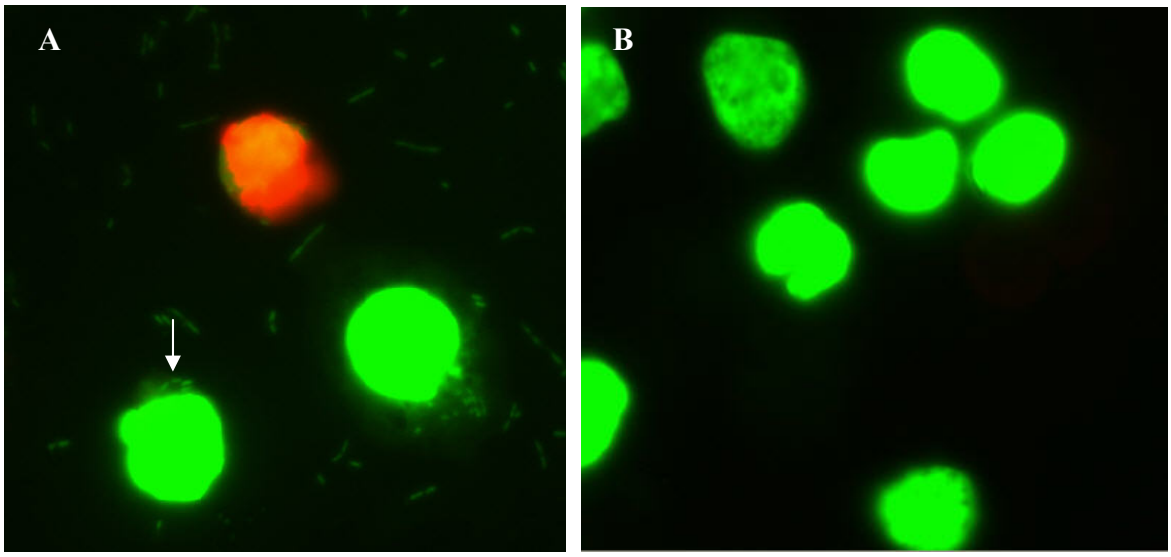


Fig.13: MNC internalize Hi in vitro. Live/dead staining was conducted on MNC after coincubation with Hi in a 1:1 ratio (A) or in medium alone (B) for 24 h, The arrows show ingested Hi in MNC (magnification, x1000). Representative micrographs of three independent experiments are shown.

3.11 Coincubation with Hi and CL097 leads to dead of MNC

After incubation for 24 h with or without Hi and CL097, the samples were washed and slides were prepared in the Live/dead staining described above. Using these criteria the

ratio of dead cells was determined in MNC cultured *in vitro* in the absence or the presence of Hi and or CL097. After 24 h, only 4.2 ± 1.5% of MNC cultured in medium alone were dead. 9.4 ± 0.8% of the cells or 16.6 ± 2.8% of the cells are dead after 24 h of incubation with CL097 or Hi. In contrast, the rate of cell death was strongly increased when MNC were coincubated with Hi and CL097 (Fig.14). A representative live/dead staining is shown in Fig.15.

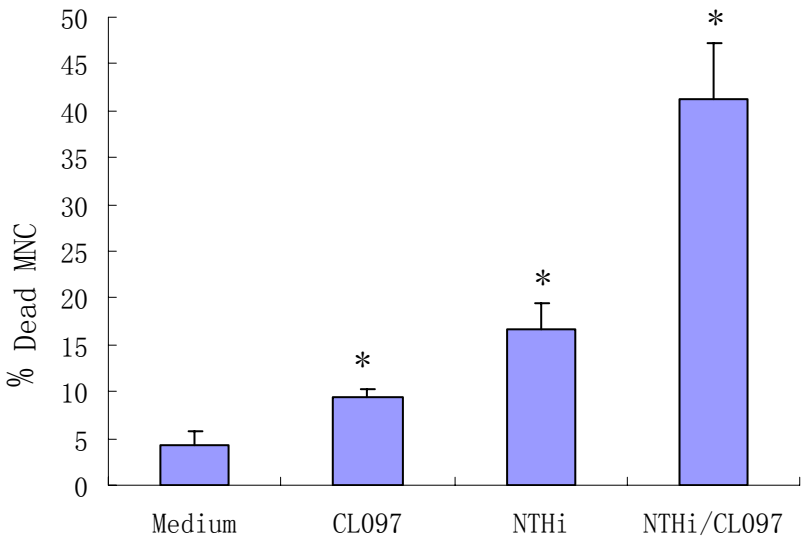
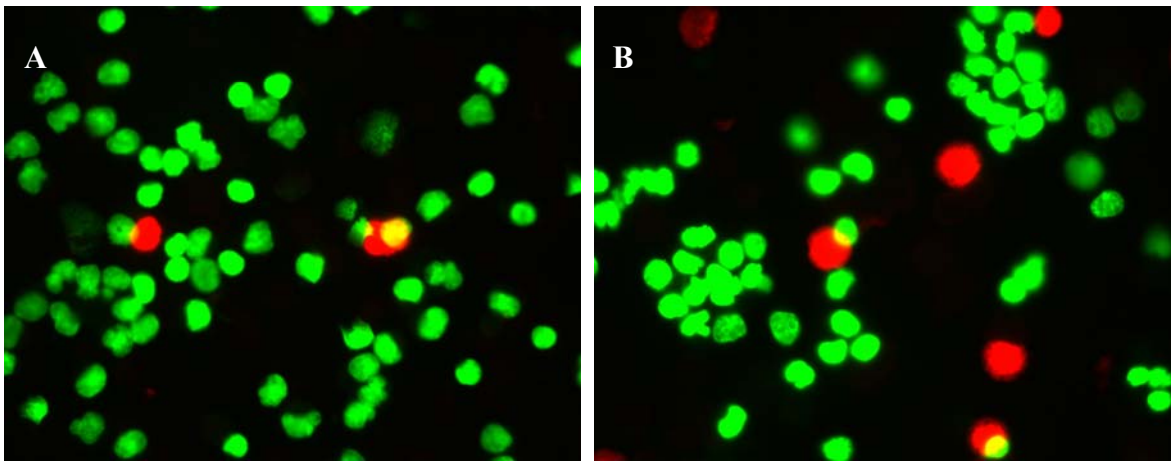


Fig. 14. The ratio of dead MNCs in response to Hi and CL097. Freshly isolated MNC were stimulated *in vitro* in medium alone, with CL097(1 ug/ml), or with Hi(10^6 cfu/ml), or with Hi/CL097 coincubated. Cytocentrifuge preparations were stained with live/dead staining. The percentage of dead cells was determined by microscopic evaluation of ⩾300 cells. The data represent the mean ± SEM for five independent experiments. *, $p < 0.05$.



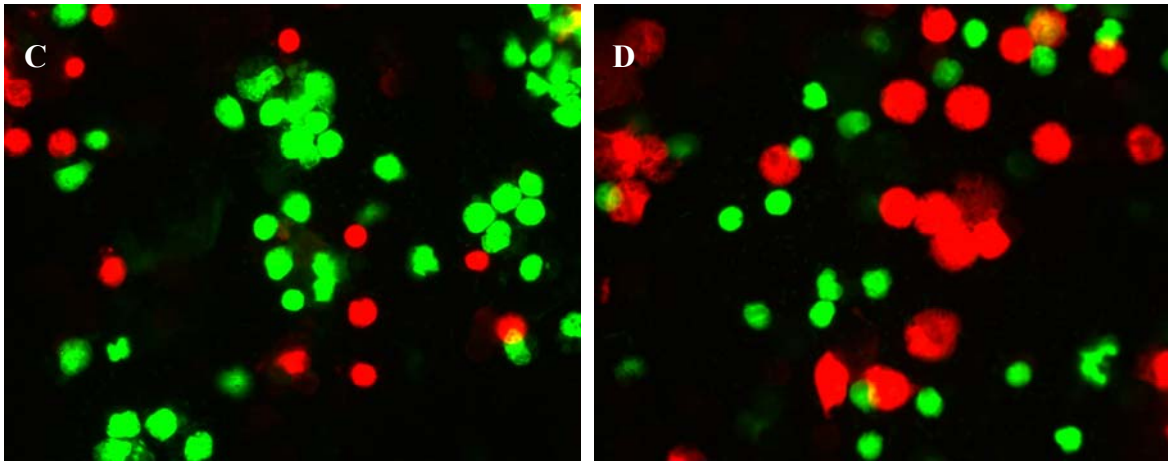


Fig.15: Coincubation with Hi and CL097 leads to dead of MNC. Live/dead staining was conducted on MNC after coincubation with medium(A), or with CL097(1 ug/ml)(B), or with Hi(10^6 cfu/ml) (C), or with Hi/CL097(D) for 24 h, A representative live/dead staining is shown (5 independent experiments).

4. Discussion

The airway epithelium plays an important role in regulating the host immune response and provides the first line of defense against inhaled microorganisms(Bals and Hiemstra.,2004). In this study, we demonstrated that Hi infection induced significant chemokine production by AEC-II and HBEpC cells. Airway epithelial cells form a complex physical barrier that defends against harmful substances and microbial pathogens. Furthermore, they express pattern recognition receptors (PRR), which recognize multiple types of organisms. Binding of pathogens to their cognate receptors induces the airway epithelium to produce inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-8, IL-6, and RANTES. All major diseases of the lung involve mechanisms of the innate or adaptive immune system. Cytokines and other mediators secreted from the airway epithelium are likely to play a critical role in chronic inflammatory airway diseases as asthma and COPD. Hi is the most common bacterial pathogen isolated in sputum samples obtained during exacerbations of COPD as well as during the stable phase of this disease (Sethi et al.,2002), which often coincide with viral respiratory infections. Viral and bacterial pathogens cause inflammation via TLR signaling. TLR2 and TLR4 are involved in Hi-induced inflammatory responses in the lung (Lorenz et al., 2005; Wieland et al.,2005). Several key inflammatory mediators including IL-1β, IL-8, and TNF-α are up-regulated by Hi. Our data are consistent with these studies.

Among the TLR ligands that we tested in this study, poly(I:C), a synthetic analog of viral dsRNA and a well characterized ligand for TLR3（Alexopoulou et al.,2001）mediated the most potent proinflammatory effects in both human alveolar epithelial cell (AEC) line A549 cells and HBEpC cells. These data demonstrate selective responses to TLR agonists in tissue cells and widespread functional expression of TLR3 in epithelial cells. We also observed that both A549 cells and HBEpC cells failed to respond to TLR7/8 agonist. In contrast, Poly(I:C) failed to stimulate rapid production of TNF-α with minimal amounts detected in the supernatants of MNCs after 24 h. In marked contrast, CL097, an agonist of

human TLR7 and TLR8 was extremely potent at inducing TNF-α from MNCs. This may reflect different signaling through TLR7/8 and TLR3. TLR3 is not widely expressed by PBMC (Muzio et al.,2000). TLR3, TLR7 and TLR8 respond to forms of RNA and have been implicated in antiviral responses (Alexopoulou et al., 2001; Heil et al.,2004). Bronchial epithelial cells have been shown to express functional TLRs, notably TLR1–6 and TLR9 (Mayer et al.,2007). TLR3 expression was observed in lung epithelial cell lines and endothelial cells, demonstrating a wide tissue expression of this receptor (Guillot et al.,2005; Tissari et al.,2005). Ritter et al demonstrate that poly(I:C) mediates the strongest proinflammatory effects in primary small airway epithelial cells (SAEC) with respect to cytokine and chemokine secretion and matrix metalloproteinase (MMP) release of the cells. These inflammatory features of poly(I:C) are thought to contribute to viral exacerbations of pulmonary inflammations including COPD and asthma (Ritter et al.,2005). However, TLR7 and TLR8 expression was not detected in A549 epithelial cells and no response of these cells to the TLR7/8 agonist was found in a previous study (Tissari et al.,2005). Our results confirm these studies.

Bacterial and viral exacerbations play a crucial role in a variety of lung diseases including COPD or asthma most probably due to a biased release of pro-inflammatory mediators. In this study, we found that poly(I:C) incubation significantly increases Hi-infected IL-8 and IL-6 expression in both epithelial cell types. Additive increases in IL-8 expression were observed as early as 3 h after Hi and poly(I:C) stimulation (data not shown). Hi infection induced only minor changes in IL-8 expression in HBEpC cells over the period studied. However addition of poly(I:C) strongly enhanced IL-8 release by HBEpC cells relative to poly(I:C) alone (Fig. 2B). The cooperative effects of Hi and TLR3 agonist also can be observed in the A549 cells. These findings indicates that costimulation can greatly alter the level of epithelial proinflammatory cytokine production. We also found an additive effect between Hi infection and CL097 in their ability to stimulate MNCs to release TNF-α. These results demonstrate that costimulation by viral ligands and Hi infection causes host cells to mount an increased inflammatory response. The precise molecular mechanisms underlying this process are not yet known. Recent data provide evidence that Hi enhances

host antiviral responses via TLR2-dependent up-regulation of TLR7 expression in human airway epithelial cells *in vitro* and mouse lung tissue *in vivo*. Moreover, Hi induces TLR7 expression via a NF-κB-dependent mechanism (Sakai et al.,2007). Another study found that infection of airway epithelial cells with Hi increases expression of the RV receptors ICAM-1 and TLR3, leading to increased RV binding and exaggerated RV-induced chemokine responses (Sajjan et al.,2006). Furthermore, poly(I:C) modulates the gene expression of other TLRs (TLR1, TLR2 and TLR3) and may increase the gene expression of the general TLR adaptor MyD88 and IRAK-2 in primary small airway epithelial cells. The effects of poly(I:C) on the expression of Toll-like receptors and molecules involved in TLR signaling is assumed to influence the immune response of the lung epithelium to viral and bacterial infections (Ritter et al.,2005). In addition, the regulation of proinflammatory cytokine production is multifactorial, therefore further yet undefined factors likely contribute to the inflammatory response. Together, our data provide additional evidence that Hi infection and viral ligands can produce cooperative effects on host cell chemokine expression and describe a cellular mechanism for virus and bacterium-induced exacerbations of asthma and COPD.

Proinflammatory cytokines expression is tightly controlled by complex signaling pathways. Ubiquitously expressed members of the mitogen-activated protein kinase (p38 MAPK, JNK, ERK1/2) family contribute to this regulation (Chang and Karin.,2001). Especially p38 MAPK is thought to be important for proinflammatory mediator expression under various conditions of infectious disease. In contrast, ERK is activated by mitogenic stimuli and plays a central role in cell proliferation and differentiation. However, recent studies have suggested that ERK and JNK also play important roles in the signalling cascades leading to the induction of various inflammatory mediators. Many extracellular stimuli activate p38 MAPK and ERK. These kinases were selectively inhibited with SB 203580 and U0126, respectively, to elucidate the biological functions of activated p38 MAPK and ERK. Although the function of p38 MAPK and ERK in host cells during exposure to Hi has been described previously, the precise role that p38 MAPK and ERK play in MNC upon Hi/CL097 coinfection remains to be clarified. We demonstrated that p38 MAPK and

p42/44MAPK phosphorylation and proinflammatory cytokine production were increased in the setting of stimulation by Hi or CL097. Either SB 203580 or U0126 significantly inhibited the expression of proinflammatory cytokines. These findings show that both ERK and p38 MAPK are involved in TNF-α release in MNCs stimulated by CL097 and Hi. Inhibition of proinflammatory cytokine expression with SB 203580 and U0126 indicates that a common downstream pathway involving both p38 MAPK and ERK might play a central role in pathogen induced cytokine expression.

Mcl-1 contributes to the control of mitochondrial integrity which is critical for maintaining cell viability. Mcl-1 expression is rapidly degraded in response to cell death signals and is immediately re-induced by survival stimuli (Yang-Yen.,2006). Our data show that the level of Mcl-1 is low and biphasic response in resting MNCs, but is continuously increased following Hi and or CL097 exposure. Maximal expression of Mcl-1 occurred by 21 h. These changes in Mcl-1 expression may have a role in the response to infection since it was demonstrated that expression of Mcl-1 delayed mitochondrial membrane permeabilization and impaired bacterial clearance *in vitro* (Marriott et al.,2005). Mcl-1 transgenic mice had significantly fewer apoptotic alveolar macrophages (AMs) and significantly less efficient clearance of *S. pneumoniae* from the lungs *in vivo* (Marriott et al.,2005). In pneumococcal infection it was shown that induction of apoptosis facilitates bacterial clearance and minimizes inflammation (Dockrell et al.,2003). These findings demonstrate the importance of an Mcl-1–regulated switch from cell viability to apoptosis for optimal host defense. Mcl-1 is thus an ideal regulator of cell viability during rapidly evolving conditions of cell stress and could allow the dynamic changes in cell viability required during bacterial infection.

Many signalling pathways have been implicated in the regulation of Mcl-1 expression in different cells under different physiological circumstances including MEK/ERK, p38 MAPK, PI3K/Akt (protein kinase B) and JAK/STAT3. MAPKs have been implicated in modulating phosphorylation of the Bcl-2 protein (Deng et al.,2000). Activation of the distinct MAPK subtype cascade is dependent on the types of cells and the stimuli used. Our

data confirmed that Mcl-1 was upregulated by Hi and /or CL097, hence the next step was to determine whether kinase signaling pathways are activated by Hi and or CL097. In this study, we analyzed two distinct signal transduction pathways previously characterized to impact Mcl-1 expression, including the ERK and p38 MAPK pathways. The results show that phosphorylation of p38 MAPK and p42/44 MAPK in MNCs is significantly increased within 15 to 30 min after addition of Hi and/or CL097. The magnitude and duration of p38 MAPK and p42/44 MAPK phosphorylation were both increased in Hi-infected and or CL097-exposed MNCs. Our findings show that p38 MAPK and p42/44 MAPK are activated in MNCs in response to Hi and/ or CL097. However, the functional role of MAPKs in cell survival or cell death is still uncertain and may be different according to the types of cells and stimuli. For example, the role of p38-MAPK in the control of neutrophil apoptosis is controversial, with some reports suggesting that it generates a survival signal (Villunger et al.,2000; Alvarado- Kristensson et al.,2004) and some contrasting reports suggesting that it triggers a death signal (Frasch et al.,1998; Aoshiba et al.,1999). The functional role of MAPK in apoptosis in Hi- and CL097-induced MNC is also unclear.

Having shown that Hi and CL097 treatment greatly increased Mcl-1 expression and also activated p38 MAPK and p42/44 MAPK kinase in MNCs, we next investigated whether these signaling pathways contribute to Mcl-1 expression. Several lines of evidence indicate that both the p38 MAPK and p42/44 MAPK signalling cascades are important pathways leading to apoptosis. However, the relationship between the Mcl-1 and MAPK pathways in mixed infections of bacteria and virus remains unclear. p38 MAPK inhibition has been reported to enhance apoptosis by upregulation of p53 and downregulation of antiapoptotic Bcl-X_L and Mcl-1 protein in multiple myeloma (MM) cells (Navas et al.,2006). Blocking the activities of ERK/MEK and PI3K, respectively, completely abrogated the Granulocyte-macrophage colony-stimulating factor (GM-CSF)-mediated increase in Mcl-1 stability and apoptosis delay (Derouet et al.,2004). We tested the role of MAPKs by using the p38-MAPK inhibitor, SB203580 and the p42/44-MAPK inhibitor, U0126. In this study, we found that the addition of any inhibitor in Hi-infected and Hi/CL097-cotreated MNC greatly decreased the cellular levels of Mcl-1. Remarkably, the presence of the both

inhibitors resulted in almost undetectable levels of this protein. These data indicate that activation of MAPK function can increase Mcl-1 stability. Altogether, these experiments suggest that the early activation and duration of p38MAPK and/or p42/22MAPK kinase may be important determinants of the cellular survival response of MNCs through altering Mcl-1 turnover following Hi and/or CL097 stimulation. We hypothesize that upregulation of p38 MAPK and p42/44MAPK expression would result in a coordinate increase in available Mcl-1 contributing to further downregulation of proapoptotic signals in Hi/CL097-treated MNCs.

In this paper, we studied the effect of costimulation with Hi/ viral ligands on host cell viability. A widely used method for quantification of viable or dead cells is live/dead staining by SYTO-16, which is a sensitive, simple, inexpensive method for the discrimination of live, apoptotic and necrotic cells and is more sensitive for detecting apoptosis in MNCs than Annexin-V (Sparrow and Tippett.,2005). There is now much evidence demonstrating the central role of Mcl-1 in regulating neutrophil and other cells survival in bacterial infection. We tested the hypothesis that Mcl-1 is critical to cell viability or apoptosis when treated with Hi and CL097. Unexpectedly, we showed for the first time that costimulation of cells with Hi/CL097 induced more apoptotic/necrotic cell death than in Hi-infected, CL097-treated alone or untreated cells. This indicates that Hi induced MNC apoposis/necrosis by other signaling mechanisms besides Mcl-1. In contrast to neutrophils Mcl-1 may not have such a central role in MNCs since other antiapoptotic molecules are available. Alternatively, direct or indirect toxic effects of the pathogen may lead to primary necrosis of the host cell. Finally, despite the induction of the anti- apoptotic Mcl-1 in Hi and Hi/CL097 treated cells, Hi and CL097 may also induce an additional pro-apoptotic factor and interfere with the balance of pro- and antiapoptotic protein. The effect of Hi and CL097 on MNC apoptosis/necrosis is a multistep process. This process appears to be mediated in part by the p38 MAPK and p44/42MAPK signal transduction pathway. Later, autocrine/paracrine mechanism related to IL-8, TNF-α or other CXCL chemokines may be involved.

In live/dead staining, viable bacteria were found intracellularly in MNC. The mechanisms for persistence need further study. Hi is usually regarded as an extracellular mucosal pathogen. However, previous observations suggest that Hi can invade eukaryotic cells. Hi entered human monocytic and epithelial cells via a receptor-mediated endocytosis involving mainly a beta-glucan receptor that could be blocked by laminarin (Ahrén et al.,2001). Our results are consistent with these studies. The possibility that Hi enter mammalian cells and thus escape the local immune system suggests that the host cells may act as a reservoir. This might be a feasible explanation for recurrent infections especially in patients with chronic obstructive pulmonary disease (Murphy et al.,2004). Colonizing Hi produce significant airway inflammation, which is increased during exacerbations (Chin et al.,2005).

Based on the experiments presented in this report, we support the notion that direct cellular damage and the consequences of proinflammatory cytokines release contribute to the pathogenesis of Hi infection. Moreover, costimulation with viral ligands and Hi infection causes host cells to mount a strong inflammatory response and induces more apoptotic/necrotic cell death. More investigation of the mechanisms that facilitate ongoing apoptosis and/or necrosis of infected host cells are warranted.

5. Summary

There is a growing body of evidence that a significant proportion of COPD patients has exacerbations with mixed infections of bacteria and virus. However the interactions between bacteria and viruses and the mechanisms that underlie the combined effect are poorly understood. We used the synthetic TLR3 ligands polyinosinic acid-cytidylic acid (poly(I:C)) and TLR7/8 ligand CL097 to model respiratory virus infections and wanted to investigate the interaction between Nontypeable Haemophilus influenzae (Hi) and viral ligands in inflammatory response and apoptosis/necrosis in mononuclear cells (MNC) and respiratory epithelial cells (A549 cell and Human bronchial epithelial cells (HBEpC)). MNCs were isolated from Buffy Coats with Ficoll Isopaque. Cells were stimulated with or without CL097 (1ug/ml), poly(I:C) (25ug/ml), and Hi (10^6 cfu/ml). In selected experiments, MNCs were preincubated with 10μM UO126 (p44/42 MAPK inhibitor) and or 20uM SB203580 (p38 MAPK inhibitor) for 1h before being stimulated with Hi and CL097. After 3,8 and 24h supernatants were harvested for ELISA(IL-8, IL-6 and TNF-α). Cells were collected in lysis buffer for Western blot analysis for p-p44/42 MAPK, p-p38 MAPK and Mcl-1. Cytocentrifuge preparations were stained with live dead staining and viewed by fluorescence microscopy to evaluate the apoptosis in MNCs.

Additive induction of proinflammatory cytokine expression was observed *in vitro* in response to Hi and viral ligands. Inhibition of p38MAPK and p44/42 MAPK signaling by chemical inhibitors blocked the effects of cytokines expression in Hi- and CL097-treated MNC. p38 MAPK and p42/44 MAPK were activated in parallel with increased Mcl-1 stability in MNCs in response to Hi and /or CL097. Inhibition of MAPK function resulted in a greatly increased rate of Mcl-1 turnover. Coincubation with Hi and CL097 led to cell death of MNCs. Based on these experiments, we support the notion that direct cellular damage and the consequences of excessive release of proinflammatory cytokines contribute to the pathogenesis of pathogen induced COPD exacerbations. Moreover, costimulation with viral ligands and Hi infection causes host cells to mount a strong inflammatory response.

6. References

Ahrén IL, Williams DL, Rice PJ, Forsgren A, Riesbeck K: The importance of a beta-glucan receptor in the nonopsonic entry of nontypeable Haemophilus influenzae into human monocytic and epithelial cells. J Infect Dis 184, 150-158 (2001)

Akgul C, Moulding DA, White MR, Edwards SW: In vivo localisation and stability of human Mcl-1 using green fluorescent protein (GFP) fusion proteins. FEBS Lett 478, 72-76 (2000)

Akira S, Takeda K: Toll-like receptor signaling Nat. Rev. Immunol 4, 499-511 (2004)

Akira S, Uematsu S, Takeuchi O: Pathogen recognition and innate immunity. Cell 124, 783–801 (2006)

Alemán M, Schierloh P, de la Barrera SS, Musella RM, Saab MA, Baldini M, Abbate E, Sasiain MC: Mycobacterium tuberculosis triggers apoptosis in peripheral neutrophils involving toll-like receptor 2 and p38 mitogen protein kinase in tuberculosis patients.Infect Immun 72, 5150-5158 (2004)

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA: Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413, 732-738 (2001)

Allen L, Dockrell DH, Pattery T, Lee DG, Cornelis P, Hellewell PG, Whyte MK: Pyocyanin production by Pseudomonas aeruginosa induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. J Immunol 174, 3643–3649 (2005)

Alvarado-Kristensson M, Melander F, Leandersson K, Rönnstrand L, Wernstedt C, Andersson T: p38-MAPK signals survival by phosphorylation of caspase-8 and caspase-3

in human neutrophils. J Exp Med 199, 449-458 (2004)

Aoshiba K, Yasui S, Hayashi M, Tamaoki J, Nagai A: Role of p38-mitogen-activated protein kinase in spontaneous apoptosis of human neutrophils. J Immunol 162, 1692-1700 (1999)

Avadhanula V, Rodriguez CA, Ulett GC, Bakaletz LO, Adderson EE: Nontypeable Haemophilus influenzae adheres to intercellular adhesion molecule 1 (ICAM-1) on respiratory epithelial cells and upregulates ICAM-1 expression. Infect Immun 74, 830-838 (2006)

Ballif BA, Blenis J: Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Differ 12, 397-408 (2001)

Bals R, Hiemstra PS: Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. Eur Respir J 23, 327-333 (2004)

Bhowmik, A, Seemungal, TAR, Sapsford, RJ, et al Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. Thorax 2000;55,114-120

Boyton RJ, Openshaw PJ: Pulmonary defences to acute respiratory infection. Br Med Bull 61, 1-12 (2002)

Bresser P, Out TA, van Alphen L, Jansen HM, Lutter R: Airway inflammation in nonobstructive and obstructive chronic bronchitis with chronic haemophilus influenzae airway infection. Comparison with noninfected patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 162, 947-952 (2000)

Chang L, Karin M: Mammalian MAP kinase signalling cascades. Nature 410, 37-40 (2001)

Chaudhuri N, Whyte MK, Sabroe I: Reducing the toll of inflammatory lung disease. Chest 131, 1550-1556 (2007)

Chin CL, Manzel LJ, Lehman EE, Humlicek AL, Shi L, Starner TD, Denning GM, Murphy TF, Sethi S, Look DC: Haemophilus influenzae from patients with chronic obstructive pulmonary disease exacerbation induce more inflammation than colonizers. Am J Respir Crit Care Med 172, 85–91 (2005)

Choi KS, Park JT, Dumler JS: Anaplasma phagocytophilum delay of neutrophil apoptosis through the p38 mitogen-activated protein kinase signal pathway. Infect Immun 73, 8209-8218 (2005)

Chuang TH, Ulevitch RJ: Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur Cytokine Netw 11, 372-378 (2000)

DeLeo FR. Modulation of phagocyte apoptosis by bacterial pathogens. Apoptosis 9, 399–413 (2004)

Deng X, Ruvolo P, Carr B, May WS Jr: Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. Proc Natl Acad Sci USA 97, 1578-1583 (2000)

Derouet M, Thomas L, Cross A, Moots RJ, Edwards SW: Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1. J Biol Chem 279, 26915-26921 (2004)

Derouet M, Thomas L, Cross A, Moots RJ, Edwards SW: Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis

by increasing the stability of Mcl-1. J Biol Chem 279, 26915-26921 (2004)

Derouet M, Thomas L, Moulding DA, Akgul C, Cross A, Moots RJ, Edwards SW: Sodium salicylate promotes neutrophil apoptosis by stimulating caspase-dependent turnover of Mcl-1. J Immunol 176, 957–965 (2006)

Dessing MC, Florquin S, Paton JC, van der Poll T: Toll-like receptor 2 contributes to antibacterial defence against pneumolysin-deficient pneumococci. Cell Microbiol. 10, 237-246 (2008)

Deszcz L, Gaudernak E, Kuechler E, Seipelt J: Apoptotic events induced by human rhinovirus infection. J Gen Virol 86, 1379-1389 (2005)

Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, Bebien M, Lawrence T, van Rijt LS, Lambrecht BN, Sirard JC, Hussell T: Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. J Exp Med 205, 323-329 (2008)

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C: Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529-1531 (2004)

Dockrell DH, Marriott HM, Prince LR, Ridger VC, Ince PG, Hellewell PG, Whyte MK: Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. J Immunol 171, 5380-5388 (2003)

Droemann D, Aries SP, Hansen F, Moellers M, Braun J, Katus HA, Dalhoff K: Decreased apoptosis and increased activation of alveolar neutrophils in bacterial pneumonia. Chest 117, 1679-1684 (2000).

Edwards MR, Hewson CA, Laza-Stanca V, Lau HT, Mukaida N, Hershenson MB, Johnston SL: Protein kinase R, IkappaB kinase-beta and NF-kappaB are required for human rhinovirus induced pro-inflammatory cytokine production in bronchial epithelial cells. Mol Immunol 44, 1587-1597 (2007)

Edwards SW, Moulding DA, Derouet M, Moots RJ. Regulation of neutrophil apoptosis. Chem Immunol Allergy 83, 204–224 (2003)

Fan M, Chambers TC: Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. Drug Resist Updat 4, 253-267 (2001)

Frasch SC, Nick JA, Fadok VA, Bratton DL, Worthen GS, Henson PM: p38 mitogen-activated protein kinase-dependent and –independent intracellular signal transduction pathways leading to apoptosis in human neutrophils. J Biol Chem 273, 8389-8397 (1998)

Galdiero M, Galdiero M, Finamore E, Rossano F, Gambuzza M, Catania MR, Teti G, Midiri A, Mancuso G: Haemophilus influenzae porin induces Toll-like receptor 2-mediated cytokine production in human monocytes and mouse macrophages. Infect Immun 72, 1204-1209 (2004)

Ge Y, Yoshiie K, Kuribayashi F, Lin M, Rikihisa Y: Anaplasma phagocytophilum inhibits human neutrophil apoptosis via upregulation of bfl-1, maintenance of mitochondrial membrane potential and prevention of caspase 3 activation. Cell Microbiol 7, 29–38 (2005)

Germain M, Duronio V: The N terminus of the anti-apoptotic BCL-2 homologue MCL-1 regulates its localization and function. J Biol Chem 282, 32233-32242 (2007)

Green DR: Apoptotic pathways: ten minutes to dead. Cell 121, 671-674 (2005)

Greve JM, Davis G, Meyer AM, Forte CP, Yost SC, Marlor CW, Kamarck ME, McClelland A: The major human rhinovirus receptor is ICAM-1. Cell 56, 839-847 (1989)

Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M: Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem 280, 5571-5580 (2005)

Gustafsson AB, Gottlieb RA: Bcl-2 family members and apoptosis, taken to heart. Am J Physiol Cell Physiolan 292, C45-51 (2007)

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S: Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526-1529 (2004)

Hewson CA, Jardine A, Edwards MR, Laza-Stanca V, Johnston SL: Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. J Virol 79, 12273-12279 (2005)

Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W, Beckett P, Al Ali M, Chauhan A, Wilson SJ, Reynolds A, Davies DE, Holgate ST: Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. Thorax 60, 1012-1018 (2005)

Howie HL, Shiflett SL, So M: Extracellular signal-regulated kinase activation by Neisseria gonorrhoeae downregulates epithelial cell proapoptotic proteins Bad and Bim. Infect Immun 76, 2715-2721 (2008)

Iannello A, Debbeche O, Martin E, Attalah LH, Samarani S, Ahmad A: Viral strategies for evading antiviral cellular immune responses of the host. J Leukoc Biol 79, 16-35 (2006)

Iwamura C, Nakayama T: Toll-like receptors in the respiratory system: their roles in inflammation. Curr Allergy Asthma Rep 8, 7-13 (2008)

Jiang X, Wang X: Cytochrome C-mediated apoptosis. Annu Rev Biochem 73, 87-106 (2004)

Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S: Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol 3, 499 (2002)

Khair OA, Davies RJ, Devalia JL: Bacterial-induced release of inflammatory mediators by bronchial epithelial cells. Eur Respir J 9, 1913-1922 (1996)

Lee SM, Kleiboeker SB: Porcine reproductive and respiratory syndrome virus induces apoptosis through a mitochondria-mediated pathway. Virology 365, 419-434 (2007)

Le Goffic R, Balloy V, Lagranderie M, Alexopoulou L, Escriou N, Flavell R, Chignard M, Si-Tahar M: Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. PLoS Pathog 2, e53 (2006)

Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, Chignard M, Si-Tahar M: Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. J Immunol 178, 3368-3372 (2007)

Lindemans CA, Coffer PJ, Schellens IM, de Graaff PM, Kimpen JL, Koenderman L: Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism. J Immunol 176, 5529-5537 (2006)

Liu H, Perlman H, Pagliari LJ, Pope RM: Constitutively activated Akt-1 is vital for the survival of human monocyte-differentiated macrophages. Role of Mcl-1, independent of

nuclear factor (NF)-kappaB, Bad, or caspase activation. J Exp Med 194, 113–126 (2001)

Lorenz E, Chemotti DC, Jiang AL, McDougal LD: Differential involvement of toll-like receptors 2 and 4 in the host response to acute respiratory infections with wild-type and mutant Haemophilus influenzae strains. Infect Immun 73, 2075-2082 (2005)

Marriott HM, Bingle CD, Read RC, Braley KE, Kroemer G, Hellewell PG, Craig RW, Whyte MK, Dockrell DH: Dynamic changes in Mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance. J Clin Invest 115, 359-368 (2005)

Marsden VS, Strasser A: Control of apoptosis in the immune system. Bcl-2, BH3-only proteins and more.Annu Rev Immuno l21,71-105(2003)

Martin HJ, Lee JM, Walls D, Hayward SD: Manipulation of the toll-like receptor 7 signaling pathway by Epstein-Barr virus. J Virol 81, 9748-9758 (2007)

Mayer AK, Muehmer M, Mages J, Gueinzius K, Hess C, Heeg K, Bals R, Lang R, Dalpke AH: Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells. J Immunol 178, 3134-3142 (2007)

McLean JE, Ruck A, Shirazian A, Pooyaei-Mehr F, Zakeri ZF: Viral manipulation of cell death. Curr Pharm 14, 198-220 (2008)

McNamee LA, Harmsen AG: Both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary Streptococcus pneumoniae infection. Infect Immun 74, 6707-6721 (2006)

Michels J, Johnson PW, Packham G: Mcl-1.Int J Biochem Cell Biol 37, 267-271 (2005)

Mogensen TH, Paludan SR, Kilian M, Ostergaard L: Live Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. J Leukoc Biol 80, 267-277 (2006)

Monick MM, Cameron K, Staber J, Powers LS, Yarovinsky TO, Koland JG, Hunninghake GW: Activation of the epidermal growth factor receptor by respiratory syncytial virus results in increased inflammation and delayed apoptosis. J Biol Chem 280, 2147-2158 (2005)

Morris GE, Parker LC, Ward JR, Jones EC, Whyte MK, Brightling CE, Bradding P, Dower SK, Sabroe I: Cooperative molecular and cellular networks regulate Toll-like receptor-dependent inflammatory responses. FASEB J 20, 2153-2155 (2006)

Moulding DA, Akgul C, Derouet M, White MR, Edwards SW: BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. J Leukoc Biol 70, 783–792 (2001)

Murphy TF, Brauer AL, Schiffmacher AT, Sethi S: Persistent colonization by *Haemophilus influenzae* in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 170, 266–272 (2004)

Muzio M, Bosisio D, Polentarutti N, D'amico G, Stoppacciaro A, Mancinelli R, van't Veer C, Penton-Rol G, Ruco LP, Allavena P, Mantovani A: Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol 164, 5998-6004 (2000)

Najib S, Sánchez-Margalet V: Human leptin promotes survival of human circulating blood monocytes prone to apoptosis by activation of p42/44 MAPK pathway. Cell Immunol 220, 143-149 (2002)

Navas TA, Nguyen AN, Hideshima T, Reddy M, Ma JY, Haghnazari E, Henson M, Stebbins EG, Kerr I, O'Young G, Kapoun AM, Chakravarty S, Mavunkel B, Perumattam J, Luedtke G, Dugar S, Medicherla S, Protter AA, Schreiner GF, Anderson KC, Higgins LS: Inhibition of p38alpha MAPK enhances proteasome inhibitor-induced apoptosis of myeloma cells by modulating Hsp27, Bcl-X(L), Mcl-1 and p53 levels in vitro and inhibits tumor growth in vivo. Leukemia 20, 1017-1027 (2006)

Naylor EJ, Bakstad D, Biffen M, Thong B, Calverley P, Scott S, Hart CA, Moots RJ, Edwards SW: Haemophilus influenzae induces neutrophil necrosis: a role in chronic obstructive pulmonary disease. Am J Respir Cell Mol Biol 37, 135-143 (2007)

Newcomb DC, Sajjan US, Nagarkar DR, Goldsmith AM, Bentley JK, Hershenson MB: Cooperative effects of rhinovirus and TNF-{alpha} on airway epithelial cell chemokine expression. Am J Physiol Lung Cell Mol Physiol 293, 1021-1028 (2007)

N'Guessan PD, Schmeck B, Ayim A, Hocke AC, Brell B, Hammerschmidt S, Rosseau S, Suttorp N, Hippenstiel S: Streptococcus pneumoniae R6x induced p38 MAPK and JNK-mediated caspase-dependent apoptosis in human endothelial cells. Thromb Haemost 94, 295-303 (2005)

Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ: Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1 Nature 426, 671– 676 (2003)

Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, Fabbri LM, Johnston SL: Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. Am J Respir Crit Care Med 173, 1114-1121 (2006)

Passariello C, Schippa S, Conti C, Russo P, Poggiali F, Garaci E, Palamara AT:

Rhinoviruses promote internalisation of Staphylococcus aureus into non-fully permissive cultured pneumocytes. Microbes Infect 8, 758-766 (2006)

Petlickovski A, Laurenti L, Li X, Marietti S, Chiusolo P, Sica S, Leone G, Efremov DG: Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. Blood 105, 4820-4827 (2005)

Phipps S, Lam CE, Mahalingam S, Newhouse M, Ramirez R, Rosenberg HF, Foster PS, Matthaei KI: Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. Blood 110, 1578-1586 (2007)

Proud D, Chow CW: Role of viral infections in asthma and chronic obstructive pulmonary disease. Am J Respir Cell Mol Biol 35, 513-518 (2006)

Punturieri A, Copper P, Polak T, Christensen PJ, Curtis JL: Conserved nontypeable Haemophilus influenzae-derived TLR2-binding lipopeptides synergize with IFN-beta to increase cytokine production by resident murine and human alveolar macrophages. J Immunol 177, 673-80 (2006)

Ritter M, Mennerich D, Weith A, Seither P: Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. J Inflamm (Lond) 29, 2-16 (2005)

Roux PP, Blenis J: ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68, 320-344 (2004)

Rudd BD, Burstein E, Duckett CS, Li X, Lukacs NW: Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. J Virol 79, 3350-3357 (2005)

Sajjan US, Jia Y, Newcomb DC, Bentley JK, Lukacs NW, LiPuma JJ, Hershenson MB: H influenzae potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression. FASEB J 20, 2121-2123 (2006)

Sakai A, Koga T, Lim JH, Jono H, Harada K, Szymanski E, Xu H, Kai H, Li JD: The bacterium, nontypeable Haemophilus influenzae, enhances host antiviral response by inducing Toll-like receptor 7 expression: evidence for negative regulation of host anti-viral response by CYLD. FEBS J 274, 3655-3668 (2007)

Saffar AS, Dragon S, Ezzati P, Shan L, Gounni AS: Phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase regulate induction of Mcl-1 and survival in glucocorticoid-treated human neutrophils. J Allergy Clin Immunol 121, 492-498 (2008)

Sethi S, Muscarella K, Evans N, Klingman KL, Grant BJ, Murphy TF: Airway inflammation and etiology of acute exacerbations of chronic bronchitis. Chest 118, 1557–1565 (2000)

Sethi S, Evans N, Grant BJ, Murphy TF: New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. N Engl J Med 347, 465-471 (2002)

Sethi S, Evans N, Grant BJB, Murphy TF: Acquisition of a new bacterial strain and occurrence of exacerbations of chronic obstructive pulmonary disease. N Engl J Med; 347, 465–471 (2002)

Sharma K, Wang RX, Zhang LY, Yin DL, Luo XY, Solomon JC, Jiang RF, Markos K, Davidson W, Scott DW, Shi YF: Death the Fas way: regulation and pathophysiology of CD95 and its ligand. Pharmacol Ther 88, 333-347 (2000)

Sparrow RL, Tippett E: Discrimination of live and early apoptotic mononuclear cells by the fluorescent SYTO 16 vital dye. J Immunol Methods 305, 173-187 (2005)

Speshock JL, Doyon-Reale N, Rabah R, Neely MN, Roberts PC: Filamentous influenza A virus infection predisposes mice to fatal septicemia following superinfection with Streptococcus pneumoniae serotype 3. Infect Immun 75, 3102-3111 (2007)

Stankiewicz W, Dabrowski MP, Chcialowski A, Plusa T: Cellular and cytokine immunoregulation in patients with chronic obstructive pulmonary disease and bronchial asthma. Mediators Inflamm 11, 307-312 (2002)

Tan YJ, Lim SG, Hong W: Regulation of cell death during infection by the severe acute respiratory syndrome coronavirus and other coronaviruses. Cell Microbiol 9, 2552-2261 (2007)

Thorburn K, Harigopal S, Reddy V, Taylor N, van Saene HK: High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis. Thorax 61, 611-615 (2006)

Tissari J, Sirén J, Meri S, Julkunen I, Matikainen S: IFN-alpha enhances TLR3-mediated antiviral cytokine expression in human endothelial and epithelial cells by up-regulating TLR3 expression. J Immunol 174, 4289-4294 (2005)

Uren RT, Dewson G, Chen L, Coyne SC, Huang DC, Adams JM, Kluck RM: Mitochondrial permeabilization relies on BH3 ligands engaging multiple prosurvival Bcl-2 relatives, not Bak. J Cell Biol 177, 277-287 (2007)

van Delft MF, Huang DC: How the Bcl-2 family of proteins interact to regulate apoptosis. Cell Res 16, 203-213 (2006)

van Zandbergen G, Gieffers J, Kothe H, Rupp J, Bollinger A, Aga E, Klinger M, Brade H, Dalhoff K, Maass M, Solbach W, Laskay T: Chlamydia pneumoniae multiply in neutrophil

granulocytes and delay their spontaneous apoptosis. J Immunol 172, 1768-1776 (2004)

Villunger A, O'Reilly LA, Holler N, Adams J, Strasser A: Fas ligand, Bcl-2, granulocyte colony-stimulating factor, and p38 mitogen-activated protein kinase: Regulators of distinct cell death and survival pathways in granulocytes. J Exp Med. 192, 647-658 (2000)

Wagner H: The immunobiology of the TLR9 subfamily Trends. Immunol 25, 381-386 (2004)

Wesche-Soldato DE, Swan RZ, Chung CS, Ayala A: The apoptotic pathway as a therapeutic target in sepsis. Curr Drug Targets 8, 493-500 (2007)

Wieland CW, Florquin S, Maris NA, Hoebe K, Beutler B, Takeda K, Akira S, van der Poll T: The MyD88-dependent, but not the MyD88-independent, pathway of TLR4 signaling is important in clearing nontypeable haemophilus influenzae from the mouse lung. J Immunol 175, 6042-6049 (2005)

Wilkinson TM, Hurst JR, Perera WR, Wilks M, Donaldson GC, Wedzicha JA: Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD. Chest 129, 317-324 (2006)

Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM, Huang DC: Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 315, 856-859 (2007)

Wong WW, Puthalakath H: Bcl-2 family proteins: The sentinels of the mitochondrial apoptosis pathway. IUBMB Life 60, 390-397 (2008)

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270, 1326-1331 (1995)

Yang-Yen HF: Mcl-1: a highly regulated cell death and survival controller. J Biomed Sci 13, 201-204 (2006)

Zhang N, Hartig H, Dzhagalov I, Draper D, He YW: The role of apoptosis in the development and function of T lymphocytes. Cell Res 15, 749–769 (2005)

Zhang Y, Ting AT, Marcu KB, Bliska JB: Inhibition of MAPK and NF-κappa B pathways is necessary for rapid apoptosis in macrophages infected with Yersinia. J Immunol 174, 7939-7949 (2005)

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