A Dissertation for the Degree of Doctor of Philosophy

The Role of TRIF and RIP2 and Inhibitory effect of *Mycobacterium tuberculosis* derived Heat shock protein 70 on Development of Allergic Airway Inflammation

알러지성 기도 염증 발생에서 TRIF과 RIP2의 역할 및 결핵균 유래 열충격 단백질70의 억제효과

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ABSTRACT

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Allergic asthma is the chronic lung disease influencing over 300 million people of all ages and characterized by airway hyperreactivity and mucous oversecretion induce the intermittent airway obstruction. Medication to control the phenotypes of allergic asthma has been major strategies for asthma treatment. However, they are not complete cure for allergic asthma.

Pattern recognition receptors (PRRs) are component of innate immunity

and initiate the immune response by recognizing the microorganism-derived materials. Previous studies reported that PRRs are related to the development of allergic asthma and there have been many report to evalulate the therapeutic functions of toll-like receptors (TLR) ligand in allergic asthma. These are suggesting that studying about the role of PRRs signaling in allergic asthma and finding effective materials are necessary for asthma treatment.

In this research, to determine the role of PRRs signaling in allergic asthma, allergic airway inflammation mice model was used. And the effect of *Mycobacterium tuberculosis* derived heat shock protein 70 (Mtb Hsp70) which is potent immune modulator and ligand of Toll-like receptor was evaulated using allergic airway inflammation mice model. In addition, bone marrow-derived dendritic cells were used for studying the role of TLR in activation of dendritic cells by Mtb Hsp70.

In chapter I, the role of TRIF, adaptor molecule of TLR3 and TLR4 signaling, in the development of allergic airway inflammation were studied. I confirmed the increased airway inflammation and Th2 immune response in OVA treated mice. However, histopathological assessment, cytokine analysis, cellular analysis in bronchoalveolar fluid, and serum immunoglobulin analysis revealed that the severity of inflammation in airway inflammation in TRIF-deficient mice

was comparable to that in WT mice.

In chapter II, the role of RIP2, adaptor molecule of Nod1 and Nod2 signaling, in the development of allergic airway inflammation were studied using mice model. Deficiency of RIP2 is not critical to develop allergic airwa inflammation in mice.

In chapter III, the beneficial effect of Mtb Hsp70 on allergic airway inflammation and their molecular mechanism were studied. In allergen induced allergic airway inflammation model, co-administration of purified recombinant Mtb Hsp70 in sensitization phase reduced indicaotors associated with allergic inflammation. However, these effects of Mtb Hsp70 were abolished in TLR2 and TLR4-deficient mice. In conclusion, our results revealed the inhibitory effect of Mtb Hsp70 on allergic airway inflammation and TLR2 and TLR4 signaling involed in these effects.

In chapter IV, dendritic cells were used for studying the role of TLR2 and TLR4 signaling in effect of Mtb Hsp70. Both TLR2 and TLR4 were required for activation of dendritic cells by purified recombinant Mtb Hsp70. In addition, TRIF and MyD88 are also requried for activation of dendritic cells by Mtb Hsp70.

In chapter V, Nod2 signaling has synergistic effect on immune activation induced by TLRs activation. So, the effect of MDP, a ligand of Nod2, on dendritic

cell activation by Mtb Hsp70 was studied. *In vitro* experiments revealed that MDP enhanced the effect of Mtb Hsp70 on activation of dendritic cells.

In this study, it was observed that TRIF and RIP2 did not have critical role in development of allergic airway inflammation in mice model. And Mtb Hsp70 attenuated the development of allergic airway inflammation and these effects are mediated by TLR2 and TLR4 signaling. Study using dendritic cells showed that TLR2 and TLR4 signaling pathway are related to the effect of Mtb Hsp70 and MDP increased the effects of Mtb Hsp70. I expect this result may help to understand the function of pattern recognition receptors in allergic asthma and develop novel therapeutic agent.

Keywords : Pattern recognition receptors, Toll-like receptors, Nod-like receptors, *Mycobacterium tuberculosis*, Heat shock protein 70, Dendritic cells, Allergic airway inflammation

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LIST OF ABBREVIATONS

BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette–Guérin
BMDCs	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	5- 6-Carboxyfluorescein diacetate succinimidyl ester
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDM	House dust mite
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mtb Hsp70	*Mycobacterium tuberculosis* derived heat shock protein 70
MSC	Mucus secreting cell
MyD88	Myeloid differentiation primary response 88
NF-κB	Myeloid differentiation primary response 88
PAMPs	Pathogen-associated molecular patterns

PAS	Periodic acid-Schiff
PI	Propidium iodide
PMB	Polymyxin B
TIR	Intracellular Toll/IL-1R
TLR	Toll like receptor
TRIF	TIR-domain-containing adapter-inducing interferon-β
OD	Optical density
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
WT	Wild type

LITERATURE REVIEW

1. Pattern Recognition Receptors (PRRs)

Pattern recognition receptors (PRRs) are responsible for sensing the presence of microorganisms by recognizing structures conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs)(Takeuchi and Akira, 2010). Currently, four classes of PRR familes, toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs), have been identified (Takeuchi and Akira, 2010). These PRRs are expressed not only in immune cells such as macrophages and DCs but also in various nonprofessional immune cells (Takeuchi and Akira, 2010) and associated to various type of immune response.

1.1. Toll-like receptors signaling pathway

TLRs are one of the best-characterized PRR family are responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes (Akira et al., 2006). TLRs are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain (Takeuchi and Akira, 2010). Ten TLRs have been identified in humans and twelve in mice (Takeuchi and Akira, 2010).

TLR2 senses the lipoproteins of bacteria and mycoplasma (Takeuchi and Akira, 2010). TLR2 recognizes its ligands by forming a heterodimer with either TLR1 or TLR6 (Takeuchi and Akira, 2010). The resulting TLR1/TLR2 and TLR6/TLR2 complexes recognize distinct ligands (triacyl and diacyl lipoproteins, respectively) (Takeuchi and Akira, 2010). The crystal structures of the extracellular domains of TLR2, TLR1, and TLR6 revealed that they form M-shaped structures and that their cognate ligands interact with internal pockets formed by the TLR1/TLR2 or TLR2/6 heterodimers (Jin et al., 2007). Activation of TLR2 induces the production of various proinflammatory cytokines in macrophages and DCs (Barbalat et al., 2009; Jin et al., 2007). And cellular responses to TLR2 ligands differ depending on the cell types involved (Barbalat et al., 2009).

TLR3 detects viral double-stranded RNA in the endolysosome (Takeuchi and Akira, 2010). Polyinosinic polycytidylic acid (poly I:C), a synthetic dsRNA analog can be recognized by TLR3 (Takeuchi and Akira, 2010). The crystal structure of TLR3 bound to dsRNA revealed that dsRNA binds to the N-terminal and C-terminal portions of TLR3 LRRs, and this ligand binding dimerizes two TLR3 molecules (Choe et al., 2005).

TLR4 recognizes lipopolysaccharide (LPS) together with myeloid

differentiation factor 2 (MD2) on the cell surface (Takeuchi and Akira, 2010). The crystal structure of a complex comprising TLR4, MD2, and LPS revealed that two complexes of TLR4-MD2-LPS interact symmetrically to form a TLR4 homodimer (Park et al., 2009a). TLR5 is highly expressed by DCs of the lamina propria (LPDCs) in the small intestin, where it recognizes flagellin from flagellated bacteria (Takeuchi and Akira, 2010).

Mouse TLR7 and human TLR7/8 recognize single-stranded (ss) RNAs from RNA viruses, as well as small purine analog compounds (imidazoquinolines) (Takeuchi and Akira, 2010). TLR7 also detects RNAs from bacteria such as Group B Streptococcus in endolysosomes in conventional DCs (cDCs) (Mancuso et al., 2009). TLR9 senses unmethylated DNA with CpG motifs derived from bacteria and viruses (Takeuchi and Akira, 2010). In addition to DNA, TLR9 also recognizes hemoxoin, a crystalline metabolite of hemoglobin produced by the malaria parasite (Coban et al., 2005).

TLR10 is related to TLR1 and TLR6 based on sequence similarity (Takeuchi and Akira, 2010). TLR10 seems to be functional in humans but mouse TLR10 is not functional (Takeuchi and Akira, 2010). And the ligand for TLR10 has not been identified yet (Takeuchi and Akira, 2010).

TLR11, which is present in mice but not in humans, shows close

homology to TLR5 (Takeuchi and Akira, 2010). TLR11 recognizes uropathogenic bacteria and a profilin-like molecule derived from the intracellular protozoan *Toxoplasma gondii* (Yarovinsky et al., 2005).

Recruitment of TIR domain-containing adaptor molecules is required to signal cascades of TLRs signaling (Akira et al., 2006). There are five TIR domain-containing adaptors including Myeloid differentiation primary response 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF; also known as TICAM-1), toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP), TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM) (Takeuchi and Akira, 2010).

Signal transduction of TLRs is usually classified into two pathways depending on the adaptor molecules, MyD88 and TRIF. MyD88 is an adapter molecule of various TLRs, except TLR3 (Takeuchi and Akira, 2010). MyD88 is composed of a death domain (DD) in addition to a TIR domain (Takeuchi and Akira, 2010). TLR2 and TLR4 signaling requires TIRAP for bridging between TLR and MyD88 (Takeuchi and Akira, 2010). MyD88 interacts with IL-1R-associated kinase (IRAK)-4, a serine/threonine kinase with an N-terminal death domain, which activates other IRAK family members, IRAK-1 and IRAK-2 (Kawagoe et al., 2008; Takeuchi and Akira, 2010). The IRAKs then dissociate

from MyD88 and interact with TNFR-associated foactor 6 (TRAF6), which acts as an E3 ubiquitin protein ligase (Takeuchi and Akira, 2010). Together with an E2 ubiquitin-conjugating enzyme complex comprising Ubc13 and Uev1A, TRAF6 αcatalyzes the formation of a lysine 63 (K63)-linked polyubiquitin chain on TRAF6 itself as well as the generation of an unconjugated free polyubiquitin chain (Xia et al., 2009).

A complex of TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3 is activated by the unconjugated free K63 polyubiquitin chain and phosphorylates IκB kinase (IKK)-β and MAP kinase kinase 6 (Takeuchi and Akira, 2010). Subsequently, the IKK complex, composed of IKK-α, IKK-β, and NF-κB essential modulator (NEMO), phosphorylates IκBa, and NF-κB inhibitory protein (Takeuchi and Akira, 2010). Phosphorylated IκB undergoes degradation by the ubiquitin-proteasome system, thereby freeing NF-κB to translocate into the nucleus and activate expression of proinflammatory cytokine genes (Takeuchi and Akira, 2010). Activation of the MAP kinases cascade is responsible for the formation of another transcription factor complex, AP-1, that targets cytokine genes (Takeuchi and Akira, 2010).

TIR-domain-containing adapter-inducing interferon-β (TRIF) is recruited to TLR3 and TLR4 and activates an alternative pathway that triggers the

activation of NF-κB, mitogen-activated protein kinases (MAPKs), and interferon regulatory factor 3 (IRF3) (Kawai and Akira, 2006; Kumar et al., 2009). TLR4 requires TRAM for activating TRIF (Takeuchi and Akira, 2010). TRIF associates with TRAF3 and TRAF6 through TRAF-binding motifs present in its N-terminal portion (Takeuchi and Akira, 2010). TRIF also contains a C-terminal receptor-interacting protein (RIP) homotypic interaction motif (RHIM) and interacts with RIP1 and RIP3 viathis motif (Takeuchi and Akira, 2010). TRAF3 is important for activating two IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKK-*i* (also known as IKK-ε) (Hacker et al., 2006). TRAF3 undergoes K63-linked auto-ubiquitination in response to TLR3 and acts as an E3 ubiquitin ligase (Takeuchi and Akira, 2010). TBK1 and IKK-i phosphorylate IRF3 and IRF7; IRF3 and IRF7 dimers translocate to the nucleus, resulting in induction of type I IFN-inducible genes (Takeuchi and Akira, 2010).

1.2. Nod1 and Nod2 signaling pathway

Nucleotide-binding oligomerization domain-containing protein 1 (Nod1) and nucleotide-binding oligomerization domain-containing protein 2 (Nod2) are cytoplasmic receptors that are composed of a central nucleotide-binding domain (NOD) and C-terminal LRRs (Inohara et al., 2005). Initial searches of genomic

databases for proteins with homology to the apoptosis regulator, apoptotic protease activating factor (Apaf)-1, and its nematode homolog, cell death protein (CED)-4, revealed two related proteins, Nod1 (CARD4) and Nod2 (CARD15) (Inohara et al., 2005). Like Apaf-1 and CED-4, Nod1 and Nod2 contain amino-terminal caspase-recruitment domain (CARDs) linked to a centrally placed NOD domain (Inohara et al., 2005). But, unlike Apaf-1, Nod1 and Nod2 possess LRRs in their carboxyl termini (Inohara et al., 2005).

Nod1 and Nod2 recognize the strucures of bacterial peptidoglycans, g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively (Hasegawa et al., 2008; Takeuchi and Akira, 2010). RIP2 (also called RICK and CARDIAK) a serine/threonine kinase mediates Nod1 and Nod2 signaling via CARD-CARD interaction with Nod1 or Nod2. Upon peptidoglycan detection, binding of Nod1 or Nod2 and the adaptor protein Rip2 via CARD-CARD interaction triggers proinflammatory pathways such as NF-κB and the mitogen-activated protein (MAP) kinase p38, JNK and ERK for production of proinflammatory mediators (Chen et al., 2009; Inohara et al., 2000; Kobayashi et al., 2002).

2. Allergic Airway Inflammation

2.1. Allergic Asthma

Asthma is a chronic airway inflammatory disease characterized by airway obstruction (Busse and Lemanske, 2001; Masoli et al., 2004; Shifren et al., 2012; To et al., 2012). Based on clinical and laboratory findings, bronchial asthma can be classified into allergic and nonallergic forms (Walker et al., 1992). Although this concepts are probably the most commonly used, other catergories were proposed by Wenzel at 2006 can give the more detail explanation about the character of allergic asthma (Wenzel, 2006). First catergory is defined by clinical or physiological phenotypes relevant to asthma; level of severity (from mild to severe), the frequency of exacerbations, the presence of chronic airflow restriction, response to treatment such as resistance to steroids, and the age of asthma onset (Wenzel, 2006). Second catergory can also be defined on the basis of specific triggers including exercise, environmental allergens, occupational allergens and irritants, drugs (such as aspirin), and menses (Wenzel, 2006). Last, patterns of inflammation such as the presence or absence of particular inflammatory cell types (e.g. eosinophils or neutrophils) can be catergories (Wenzel, 2006).

Asthma has different character depending on the the age of the phenotype

onset. Allergic asthma usually starts during childhood and is characterized by allergen-dependent, often seasonal symptoms with positive skin tests to allergens and elevated total and allergen-specific serum IgE (Walker et al., 1992). In contrast, nonallergic asthma usually begins in adulthood, is more severe, has no elevation in serum IgE, and is associated with sinusitis and nasal polyposis (Walker et al., 1992).

Allergic asthma is associated with active T-cell immune responses to inhaled allergens that are skewed toward the Th2 phenotype associated of production of Th2 cytokine such as IL-4, IL5, IL-9, and IL-13 (Agrawal and Shao, 2010). These Th2 cytokines enhanced allergic inflammatory cytokine responses. IL-4 or IL-13 binding to B cells induced the IgE production via STAT-6 mediated pathway (Wills-Karp et al., 1998). Binding of allergen specific IgE to its receptors on the surface of mast cells, basophils, lymphocytes, eosinophils, platelets and macrophages leads the inflammatory response in tissue (Busse and Lemanske, 2001). These specific immune responses to allergen cause the characteristic phenotype and symptoms of allergic asthma. Pathogenesis of allergic asthma can be explained by modification of airway such as bronchoconstriction, airway hyperresponsiveness and airway remodeling and specific immune response associated to airway inflammation.

Dominant symptoms in asthma is airway narrowing causing the interference of airflow (National Heart and Asthma, 2007). Exposure to allegen induced the acute exacerbations of asthma such as bronchial smooth muscle contraction (National Heart and Asthma, 2007). Allergen-induced acute bronchoconstriction is related to the mediators (histamine, tryptase, leukotrienes, and prostaglandins) released from mast cell by IgE-dependent pathway directly contract airway smooth muscle (Busse et al., 1999). In persistent stage, edema and structural changes in airway can be the cause of limitation of air flow (National Heart and Asthma, 2007). And these changes may not respond to usual treatment (National Heart and Asthma, 2007).

Inflammation is underlying mechanism of pathogenesis of allergic asthma which various inflammatory cells, mediators, and immunoglobulins are involved in (National Heart and Asthma, 2007). Almost every type of inflammatory cells are involved in symptoms of allergic asthma. Lymphocyte associated to production of Th2 cytokine (e.g., IL-4, IL-5, and IL-13) and allergen induced IgE are related to phenotyep of allergic asthma (Akbari et al., 2006; Cohn et al., 2004; Larche et al., 2003; Robinson, 2000). Mast cells releases bronchoconstrictor mediators (histamine, cysteinyl-leukotrienes, prostaglandin D2) (Boyce, 2003; Galli et al., 2005; Jarjour and Kelly, 2002; Robinson, 2004). Increasing of

eosinophils in the airways of asthma patients is observed and often correlate with severity of asthma (Chu and Martin, 2001; Leckie et al., 2000; Sampson, 2000; Williams, 2004). They contain inflammatory enzymes, generate leukotrienes, and express a wide variety of pro-inflammatory cytokines (Leckie et al., 2000). Neutrophils are increased in the airways and sputum of persons who have severe asthma (Fahy et al., 1995). Macrophages are the most numerous cells in the airways and also can be activated by allergens through low-affinity IgE receptors to release inflammatory mediators and cytokines that amplify the inflammatory response (Peters-Golden, 2004).

Inflammatory mediators such as cytokines have pivotal role in pathogenesis of allergic asthma. IL-5 is needed for eosinophil differentiation and survival, IL-4 is important for Th2 cell differentiation, and IL-13 is important for IgE formation. Cysteinyl-leukotriene are potent bronchoconstrictors derived mainly from mast cells and leukotriene B4 contribute to recruitment of neutrophils (Busse, 1996; Gelfand and Dakhama, 2006; Leff, 2001). Serum IgE levels are corellated with asthma (Burrows et al., 1989). Inhaled allergens cross-link IgE bound to these effector cells, which results in aggregation of the receptors (Chang, 2000). This cross-linking and aggregation causes the immediate release of mediators such as histamine, arachidonic acid, and cytokines such as TNF-a,

IL-4, and IL-5 (Williams and Galli, 2000). Primary sensitization also leads to production of IgG_1 (Williams et al., 2012). This immunoglobulin play a role in allergic asthma via FcγR on the cell surface (Williams et al., 2012).

Medication have been fundamental strategy for allergic asthma treatment (Fanta, 2009). Quick-acting beta-adrenergic agonists administratered by inhalation are the most effective therapy for rapid reversal of airflow obstruction and prompt relief of asthmatic symptoms (Fanta, 2009). Most widely used are the short-acting, beta2-selective, adrenergic agonists : albuterol (commonly known as salbutamol), levalbuterol, and pirbuterol (Fanta, 2009). The short-acting beta-agonists all have an onset of action in 5 minutes or less, with a peak effect in 30 to 60 minutes and a duration of action of 4 to 6 hours (Nelson, 1995). Inhaled corticosteroids has the greatest effect in helping patients achieve well-controlled asthma (Fanta, 2009). The inhaled long-acting beta-agonists, salmeterol and formoterol,have largely replaced the older long-acting bronchodilators-orally administered, slow-releas albuterol and theophylline (Fanta, 2009). Cysteinyl leukotriene-receptor antogonists, motelukast (Singulair), zafirlukast (Accolate), and pranlukast (Zyflo) block the action of leukotriene C4, D4, and E4 at the type 1 cysteinyl leukotriene receptor are an alternative treatment for mild persistent asthma (Drazen et al., 1999; Fanta, 2009). The anti-IgE monoclonal antibody, omalizumab, is the first

biologic immunoregulatory agent available to treat asthma (Fanta, 2009). It binds to the portion of IgE that recognizes its high-affinity receptor (FceR1) on the surface of mast cells and basophils (Fanta, 2009).

2.2. Effects of TLR signalings on Allergic Asthma

Activation of TLRs signaling is involved in pathogenesis of allergic asthma. And they are associated with the basal mechanism connecting the infection and allergic disease (Bortolatto et al., 2008; Eisenbarth et al., 2002; Hammad et al., 2009; Hollingsworth et al., 2004; Shalaby et al., 2013; Stowell et al., 2009; Torres et al., 2010)

Pam3CSK4, ligand of TLR2, ameliorates established allergic airway inflammation by promoting Th1 responses such as IFN-γ and IL-12 production (Patel et al., 2005). Double-stranded RNA (dsRNA) increased the lung inflammation, airway hyperresponsiveness, and antigen-specific Th2 responses in OVA-sensitized mice through TLR3-TRIF (Toll/IL-1R domain-containing adaptor-inducing IFN-β) pathway (Torres et al., 2010). Effects of LPS, ligands of TLR4, were very various depend on the concentration, administration period and genetic background of host. Exposure to airborne endotoxin LPS in infancy may protect against asthma by promoting enhanced Th1 response and tolerance to

allergens. On the other hand, later in life, it adversely affects patient with asthma (Reed and Milton, 2001). LPS enhanced the Th2 response to inhaled antigen (Eisenbarth et al., 2002; Kim et al., 2007). Administration of LPS with OVA in challenge stage enhanced airway eosinophilia, without affecting IgE levels of AHR (Delayre-Orthez et al., 2004). However, exposure to LPS during sensitization prevented the further endotoxin induced exacerbation of airway inflammation (Delayre-Orthez et al., 2005; Delayre-Orthez et al., 2004). Intranasal administration of a TLR7 agonist R848 suppressed experimental asthma by inducing type I interferon production and inhibiting Th2 responses (Xirakia et al., 2010). Oral administration of CpG-ODN, a TLR9 agonist, could prevent eosinophilic airway inflammation (Kitagaki et al., 2006).

Currently, ther have been many research to develop novel therapeutic agent with TLR agonist. Monophosphoryl lipid A derived from LPS of Salmonella minnesota R595 has been investigated in allergic rhinitis and immunotherapy (Casale et al., 2006). Effect of intranasal treatment of VTX-1463, TLR8 agonist, on allergic patients was studied (Horak et al., 2011). CpG oligodeoxynucleotides , ligand of TLR8, have been studied as adjuvants for immuno therapy for allergic asthma (Nguyen and Casale, 2011).

3. Heat shock proteins

3.1. Function of Heat shock protein 70

Heat shock proteins are molecular chaperones inside cells, regulating conformational change, translocation, assembly and degradation of cellular proteins (Torigoe et al., 2009). They have important roles in cellular protection against various stress such as ischemia, heat stress and oxidative stress (Lanneau et al., 2008). Mammalian Hsps have been classified according to their molecular size : Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps (Schmitt et al., 2007). Hsp90s is involved in signaling by acting at a late stage of folding of substrates. Hsp60 play a role in early stages of folding. Hsp100 have a role in degradation and disaggregation cooperating with protease ring and Hsp70 respectively (Saibil, 2013). Hsp70 is 70kDa size heat shock protein and implicated in protein transport assembly and synthesis (Brenu et al., 2013; Wang et al., 2001b). Hsp70 is comprised of an N-terminal nucleotide-binding domain with ATPas activity and a C terminal containing a substrated-binding domain (Cegielska and Georgopoulos, 1989; Schlecht et al., 2011). Human Hsp70 family consists of at least eight members and can be categorized by differences in subcellular localization, tissue-specific expression and stress-induced expression (Murphy, 2013; Ritossa, 1962;

Saibil, 2013). Hsp70 have been highly conserved, showing 60~78% identity among eukaryotes and 40~60% identity between *E. coli* Hsp70 and the eukaryotic Hsp70s (Craig et al., 1990). In addition to role as molecular chaperone, Hsp70 regulates apoptotic pathway (Brenu et al., 2013). Hsp70 inhibits apoptosis induced by interferon-inducible double-stranded RNA-dependent protein kinase (PKR) (Pang et al., 2002) and over-expression of Hsp70 increased antiapoptotic protein, Bcl-2 (Yenari et al., 2005). Hsp70 interacts with the mitochondria through death receptor signaling where is binds to death receptors DR4 and DR5 impeding TNF-related apoptosis inducing ligand (TRAIL) (Pang et al., 2002).

3.2. Effect of Hsp70 on Immune Response

Including their roles in the cytosol, Hsps play roles in the stimulation of the immune system when located in the extracellular space or on the plasma membrane (Schmitt et al., 2007). Exogenous Hsp stimulate IL-1β, TNF-α, and IL-6 production in human monocyte (Asea et al., 2000) and induced the release of nitric oxide by macrophages and dendritic cells (Panjwani et al., 2002). CD40, LOX-1, CD36, Toll-like receptors (TLRs), and SR-A of antigen presenting cells (APCs) act as receptors for Hsps (Binder et al., 2004). Ligation of Hsps with these receptor lead to receptor mediated endocytosis or cytokine production and

expression of co-stimulatory molecules via activation of intracellular signaling pathway in APCs (Arnold-Schild et al., 1999; Binder et al., 2004). Mycobacterium tuberculosis derived Hsp70 (MTB Hsp70) is well characterized and functions as an adjuvant in stimulating the host immune response (Wang et al., 2001b). MTB hsp70 stimulate RANTES and MIP-1α production in THP-1 cell and TNF-α production and expression of CD80, CD83, CD86, and HLA-DR in PBMC-derived human dendritic cells (Wang et al., 2002). Conjugation with antigenic protein, MTB Hsp70 have adjuvant effect and induced both MHC class I and MHC class II restricted T cell response (Huang et al., 2000; Suzue and Young, 1996).

4. General Introduction: Purpose of This Study

Pattern recognition receptors (PRRs) sense the presence of microorganisms by recognizing structures of microbial species called pathogen-associated molecular patterns (PAMPs) (Takeuchi and Akira, 2010). Although they are commonly classified the member of innate immunity, their role is not restricted to production of proinflammatory cytokines in response to bacterial and viral infection. Currently studying about the role of PRRs help to understand the mechanisms related to pathogenesis of allergic asthma related to Th2 immune respone. However, the role of PRRs signaling in allergic asthma is not fully understood. TRIF and RIP2 are important molecules mediating PRRs signaling. Although there are many evidence suggesting the possiblity of function of TRIF and RIP2 in develpment of allergic asthma (Hsia et al., 2014; Moon et al., 2011; Shalaby et al., 2013), their pecise roles are not studied sufficiently.

Heat shock protein 70 of *Mycobacterium tuberculosis* is potent immune activator and have activity as ligands of TLRs (Vabulas et al., 2002; Wang et al., 2002). Many studies showed that infection of Mycobacterium have attenuation effect on allergic airway inflammation (Erb et al., 1998; Yang et al., 1999). And administration of protein derived from *Mycobacterium leprae* reduced airway hyperresponsiveness and inflammation in murine allergic airway inflammation

model (Rha et al., 2002). In addition, ligands of TLRs have therapeutic effect on allergic asthma (Casale et al., 2006; Horak et al., 2011; Nguyen and Casale, 2011). These results are suggesting that Mtb Hsp70 may have an effect on the development of allergic airway inflammation.

In order to study the role of molecules involved in PRRs signaling in development of allergic asthma, effect of Mtb Hsp70 on allergic asthma and function of TLRs in effect of Mtb Hsp70, allergen-induced airway inflammation mice model and bone marrow derived-dendritic cells were used.

CHAPTER I

Role of TRIF in Development of Allergic Airway Inflammation

1.1. Abstract

Toll-like receptors (TLRs) signaling are closely associated with asthma and have emerged as a novel therapeutic target in allergic disease. The functions of TLR3 and TLR4 in allergic airway inflammation have been studied; however, the precise role of TIR-domain-containing adapter-inducing interferon-β (TRIF), the adaptor molecule for both TLR3 and TLR4, is not yet fully understood. To investigate this, I induced allergic airway inflammation which is one of the major symptoms of allergic asthma and compared the severity of allergic airway inflammation in WT and $TRIF^{-/-}$ mice. Histopathological assessment revealed that the severity of inflammation in airway inflammation in TRIF-deficient mice was comparable to that in WT mice. The total number of cells recovered from bronchoalveolar lavage fluid did not differ between WT and TRIF-deficient mice. Moreover, TRIF deficiency did not affect Th1 and Th2 cytokine production in lung tissue and the level of serum OVA-specific IgE, IgG_1 and IgG2c. These findings suggest that TRIF-mediated signaling may not be critical for the development of allergic airway inflammation.

1.2. Introduction

TLR3 and TLR4 signaling affect the development of allergic asthma. Activation of TLR3 results in Th2 cytokine production and influx of eosinophils, myeloid DCs, and inflammatory T cells and increases in the total number of cells in bronchoalveolar lavage (BAL) fluid (Stowell et al., 2009; Torres et al., 2010). And stimulation of TLR4 promotes cytokine production that mediates the maturation of Th2-polarized lung DC (Hollingsworth et al., 2004).

In toll-like receptor signaling pathway, TIR-domain-containing adapter-inducing interferon-β (TRIF) mediates the signal transduction of TLR3 and TLR4 (Kawai and Akira, 2006; Kumar et al., 2009). It has been reported that the role of TRIF in development of Th2 immune response induced by viral infection or LPS (Abston et al., 2012; Kaisho et al., 2002). These evidences suggested that TRIF may have a critical role of developmt of allergic asthma.

Although, the role of TRIF in allergic asthma has been studied (Sahiner et al., 2014; Shalaby et al., 2013; Torres et al., 2010), its precise role in allergic asthma is controversial. Deficiency of TRIF shows contrasting effects on asthma, depending on the animal model and experimental conditions. In a pollen-induced asthma model, TRIF deficiency results in the exacerbation of airway inflammation by augmenting the total number of BAL inflammatory cells and increasing of

chemokines and eotaxin in bronchoalveolar lavage (BAL) fluid compared with those in BAL fluid of WT mice (Shalaby et al., 2013). In contrast, TRIF deficiency cause the reduction of IL-17 associated with neutrophilic asthma in an OVA-induced model of allergic airway inflammation (Hsia et al., 2014). Interestingly, in some studies, TRIF deficiency did not have any effect on airway inflammation or the asthmatic phenotype in murine models of OVA-induced asthma (Bortolatto et al., 2008; Torres et al., 2010).

Studing about the TRIF may help to understand the role of TLRs signaling in allergic asthma and may contribute to develop novel therapeutic strategies for allergic asthma. In this study, in order to reveal the effect of TRIF in allergic asthma, I induced the allergic airway inflammation which is one of the major symptoms of allergic asthma and compared the severity of allergic airway inflammation in WT and TRIF-deficient mice.

1.3. Materials and Methods

Animals

Wild-type C57BL/6 mice, 6- to 8-weeks old, were purchased from KOATECH (Pyeongtaek, Kyounggi-do, Korea). TRIF$^{-/-}$ mice on C57BL/6 background were kindly gifted by Shizuo Akira (Osaka University, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee in Konyang University.

Airway inflammation induction

Protocols for inducing allergic airway inflammation in mice are depicted schematically in Figure 1. Briefly, wild-type (WT) mice and TRIF$^{-/-}$ mice were sensitized by administering 40 μg OVA (Sigma-Aldrich, St. Louis, MO, USA) with 4 mg aluminum hydroxide (Sigma-Aldrich) intraperitoneally (i.p.) in a volume of 200 μl on days 0 and 7. Anesthetized mice were challenged intranasally (i.n.) with 200 μg OVA diluted in PBS in a volume of 25 μl. Control groups received PBS alone. Animals were sacrificed 2 days after the final i.n. administration, and samples of lung, serum, and BAL fluid were collected for further analysis.

Measurement of the concentration of cytokines and OVA-specific serum antibodies

Concentrations of IL-5 and IL-13 in lung extract were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, UK). OVA-specific serum IgE, IgG_1, and IgG_{2c} levels were determined by ELISA. OVA (10 μg/ml) was coated onto 96-multiwell plates. Serum samples were diluted 1/10000 for IgG_1, 1/1000 for IgG_{2c} and 1/20 for IgE. Biotinylated rat anti-mouse IgE (BD Biosciences, San Jose, CA, USA) was applied, followed by Streptavidin-HRP (BD Biosciences) to quantify OVA-specific serum IgE. Peroxidase-conjugated rat anti-mouse IgG_1 and IgG_{2c} (SouthernBiotech, Birmingham, AL, USA) were used to quantify OVA-specific serum IgG_1 and $IgG2_c$ respectively.

Quantification of cells in BAL fluids

BAL fluid was prepared by washing the lungs with 0.8 ml of PBS. The cell pellets were prepared by centrifugation at 300 ×g for 3min. After discarding the supernatants, cell pellets were resuspended in RPMI 1640. Cells were stained with trypan blue, and the total number of viable cells was determined using a

hemocytometer.

Histological evaluation of tissue inflammation

To evaluate tissue inflammation, the left lung from each mouse was fixed in 10% neutral-buffered formalin (pH 7.0) for 48 hours and embedded in paraffin. Sections (2-μm thick) were prepared and stained with hematoxylin and eosin. Tissue inflammation in each sample was examined by light microscopy and was expressed as a numerical score. Tissue inflammation based on the abundance of lesions was scored as follows: 0 = non-specific lesion, 1 = slight, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe.

Statistical analysis

Differences among the mean values of the different groups were analyzed, and the values were expressed as mean ± SD. Statistical analyses were performed by Student's *t*-test by GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

1.4. Results

TRIF deficiency does not contribute to histopathological changes in an OVA-induced model of airway inflammation

Inflammation in tissues was assessed by histological examination, and the total number of cells in BAL fluid was counted (Figure 2). OVA-sensitized/challenged WT and TRIF$^{-/-}$ mice showed inflammatory cell infiltration around the airways and interstitium of the alveoli (Figure 2A.). These lesions did not appear in PBS-sensitized/challenged control WT and TRIF$^{-/-}$ mice. Scores indicating severity of inflammation were significantly increased in OVA-sensitized/challenged WT and TRIF$^{-/-}$ mice compared to PBS-sensitized/challenged WT and TRIF$^{-/-}$ mice; however, a significant difference in the scores was not observed between OVA-sensitized/challenged WT mice and OVA-sensitized/challenged TRIF$^{-/-}$ mice (Figure 2B). The total number of cells in BAL fluid was also increased in OVA-sensitized/challenged WT and TRIF$^{-/-}$ mice, and no significant difference in the total number of cells was detected between the two groups (Figure 2C).

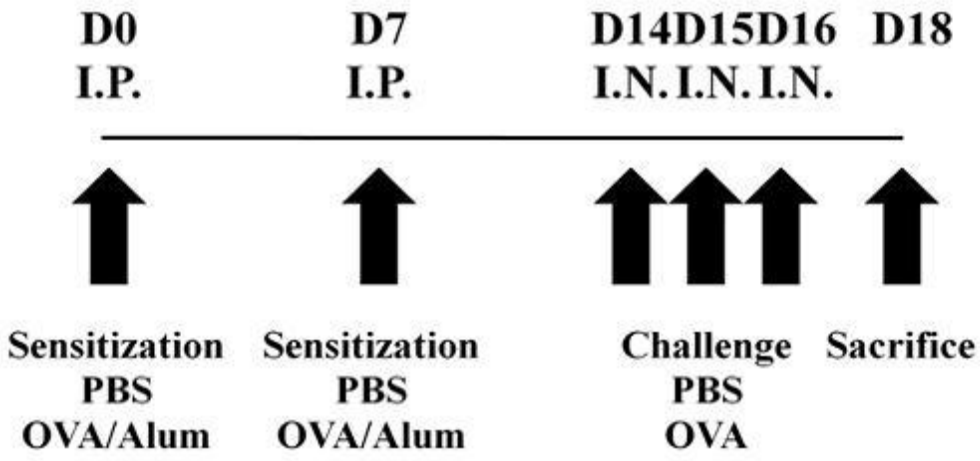

Figure 1. A schematic diagram of the experimental design of an OVA-induced model of allergic airway inflammation.

Sensitization of OVA was performed at day 0 (D0) and day 7 (D7) by intraperitoneal (i.p.) injection of OVA with aluminum hydroxide used as an adjuvant. From days 14 to 16, mice were inranasally (i.n.) challenged with OVA and were sacrificed 2 days after last challenge.

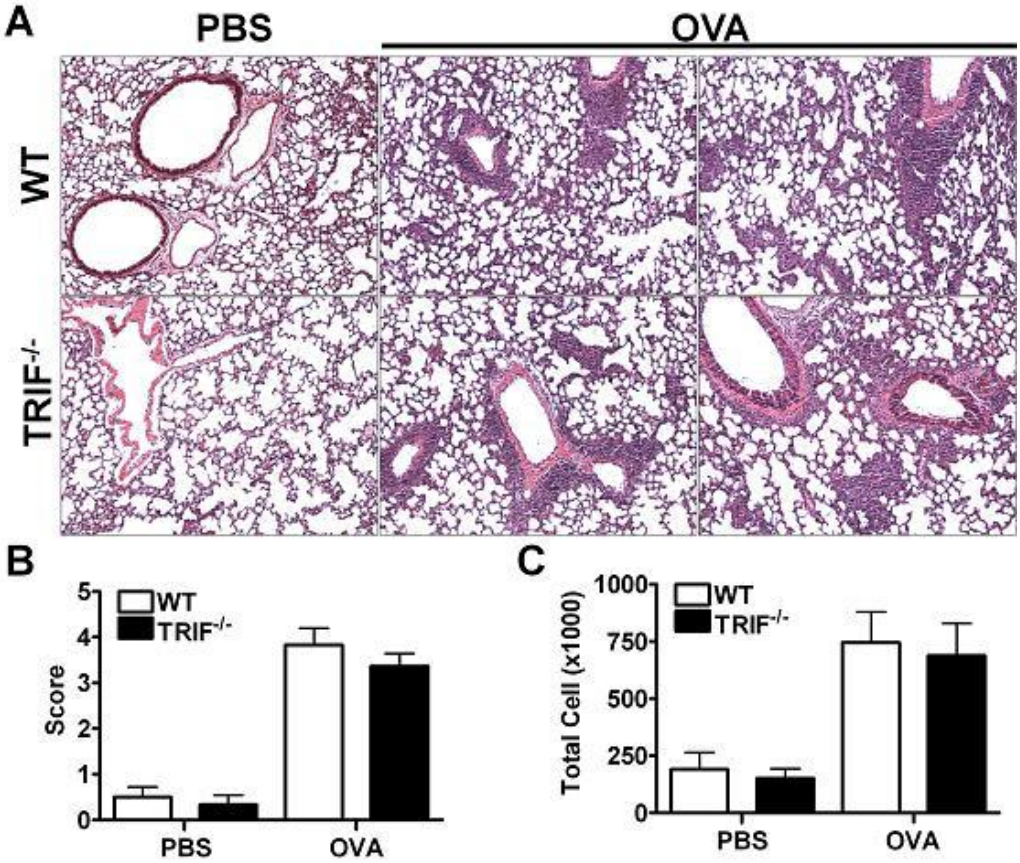

Figure 2. Histopathological analysis for development of allergic airway inflammation in WT and TRIF$^{-/-}$ mice.

(A) In microscopic analysis of hematoxylin and eosin (H&E)-stained tissue sections. (B) Histopathological scores were determined semi-quantitatively by microscopic examination. (C) The number of cells in bronchoalveolar lavage (BAL) fluid was measured. Data are shown as mean ± SD of each group (n = 5 per group).

TRIF deficiency does not change the level of inflammatory cytokines in OVA-induced allergic airway inflammation

IL-5 is the principal eosinophil-activating cytokine and also mediates eosinophil recruitment and triggers the activation of B cells (Abbas et al., 1996; Takatsu et al., 1994; Torres et al., 2010). IL-13 plays a role in the contraction of smooth muscle cells in the airway (Wills-Karp, 2004). Th2 cytokines are associated to pathogenesis of allergic response, whereas Th1 cytokines are counter balance of Th2 (Yazdanbakhsh et al., 2002). IL-12 prevents the expansion of Th2 cells, and IFN-γ is the major cytokine produced by Th1 cells (Chung, 2001; Coffman et al., 1993). To evaluate cytokine production in lung tissue, the concentration of cytokines in the supernatant of lung homogenate were measured by ELISA. The levels of IL-5 and IL-13 in lung extract were increased in both WT and $TRIF^{-/-}$ OVA-sensitized/challenged WT and TRIF-deficient mice (Figure 3A, B). The level of type 1 cytokine IL-12 was also increased in both WT and $TRIF^{-/-}$ OVA-sensitized/challenged mice, and no significant difference was observed between the groups (Figure 3C). Finally, IFN-γ was not significantly increased in OVA-sensitized/challenged mice compared to control mice (Figure 3D).

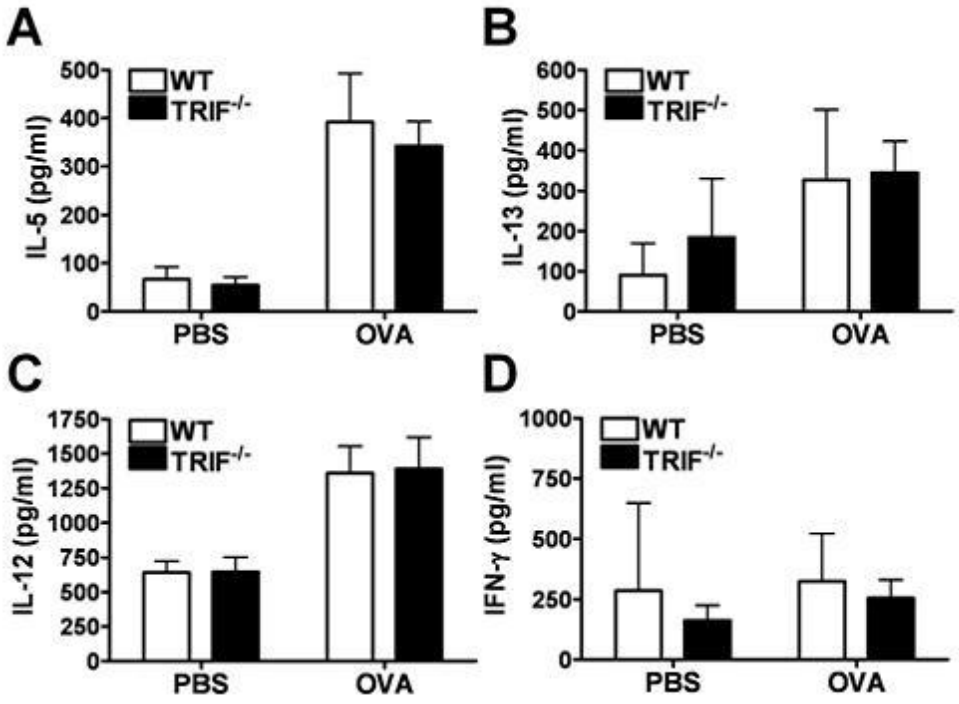

Figure 3. The production of Th1 and Th2 cytokine in the lung extract.

(A) IL-5, (B) IL-13, (C) IL-12 and (D) IFN-γ in lung extract of OVA treatment in WT and $TRIF^{-/-}$ mice were measured by ELISA. Data are shown as mean ± SD of each group (n=5 per group).

TRIF deficiency does not affect to serum levels of OVA-specific IgE, IgG_1, and IgG_{2c} in the OVA-induced model of airway inflammation

Serum levels of OVA-specific IgE, IgG_1 and IgG_{2c} were measured by ELISA. Based on our previous results, we expected that TRIF deficiency would not affect the level of allergen- specific immunoglobulin. OVA-specific IgE, $IgG_{1,}$ and IgG_{2c} levels in OVA-sensitized/challenged $TRIF^{-/-}$ mice were similar to those found in WT mice (Figure 4).

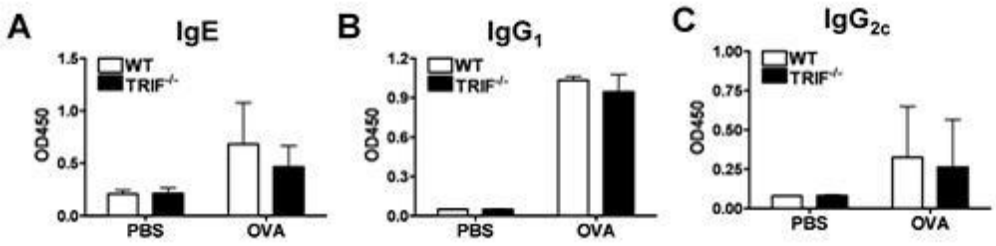

Figure 4. The level of OVA-specific IgE, IgG_1 and IgG_{2c} in serum.

The OVA-specific antibody subclasses (A) IgE, (B) IgG_1, and (C) IgG_{2c} in serum were measured by ELISA. Data are shown as mean ± SD of each group (n = 5 per group;*$p < 0.05$).

1.5. Conclusion

Infections are closely related to the pathogenesis of allergic asthma (Matsumoto and Inoue, 2014). However, the effect of infections on the development of allergic asthma is quite controversial. Viral infection of the airway commonly aggravates allergic asthma (Matsumoto and Inoue, 2014). Human rhinovirus (HRV) is associated with the exacerbation of asthma in both children and adults (Atmar et al., 1998; Nicholson et al., 1993). In addition, respiratory syncytial virus (RSV) infection induces a lower IFN-γ/IL-10 ratio and contributes to the polarization of Th2-biased immune responses and the production of IL-13-mediated AHR (Joshi et al., 2003; Matsumoto and Inoue, 2014). The role of bacterial infection in allergic asthma is controversial. Recent studies showed that bacterial infections are associated with the exacerbation of asthma and exposure to LPS enhances the Th2 response to inhaled allergens (Delayre-Orthez et al., 2004; Eisenbarth et al., 2002; Kim et al., 2007). In contrast, epidemiological studies suggest that insufficient Th1 immune response induced by infection in infancy is related to development of allergic disease (Yazdanbakhsh et al., 2002). In addition, exposure to airborne endotoxin attenuated asthma by promoting enhanced Th1 response and tolerance to allergens (Reed and Milton, 2001).

TLR signaling has been suggested as a basal mechanism that connects the infection and allergic disease and emerged as a novel therapeutic target of drugs for asthma (Bortolatto et al., 2008; Eisenbarth et al., 2002; Hammad et al., 2009; Hollingsworth et al., 2004; Stowell et al., 2009; Torres et al., 2010). To develop novel drugs for asthma, the role of molecules associated with TLR signaling in allergic asthma must be defined. Although the role of TRIF, an adaptor molecule of TLR3 and TLR4, which recognize viral RNA and bacterial LPS, respectively, in allergic asthma has been studied (Sahiner et al., 2014; Shalaby et al., 2013; Torres et al., 2010), its function is not yet fully understood. Deficiency of TRIF shows contrasting effects, depending on the animal model and experimental conditions used. In a pollen-induced asthma model, TRIF deficiency results in the exacerbation of airway inflammation by augmenting the total number of BAL inflammatory cells and increasing of chemokines KC and eotaxin in BAL fluid compared with those in BAL fluid of WT mice (Shalaby et al., 2013). However, TRIF deficiency reduces IL-17 associated with neutrophilic asthma in an OVA-induced model of allergic airway inflammation (Hsia et al., 2014). Interestingly, in some studies, TRIF deficiency did not have any effect on airway inflammation or the asthmatic phenotype in murine models of OVA-induced asthma (Bortolatto et al., 2008; Torres et al., 2010).

In our study allergic airway inflammation induced by immunization with an i.p. injection of OVA/alum and subsequent challenge with an i.n. injection of OVA solution was developed to determine the effect of TRIF deficiency on allergic airway inflammation. The histopathological scores, total inflammatory cells in BAL fluid, the production of Th1 and Th2 cytokines in lung tissue, and levels of OVA-specific immunoglobulin in $TRIF^{-/-}$ mice were comparable to those observed in WT mice. These results suggest that TRIF deficiency did not affect on the development of airway inflammation in OVA in murine models of OVA-induced asthma.

CHAPTER II

Role of RIP2 in Development of Allergic Airway Inflammation

2.1. Abstract

Receptor interacting protein 2 (RIP2), a serine/threonine kinase, is an adaptor molecule of NOD1 and NOD2. There have been many researches suggesting that Rip2 may have a role in development of allergic asthma, but its precise role is not been revealed yet. In this study, we examined the role of RIP2 in the development of allergic airway inflammation, one of the major symptoms of allergic asthma, in a mouse model. Airway inflammation was induced in mice through intranasal administration of ovalbumin (OVA) after two intraperitoneal immunizations with OVA. Lung inflammation and mucus hypersecretion were histologically examined and total cell infiltration in bronchoalveolar (BAL) fluids was determined. Levels of the Th2-related cytokines, IL-5 and IL-13, in lung extracts were measured by ELISA. The production of antigen-specific IgE and IgG1 was also examined in serum. OVA-induced lung inflammation and mucus hypersecretion was not different between WT and RIP2-deficient mice. The production of IL-5 and IL-13 in bronchoalveolar (BAL) fluids was also not impaired in RIP2-deficient mice as compared to WT mice. Moreover, RIP2 deficiency did not affect the production of OVA-specific IgG_1 and IgE in the serum. Our results suggest that RIP2 is not associated with the development of allergic airway inflammation.

2.2. Introduction

Nod1 and Nod2 signalings are involved in allergic diseases. Genetic variations of NOD1 are associated with the presence of asthma and elevation of IgE levels in humans (Eder et al., 2006; Hysi et al., 2005). Polymorphic allele of the NOD2 gene are risk factor of developing allergic rhinitis and atopic dermatitis (Kabesch et al., 2003). There is also evidence of an association between the allergic asthma and RIP2 which is adaptor molecule of Nod1 and Nod2. Nakashima *et al*. suggested that genetic variants of the RIP2 gene may be associated with the severity of asthma, even though these variants are not likely involved in asthma development (Nakashima et al., 2006). Blockade of RIP2 by a flavonoid aglycone naringenin contributed to the suppression of the production of thymic stromal lymphopoietin (TSLP) in mast cells, which play a pivotal role in allergic asthma (Moon et al., 2011). Gefinitib having regulating effect on allergic airway inflammation is one of the inhibitor of RIP2 (Hur et al., 2007). These findings suggest that RIP2 may be associated with the development of allergic asthma, and prompted us to determine the exact role of RIP2 in the development of allergic airway inflammation. We induced allergen-induced airway inflammation in WT and RIP2-deficient mice and examined the severity of lung inflammation, Th2-derived cytokine production in lung extracts, and the

production of immunoglobulins in serum.

2.3. Materials and Methods

Animals

Wild-type C57BL/6 mice, 6- to 8-weeks old, were purchased from KOATECH (Pyeongtaek, Kyounggi-do, Korea). RIP2-deficient mice on C57BL/6 background were purchased from The Jackson laboratory (Bar Habor, ME, USA). All animal experiments were approved by the Institutional Animal Care and Use Committee in Konyang University.

Airway inflammation induction

Protocols for inducing allergic airway inflammation in mice are depicted schematically in Figure 1A. WT and RIP2-deficient mice were sensitized with 40 μg OVA (Sigma-Aldrich) with 2 mg of adjuvant (Imject® Alum, Thermo scientific, Rockford, IL, USA) in 200 μl of PBS or PBS alone by intraperitoneal (i.p) injection on day 0, day 1, day 7 and day 8. On day 14, day 15, day 21 and day 22 anesthetized mice were challenged intranasally with 200 μg OVA in PBS or PBS alone in a volume of 50 μl. Animals were sacrificed 2 days after last challenge and samples were collected for analysis.

Measurement of the concentration of cytokines and OVA-specific serum antibodies

Concentrations of IL-5 and IL-13 in lung extract were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, UK). OVA-specific serum IgE, IgG_1, and IgG_{2c} levels were determined by ELISA. OVA (10 μg/ml) was coated onto 96-multiwell plates. Serum samples were diluted 1/10000 for IgG_1, 1/1000 for IgG_{2c} and 1/20 for IgE. Biotinylated rat anti-mouse IgE (BD Biosciences, San Jose, CA, USA) was applied, followed by Streptavidin-HRP (BD Biosciences) to quantify OVA-specific serum IgE. Peroxidase-conjugated rat anti-mouse IgG_1 and IgG_{2c} (SouthernBiotech, Birmingham, AL, USA) were used to quantify OVA-specific serum IgG1 and IgG2c respectively.

Quantification of cells in bronchoalveolar lavage (BAL) fluids

BAL fluid was prepared by washing the lungs with 0.8 ml of PBS. The cell pellets were prepared by centrifugation at 300 ×g for 3min. After discarding the supernatants, cell pellets were resuspended in RPMI 1640. Cells were stained with trypan blue, and the total number of viable cells was determined using a hemocytometer.

Histological evaluation of tissue inflammation

In order to evaluate tissue inflammation, left lung from each mouse was fixed in 10% neutral-buffered formalin for 48 hours and embedded in paraffin. Tissue sections (2 μm thick) were prepared and stained with Hematoxylin and Eosin or periodic acid-Schiff (PAS), and examined under light microscopy. Scoring of the tissue inflammation was based on the presence or abundance of inflammatory lesion as followed, 0: non-specific lesion, 1: mild, 2: mild to moderate, 3: moderate, 4: moderate to severe, 5: severe. For quantitating mucus staining, PAS-positive goblet cells in the airways were counted and the length of the basement membrane (BM) in each airway was measured using ImageJ version 1.44 (National Institutes of Health, Bethesda, MD, USA). The results are given as mean number of PAS-positive goblet cells per millimeter of BM (Makela et al., 2002).

Statistical analysis

The differences among the mean values of the groups were tested, and the values were expressed as the mean ± SD. All of the statistical calculation were performed by t-test using GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

2.4. Results

Role of RIP2 in the severity of OVA-induced inflammation in mouse lungs

To evaluate the role of RIP2 in development of allergic airway inflammation, we modified the protocol for OVA-induced airway inflammation. Instead of alum hydroxide, comercial alum based adjuvant was used for sensitization and airway inflammation in mice was achieved by challenge with OVA four times i.n. after immunizations, as described in Fig. 5. We first examined whether RIP2 affects the severity of airway inflammation. Intra-nasal challenge with OVA induced severe infiltration of inflammatory cells, mostly consisting of lymphocytes and granulocytes, around the bronchus and increased the thickness of the alveolar walls in both WT and RIP2 deficient mice (Figure 1B). However, when histopathological scores were assessed, RIP2 deficiency did not appear to affect the severity of lung inflammation induced by OVA (Figure 1C). In addition, total infiltrating cells were counted from BAL fluids of mice with and without i.n. challenge by OVA. Compared to PBS-treated mice, OVA challenge increased the number of infiltrating cells in BAL fluids in both WT and RIP2-deficient mice, with no significant difference between the WT and RIP2-deficient mice (Figure 1D).

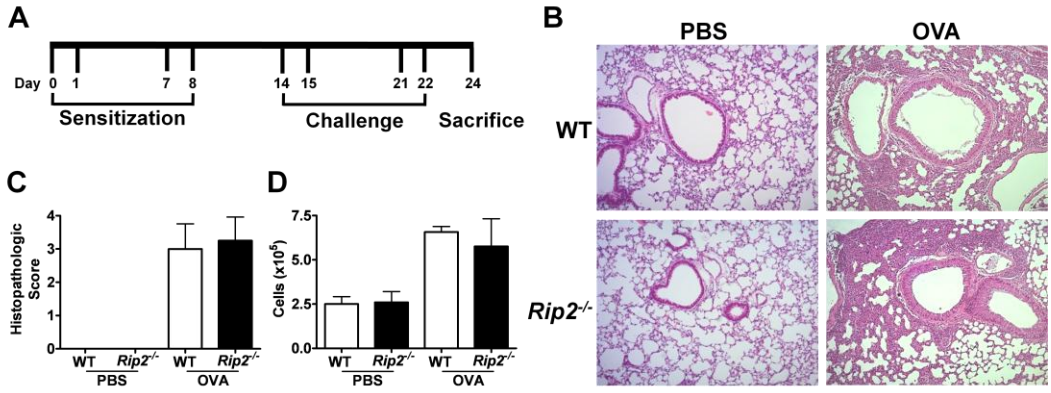

Figure 1 OVA-induced airway inflammation in WT and RIP2-deficient mice.

A schematic diagram of the experimental design (A). Mice were sensitized by i.p. administration of OVA mixed with adjuvant at days 0, 1, 7, and 8. On days 14, 15, 21, and 22, mice were challenged with OVA or PBS. Photographs of lung tissues were obtained from HE-stained sections (B) and histopathological scores were determined semi-quantitatively by microscopic examination (C). Total cell number in BAL fluids was counted (D) and differential cell count was performed using Diff-Quick staining (E). Data are expressed as means ± SDs.

Effect of RIP2 on goblet cell hyperplasia and mucus hypersecretion in the bronchus of mice challenged with OVA intranasally

To determine whether RIP2 deficiency influenced these phenomena, slide sections of lung were stained with PAS and observed under a light microscope. The number of PAS-positive cells as well as mucus secretion in the airway epithelial layer of mice was increased by intranasal challenge with OVA. However, there was no significant difference in the number of PAS-positive cells or mucus secretion between WT and RIP2-deficient mice (Figure 2A and B).

Role of RIP2 in the production of Th2-derived cytokines in the lung extracts of mice challenged intranasally with OVA

We examined OVA-induced production of IL-5 and IL-13 in lung extracts of WT and RIP2-deficient mice. Results showed that i.n. challenge with OVA increased the production of IL-5 and IL-13 in the lungs of mice (Figure 3). However, RIP2-deficiency did not affect OVA-induced production of IL-5 and IL-13 in lung extracts (Figure 3).

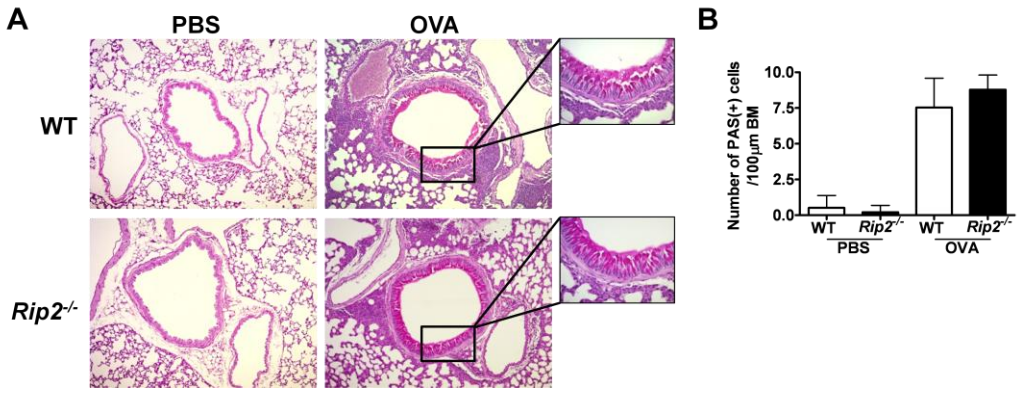

Figure 2 Hyperplasia of goblet cells in bronchus of lung tissue.

Lung sections were stained with PAS and examined under light microscopy. Areas in black boxes are shown at a higher magnification on the right of each picture (A). Numbers of PAS-positive cells in the airway epithelium were counted. In each studied airway, the length of the basement membrane (BM) was measured by image analysis software. The results are presented as mean number of PAS-positive cells per micrometer of BM (B). Data are expressed as means ± SDs.

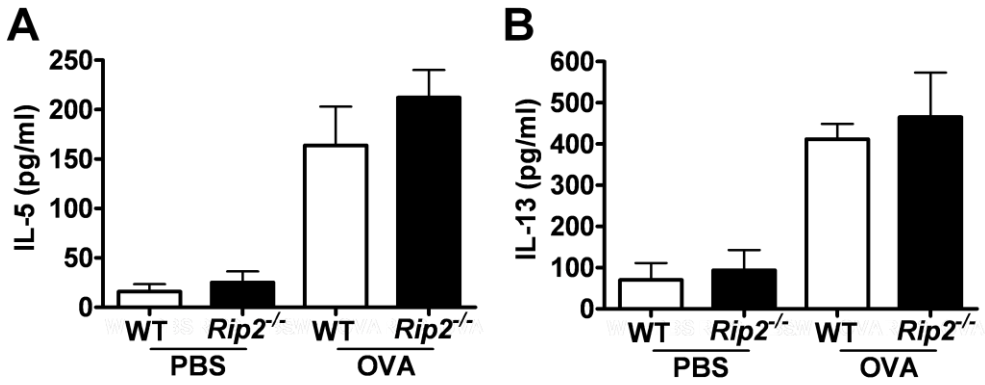

Figure 3 Cytokine production in the lung extract of WT and RIP2-deficient mice.

Lung extracts from the right lungs of sacrificed mice. Concentrations of IL-5 (A) and IL-13 (B) in lung extracts were measure by ELISA. Data are expressed as means ± SDs. Coefficients of variations (%) was provided in parenthesis.

Role of RIP2 in the production of antigen-specific immunoglobulins in the serum of mice challenged with OVA

Finally, we measured the level of antigen-specific IgE, IgG_1, and IgG_{2c} production in serum. As expected, the production of antigen-specific IgE and IgG1 in serum was increased by OVA challenge. However, there were no significant differences in the levels of OVA-specific IgE, IgG_1, and IgG_{2c} between WT and RIP2-deficient mice (Figure 4).

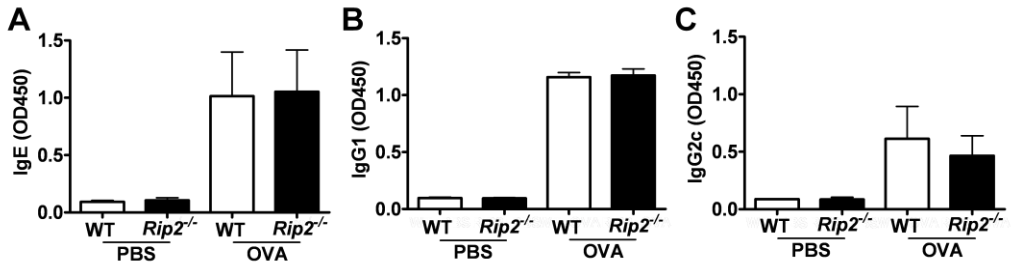

Figure 4 Levels of OVA-specific IgE, IgG_1 and IgG_{2c} in serum.

Serum was obtained from blood samples collected 48 after the last OVA challenge. Levels of OVA-specific IgE (A), IgG_1 (B) and IgG_{2c} in the serum were measured by ELISA. Data are expressed as means ± SDs.

2.5. Conclusion

In addition to their protective role in bacterial or viral infections, PRRs have been reported to be involved in allergic asthma. Toll-like receptors (TLRs), the best characterized PRRs, are known to mediate the induction of allergic airway inflammation. There is also evidence of a close association between polymorphisms in TLR genes and asthma (Basu and Fenton, 2004; Cook et al., 2004; Eder et al., 2004). These are suggesting that innate immune responses mediated by PRRs play a critical role in the development of allergic airway inflammation.

Similar to TLRs, NOD1 and NOD2 stimulation triggers the activation of NF-κB and MAPKs, which are critical factors for the production of proinflammatory cytokines; the adaptor molecule RIP2 is required for these events (Chen et al., 2009). However, in contrast to TLRs, the association between NOD1 and NOD2 signaling and allergic asthma is poorly understood. Based on several indirect lines of evidence that RIP2 may be involved in allergic asthma (Moon et al., 2011; Nakashima et al., 2006), I sought to determine the role of RIP2 in OVA-induced airway inflammation. I found no significant differences between WT and RIP2-deficient mice in the severity of lung inflammation, total cell infiltration in BAL fluid, IL-5 and IL-13 secretion in lung extracts, or antigen-

specific IgE, IgG_1 and IgG_{2c} production in the serum of mice challenged intranasally with OVA. Magalhaes *et al*. showed that RIP2 is required for NOD1- and NOD2-induced Th2 immunity (Magalhaes et al., 2011). NOD1 and NOD2 ligands increased the number of OVA-specific cells producing IL-5 or IL-5 in the splenocytes of WT mice, but not of RIP2-deficient mice (Magalhaes et al., 2011). In addition, RIP2 was essential for OVA-specific IgG_1 production mediated by Nod1 and Nod2 stimulation (Magalhaes et al., 2011). A recent study showed that serum samples from normal mice, but not from antibiotic-treated mice, had Nod1- and Nod2-stimulating activity (Clarke et al., 2010), suggesting that microbiota may steadily release Nod1- and Nod2-stimulatory factors (e.g. peptidoglycans) into body fluid. Therefore, in this study, we compared experimental parameters between WT and RIP2-deficient mice in the absence of Nod1 and Nod2 stimulation to mimic physiological conditions. Consistent with the findings of a previous study (Magalhaes et al., 2011), we found that WT and RIP2-deficient mice immunized with OVA and alum without Nod1 and Nod2 ligands did not have differences in antigen-specific IgG production in the serum. Eosinophilic infiltration into the lung by OVA was also not impaired in RIP2-deficient mice compared with WT mice (Nembrini et al., 2011). Taken together, our results indicate that under normal conditions, RIP2 deficiency is not associated with the

development of allergic airway inflammation.

Microbial infections seem to affect the development or severity of allergic asthma through TLR-mediated signaling (Conrad et al., 2009; Nembrini et al., 2011). Nod1 and Nod2 cooperate with TLRs to induce innate immune response against microbial infections (Kim et al., 2011). *Listeria*-induced production of cytokines was impaired in NOD1/2 double- or RIP2-deficient macrophages after LPS exposure (Kim et al., 2008). RIP2 deficiency also led to decreased production of cytokines in TLR-tolerized macrophages in response to *Pseudomonas* infection, and protected mice from lethality induced by the bacterial infection (Park et al., 2009b). Therefore, it is necessary to clarify whether Nod1/2 and RIP2 contribute to the control of the development of allergic diseases mediated by microbial infection.

CHAPTER III

Attenuation of Allergic Airway Inflammtion by *Mycobacterium tuberculosis*-derived Hsp70

3.1. Abstract

Mycobacterium infection counteractes to Th2 immune response and attenuates the allergic inflammation and it has been shown that *Mycobacterium tuberculosis* has an effect to enhance the immune response *in vitro* and *in vivo* via TLR2 and TLR4 signaling pathway. In this study, we examined the beneficial effect of *Mycobacterium tuberculosis* heat shock protein 70 (Mtb Hsp70) on allergic airway inflammation. In allergen induced allergic airway inflammation model, co-administration of purified recombinant Mtb Hsp70 in sensitization phase reduced lung inflammation and hyperplasia of mucus secreting goblet cells in airway. And allergen-specific IgE level in serum and the production of Th2 cytokines such as IL-4, IL-5 and IL-13 in lung extract were also decreased in Mtb hsp70 treated group. However, these effects of Mtb Hsp70 were abolished in development of allergic airway inflammation in TLR2 and TLR4-deficient mice. In conclusion, our results revealed the inhibitory effect of Mtb Hsp70 on allergic airway inflammation and TLR2 and TLR4 signaling involed in these effects.

3.2. Introduction

It is generally accepted that allergic respiratory disease in adults is associated with active T-cell immune responses to inhaled allergens that are skewed toward the Th2 phenotype (Agrawal and Shao, 2010). Manipulating of the balance between Th1 and Th2 immunity can be the potential approach to therapy for allergic airway inflammation (Agrawal and Shao, 2010). Th1 immune response induced by ligands of TLR2 and TLR4 ameliorate the allergic airway inflammation (Delayre-Orthez et al., 2005; Delayre-Orthez et al., 2004; Patel et al., 2005). *Mycobacterium tuberculosis* derived Hsp70 (Mtb Hsp70) is well characterized and functions as an adjuvant in stimulating the host immune response (Wang et al., 2001b). In addition, toll-like receptors (TLR) 2 and TLR4 signalings are involved in Hsp70-induced immune responses (Bulut et al., 2005; Qazi et al., 2007; Vabulas et al., 2002). These results suggested that Mtb Hsp70 may have effect on the development of allergic airway inflammtion.

In the present study, we sought to determine beneficial effect of Mtb Hsp70 on allergen-induced airway inflammation which is is a major symptom of allergic asthma (Agrawal and Shao, 2010) and role of TLR signalings in its effects.

3.3. Materials and Methods

Animals

Wild-type C57BL/6 and BALB/c mouse were purchased from KOATECH (Pyeongtaek, Kyeonggi-do, Korea). TLR2-, TLR4- deficient mice on C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice deficient in both TLR2 and TLR4 were generated by crossing TLR2-deficient and TLR4-deficient mice and intercrossing the F1 generation (Park et al., 2014). The animals were maintained at Konyang University medical college. All animal experiments were approved by the Institutional Animal Care and Use Committee of Konyang University.

Purificed recombinant Mtb Hsp70

To produce recombinant Rv0350 protein, the corresponding gene was amplified by PCR using Mtb H37Rv ATCC27294 genomic DNA as template and the following primers: forward, 5'-GGGCCCCATATGGCTCGTGCGGTCGGGATC -3', and reverse, 5'-GGGCCCAAGCTTCTTGGCCTCCCGGCCGTCGTC -3'. The PCR product of *Rv0350* was cut with *Nde*I and *Hind*III. Both the products were inserted into

pET22b (+) vector (Novagen, Madison, WI, USA) and the resultants were sequenced. The recombinant plasmids containing *Rv0350* were transformed into *E. coli* BL21 cells by heat-shock for 1 min at 42℃. The overexpressed Rv0350 was prepared with slight modifications as previously described (ref). Briefly, *E. coli* containing recombinant plasmid were grown at 37℃ until the optical density (OD) at 600 nm was 0.4 to 0.5 and then induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG; ELPIS-Biotech, Daejeon, South Korea). The bacterial cells were then harvested by centrifugation and suspended in 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 5 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (Sigma); and lysed by sonication. The recombinant Rv0350 was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography in accordance with the manufacturer's instructions (Qiagen, Chatsworth, CA, USA). Each purification step was analyzed by 13.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) with Coomassie brilliant blue stain and immunoblot using anti-His antibodies (Santa Cruz). The purified protein was pooled, concentrated and dialyzed against phosphate-buffered saline (PBS, pH 7.4). To remove endotoxin contamination, the dialyzed recombinant protein was incubated with polymyxin B-agarose (PmB, Sigma) for 6 h at 4℃. Lastly, purified endotoxin-free recombinant protein was filter sterilized and frozen at -

70℃. The protein concentration was estimated with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Residual LPS in the Rv0350 preparation was determined using the Limulus amoebocyte lysate (LAL) test (Lonza, Basel, Switzerland), according to the manufacturer's instructions. The purity of Rv0350 was evaluated by Coomassie brilliant blue (CB) staining and Western blot using an anti-histidine antibody. Endotoxin contamination was evaluated by an LAL assay and was less 15 pg/ml (0.1 UE/ml) in Mtb Hsp70 preparation. The protein concentration was estimated with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Generation of allergic airway inflammation

HDMs (*Dermatophagoides pteronyssimus*) were obtained from the Arthropods of Medical Importance Bank (Yonsei University, Seoul, Korea). We sensitized mice by intraperitoneal injection with 100 μg of HDM extracts in 2 mg of Imject™ Alum (Thermo Scientific, Rockford, IL, USA) in 200 μl of PBS weekly for 2 weeks. One week after the last sensitization, the mice were intranasally challenged with 30 μg of HDM extracts every day for 3 days under anesthesia. We sacrificed mice 48 h after the last challenge. For induction of

OVA-induced airway inflammation, same protocol of HDM-induced model except for immunization with 100ug of OVA (Sigma-Aldrich, St. Louis, MO, USA) and challenge with 200ug of OVA was applied.

Levels of cytokine in lung homogenates and serum immunoglobulin

Lung extracts were obtained using a tissue homogenizer. Homogenates were centrifuged at 1,000×g for 10 min. Supernatants were collected, and then stored at -70℃ for further analysis. Levels of IL-5 and IL-13 in stored supernatant were measured using ELISA kit (R&D System, Minneapolis, MN, USA). To measure HDM-specific IgE levels, 96-well ELISA plates were coated with OVA (10 μg/ml) at 4℃ overnight. After blocking with 1% BSA in PBS and serum samples (at 1:20 dilution) were added to the plate. After incubation for 2 h at room temperature, biotinylated rat anti-mouse IgE (BD Biosciences, San Jose, CA, USA) was applied, followed by streptavidin HRP (BD Biosciences). After washing, the TMB substrate reagent set (BD Biosciences) was applied according to the manufacturer's instructions and optical density (OD) was measured at 450 nm. OVA-specific IgG1 was measured using the same methods described above except peroxidase-conjugated rat anti-mouse IgG_1 (Southern Biotech, Birmingham, AL, USA) was used.

Histopathological analysis

In order to evaluate tissue inflammation, left lung from each mouse was fixed in 10% neutral-buffered formalin for 48 hours and embedded in paraffin. Tissue sections (2 μm thick) were prepared and stained with Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) and Eosin (Sigma-Aldrich, St. Louis, MO, USA) or Periodic Acid-Schiff (PAS) (PAS Kit, Sigma-Aldrich, St. Louis, MO, USA), and examined under light microscopy. Scoring of the tissue inflammation was based on the presence or abundance of inflammatory lesion. For quantification mucus staining, PAS-positive cells in the airways were counted and the length of the basement membrane (BM) in each airway was measured using ImageJ version 1.44 (National Institutes of Health, Bethesda, MD, USA). The results are given as mean number of PAS-positive cells per 100 micrometer of BM.

Statistical analysis

The significance of differences in mean values of the groups was evaluated by t-tests, and values are expressed as means ± SD. All statistical calculations were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

3.4. Results

Purification of recombinant Mtb Hsp70

Recombinant Mtb Hsp70 protein was extracted from *E. coli* by sonication, purified using Ni-NTA resin, and then dialyzed. SDS-PAGE followed by immunoblotting with an anti-histidine Ab was used to confirm both the purity and the appropriate molecular mass (~70 kDa) of the recombinant protein (Figure 1). The purity of Mtb Hsp70 was quantified using Quantity-one software (Bio-Rad), and the recombinant protein was found to have >98% purity following two passages through a Ni-NTA column.

Mtb Hsp70 alleviates HDM-induced airway inflammation and reduces Th2 immune response in mice

Stimulation with TLR agonists such as Pam3Cys and LPS during allergen sensitization suppresses asthmatic responses by reducing airway hyperreactivity, mucus production, Th2-type inflammation in the lung, and IgE production in serum (Haapakoski et al., 2013). Thus, we investigated whether Mtb Hsp70 treatment reduces allergen-induced airway inflammation in mice. In vivo experimental schedule was depicted in Figure 2A. In mice group sensitized with

only HDM/alum (HDM-sensitized mice), intranasal challenge with HDM led to severe airway inflammation (Figure 2B and 2C). However, mice sensitized with HDM/alum together with Mtb Hsp70 (Mtb Hsp70-sensitized mice) exhibited only weak inflammation in the lungs, which was less severe even as compared with LPS-sensitized mice (Figure 2B and 2C). Moreover, hyperplasia of mucus secreting cells (MSCs) was also decreased in the lungs of Mtb Hsp70-sensitized mice, as compared with HDM-sensitized mice (Figure 2B and 2D). We also measured serum IgE level and Th2 cytokines production in the lung extract. The level of Th2 cytokines such as IL-4, IL-5, and IL-13 was also increased in the lung extract from HDM-sensitized mice, but not in those from LPS- or Mtb Hsp70-sensitized mice (Figure 3A-C). In addition, antigen-specific IgE production in serum was up-regulated in HDM-sensitized mice, which was reduced in LPS- or Mtb Hsp70-sensitized mice (Figure 3D).

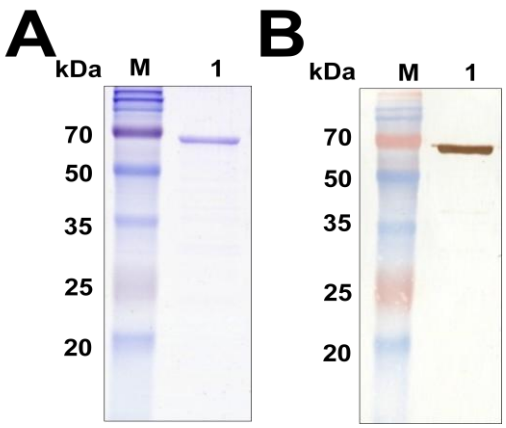

Figure 1 Purification of recombinant Mtb Hsp70

Purified recombinant Mtb Hsp70 was analyzed by 13.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) followed by staining with (A) Coomassie Brilliant Blue or (B) immunoblotting with an anti-histidine Ab (Santa Cruz, TX, USA).

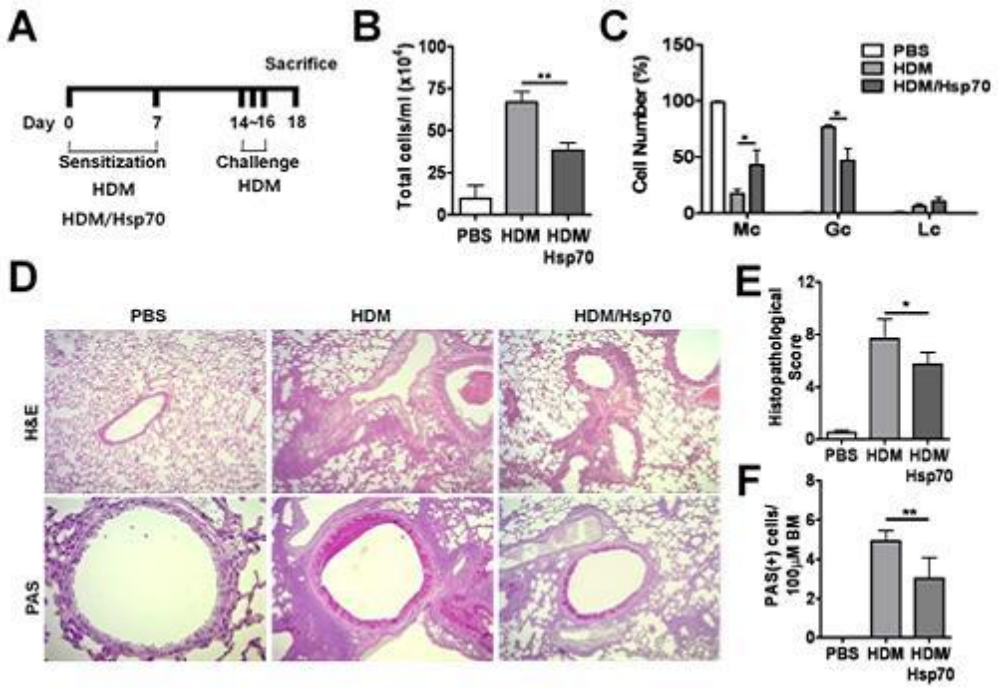

Figure 2 Attenuation of HDM-induced airway inflammation by Mtb Hsp70.

A schematic diagram of the experimental protocol is shown (A). The number of total cells (B), monocytes/macrophages (Mc), granulocytes (Gc), and lymphocytes (Lc) in the BAL fluid were determined by Diff-Quick stain. Representative photographs of lung tissues were obtained from H&E- and PAS-stained sections (D). Histopathological scores were determined semi-quantitatively by microscopic examination (E), and the mean number of PAS-positive cells per 100 μm of basement membrane (BM) in the airway epithelium was counted using image analysis software (F). Data are shown as mean ± SD of

triplicate samples from one experiment that is representative of three independent experiments (*$p < 0.05$ and **$p < 0.01$).

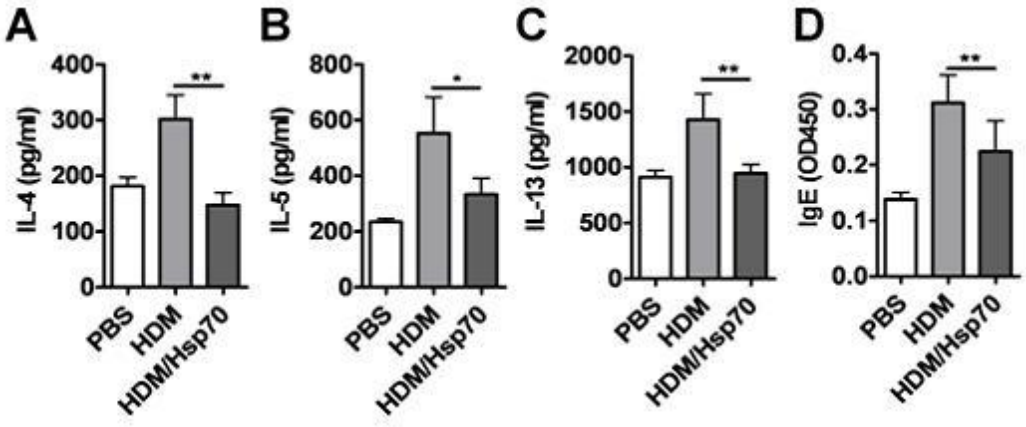

Figure 3. Reduction of Th2 cytokines and serum IgE in HDM-induced airway inflammation by Mtb Hsp70.

Allergen-induced airway inflammation was induced as described in Materials and Methods. The concentrations of IL-4 (A), IL-5 (B), and IL-13 (C) in lung extracts and the levels of HDM-specific IgE (D) in serum were measured by ELISA. Lung extracts were collected from homogenate of the right lungs of sacrificed mice. Data are shown as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments (*$p < 0.05$ and **$p < 0.01$).

Mtb Hsp70 attenuate OVA-induced airway inflammation

We sought to confirm the improvement effect of Mtb Hsp70 on allergic airway inflammation in another mice model using ovalbumin. Experimental protocol was same with above one except that OVA were used as allergen (Figure 4A). Intranasal challenge with OVA also induced severe airway inflammation and hyperplasia of MSCs in mice sensitized with only OVA/alum (OVA-sensitized mice) (Figure 4B-D). However, Mtb Hsp70 treatment at sensitization significantly reduced both airway inflammation and MSCs hyperplasia (Figure 4B-D). OVA-specific IgE level in serum was also significantly lower in Mtb Hsp70-sensitized mice, as compared with OVA-sensitized mice (Figure 4E). Taken together, Mtb Hsp70 may exert its inhibitory effect on allergic airway inflammation by reducing Th2 immune response.

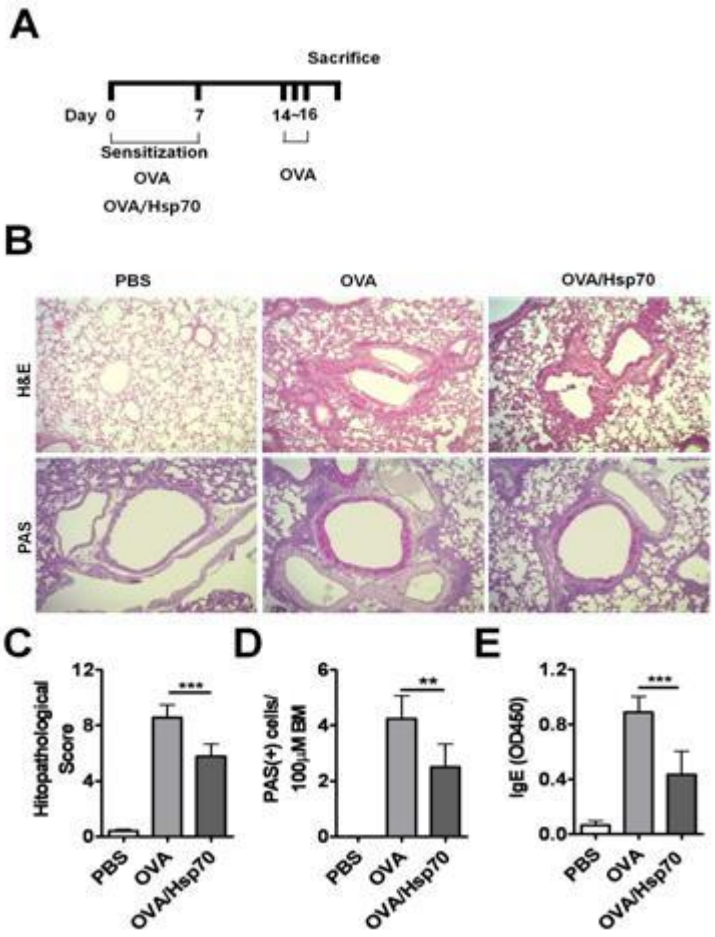

Figure 4 Attenuation of OVA-induced airway inflammation by Mtb Hsp70.

A schematic diagram of the experimental protocol is shown (A). Representative photographs of lung tissues were obtained from H&E- and PAS-stained sections (B). Histopathological scores were determined semi-quantitatively by microscopic examination (C) and the mean number of PAS-positive cells per 100 μm of basement membrane in the airway epithelium was counted using image analysis

software (D). Level of OVA-sepcific IgE in serum was measured by ELISA. Data are shown as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments (***$p < 0.001$).

TLR2 and TLR4 are essential for inhibitory effect of Mtb Hsp70 on OVA-induced airway inflammation

To determine if TLR2 and TLR4 are required for the inhibitory effects of Mtb Hsp70 in allergic airway inflammation, we performed the same *in vivo* experiment with TLR2/4 double-deficient mice. Because HDM could not induce optimal allergic inflammation in mice with TLR2 or TLR4 deficiency (Ryu et al., 2013), we used OVA as an allergen. Intranasal challenge of OVA induced airway inflammation and MSC hyperplasia in TLR2/TLR4 double-deficient mice sensitized with only OVA (Figure 5A–C). However, treatment with Mtb Hsp70 (OVA/Hsp70) during sensitization did not reduce the lung inflammation and MSC hyperplasia in TLR2/TLR4 double-deficient mice (Figure 5A–C). Moreover, neither levels of OVA-specific IgE in serum nor Th2 cytokines in lung extracts were decreased in the TLR2/TLR4 double-deficient mice treated with OVA/Mtb Hsp70 at sensitization, as compared with mice with OVA treatment alone (Figure 5D–F). These findings suggest that TLR2 and TLR4 signaling are critical for the inhibitory properties of Mtb Hsp70 on allergic airway inflammation.

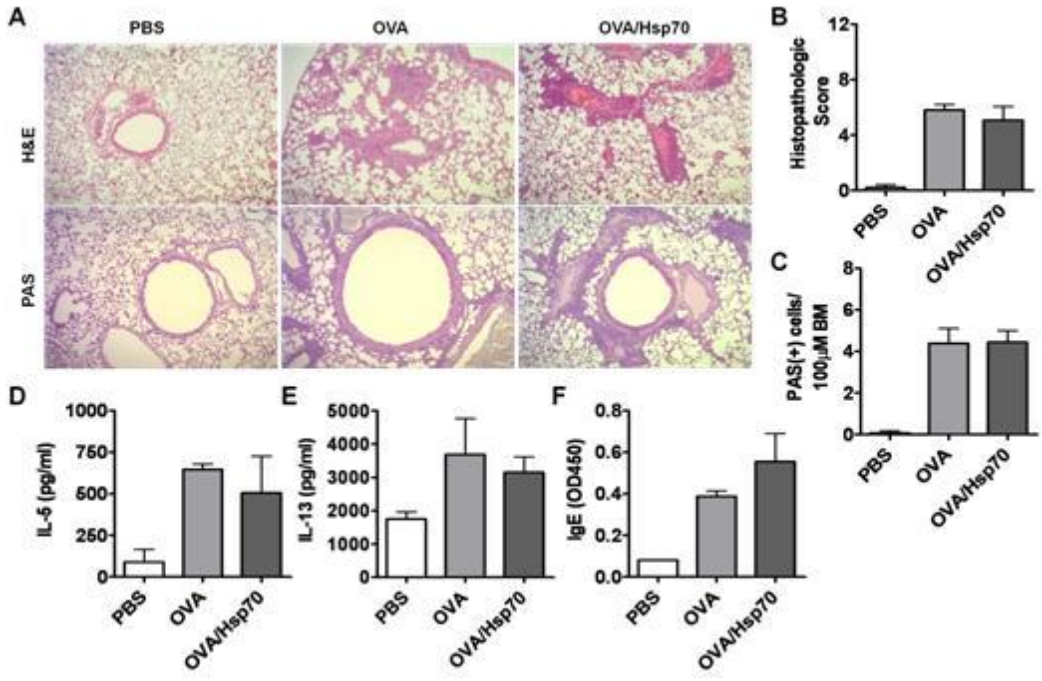

Figure 5 Effect of Mtb Hsp70 on OVA-induced airway inflammation in TLR2/4 deficient mice.

Mice deficient in both TLR2 and TLR4 were sensitized with intraperitoneal injections of 100 μg of OVA emulsified in alum on days 0 and 7. One week after the last sensitization, the mice were intranasally challenged with 200 μg of OVA extracts every day for 3 days under anesthesia and sacrificed 2 days after the last challenge. Representative photographs of lung tissues were obtained from H&E- and PAS-stained sections (A). Histopathological scores were determined semi-quantitatively by microscopic examination (B) and the mean number of PAS-positive cells per 100 μm of basement membrane (BM) in the airway epithelium

was counted using image analysis software (C). The concentrations of IL-5 (D) and IL-13 (E) in lung extracts and the levels of OVA-specific IgE in blood serum (F) were measured by ELISA. Lung extracts were collected from homogenate of the right lungs of sacrificed mice. Data are shown as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments.

3.5. Conclusion

Recent reports have shown that activation of Th1 immune response by Hsp65 from *M. leprae* attenuated airway inflammation by suppressing Th2 cytokine production and eosinophilia in BAL fluid (Erb et al., 1998; Rha et al., 2002; Yang et al., 1999). This study is suggesting that other immune modulators derived from Mycobacterium may have therapeutic effect on allergic airway inflammation.

An epidemiological study showed that exposure to airborne endotoxin in infancy may protect against asthma by promoting a Th1 response and tolerance to allergens, but exposure later in life adversely affects patient with asthma (Reed and Milton, 2001). Several animal studies have supported this epidemiological study. Administration of TLR2 or TLR4 ligands before or during allergen sensitization attenuated the level of IgE, IL-4, IL-5, and IL-13 (Delayre-Orthez et al., 2004; Gerhold et al., 2002; Haapakoski et al., 2013; Velasco et al., 2005). On the other hand, administration of TLR2 or TLR4 ligands before or during allergen challenge enhanced lung inflammation and eosinophilia in lung lavage fluid (Delayre-Orthez et al., 2004; Duechs et al., 2011). However, another report showed that intraperitoneal administration of Pam_3CSK_4 2 h after intranasal allergen challenge ameliorated the asthmatic immune response, which manifested

with an increase of IL-4, IL-5, and serum IgE, and enhanced IFN-γ production (Patel et al., 2005). Co-administration of LPS with allergen by inhalation during allergen sensitization enhanced the allergic airway inflammation response upon intranasal challenge with OVA (Eisenbarth et al., 2002; Kim et al., 2007), suggesting that the presence of the effective time to exposure an immun modulator.

In the current study, we showed the attenuation of allergic airway inflammation by Mtb Hsp70 in two different allergen-induced allergic airway inflammation mice model and TLR2 and TLR4 are involved in these effect. Our results demonstrate that Mtb Hsp70 will possibly lead to the development of new immunotherapeutic strategies for allergic airway inflammation.

CHAPTER IV

Activation of BMDCs by *Mycobacterium-tuberculosis*-derived Hsp70 via TLR signaling pathway

4.1. Abstract

As a potent immune regulator, heat shock protein 70 derived from *Mycobacterium tuberculosis* (Mtb Hsp70) has adjuvant effect and activates immune cells such as macrophages and dendritic cells (DCs). Although Toll-like receptors (TLRs) are known to involve in DCs activation by Mtb Hsp70, there is still a controversy and the underlying mechanism is not well understood. In this study, we examined whether TLR2, TLR4, TRIF, and MyD88 regulate Mtb Hsp70-induced DCs activation. Purified recombinant Mtb Hsp70 led to the production of cytokines (IL-6, IL-12, and TNF-α) in bone marrow derived dendritic cells (BMDCs) from WT mice. The production of cytokines were partially impaired in TLR4-deficient and abolished in TLR2/4-deficient BMDCs, although TLR2-deficient BMDCs could produce comparable level of cytokines with WT cells. Mtb Hsp70 also up-regulated the expression of CD80, CD86, and MHC class II, similarly which was impaired in TLR4- and TLR2/4-deficient BMDCs. Both TRIF and Myd88, the adaptor molecules of TLRs signaling, were required for the production of cytokines and the expression of CD80, CD86, and MHC class II molecules in BMDCs in response to Mtb Hsp70. In addition, Mtb Hsp70 induced phosphorylation of IκB-α, p38, ERK, and JNK via TLR2/4-, TRIF-, or MyD88-dependent manner. In mixed leukocyte reaction with naive

$CD4^+$ T cell from OT-II mouse, IFN-γ production was increased depend on the concentration of Mtb Hsp70 and partially impaired in TLR2- and TLR4-deficient BMDCs and completely depleted in TLR2/4-, TRIF-, and MyD88-deficient BMDCs. In addition, these effects of Mtb Hsp70 on DC were enhanced by co-treatment with muramyl dipeptide (MDP). In conclusion, our results revealed the TLRs signaling-mediated cellular mechanism of Mtb Hsp70-induced DC activation.

4.2. Introduction

In previous study, I observed the attenuation effects of Mtb Hsp70 on development of allergic airway inflammation and these effects are diminished in TLR2/4-deficient mouse. This results suggested the necessity of further studis about the role of TLR2 and TLR4 in Mtb Hsp70-induced immune activation.

Although TLR2 and TLR4 have been suggested as receptors for Mtb Hsp70, there is still a controversy (Asea et al., 2002; Qazi et al., 2007; Vabulas et al., 2002). IL-12 and TNF-α production by human Hsp70 was impaired in TLR4-deficient BMDCs, as compared with WT cells, whereas TLR2 deficiency did not affect the cytokines production (Vabulas et al., 2002). TLR4, but not TLR2, was also critical for IL-12 production by Mtb Hsp70 stimulation in bone marrow-derived macrophages (BMDMs) (Qazi et al., 2007). In contrast, Bulut *et al*. (2005) showed that Mtb Hsp70-induced production of IL-6 was partially impaired in TLR2-deficient macrophages and TNF-α production was abolished in TLR4-deficient cells (Bulut et al., 2005). Mtb Hsp70 also activated NF-κB in both TLR2 and TLR4 transfected HEK293 cells and co-transfection of TLR2 and TLR4 synergistically augmented the NF-kB activation (Asea et al., 2002). These are suggesting that the underlying mechanism of Mtb Hsp70-mediated immune activation in DCs remains to be elucidated (Bulut et al., 2005; Qazi et al., 2007).

In this study, we investigated whether TLR2, TLR4, TRIF and MyD88 are required for Mtb Hsp70-induced activation of DCs by evaluating cytokines production, activation of transcription factor, expression of co-stimulatory molecules, and the ability to differentiate the $CD4^+$ T cells into cytokine producing effector cells.

4.3. Materials and Methods

Animals

Wild-type C57BL/6 mouse were purchased from KOATECH (Pyeongtaek, Kyeonggi-do, Korea). TLR2-, TLR4- deficient mice, MyD88-dificient mice, and OT-II T-cell receptor transgenic mice on C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice deficient in both TLR2 and TLR4 were generated by crossing TLR2-deficient and TLR4-deficient mice and intercrossing the F1 generation (Park et al., 2014). TRIF-deficient mice on C57BL/6 background were a gift from S. Akira (Osaka University, Osaka, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Konyang University.

Purificed recombinant Mtb hsp70

To produce recombinant Rv0350 protein, the corresponding gene was amplified by PCR using Mtb H37Rv ATCC27294 genomic DNA as template and the following primers: forward, 5'-GGGCCCCATATGGCTCGTGCGGTCGGGATC -3', and reverse, 5'-GGGCCCAAGCTTCTTGGCCTCCCGGCCGTCGTC -3'. The PCR product of

Rv0350 was cut with *Nde*I and *Hind*III. Both the products were inserted into pET22b (+) vector (Novagen, Madison, WI, USA) and the resultants were sequenced. The recombinant plasmids containing *Rv0350* were transformed into *E. coli* BL21 cells by heat-shock for 1 min at 42℃. The overexpressed Rv0350 was prepared with slight modifications as previously described (ref). Briefly, *E. coli* containing recombinant plasmid were grown at 37℃ until the optical density (OD) at 600 nm was 0.4 to 0.5 and then induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG; ELPIS-Biotech, Daejeon, South Korea). The bacterial cells were then harvested by centrifugation and suspended in 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 5 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (Sigma); and lysed by sonication. The recombinant Rv0350 was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography in accordance with the manufacturer's instructions (Qiagen, Chatsworth, CA, USA). Each purification step was analyzed by 13.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) with Coomassie brilliant blue stain and immunoblot using anti-His antibodies (Santa Cruz). The purified protein was pooled, concentrated and dialyzed against phosphate-buffered saline (PBS, pH 7.4). To remove endotoxin contamination, the dialyzed recombinant protein was incubated with polymyxin B-agarose (PmB, Sigma) for 6 h at 4℃. Lastly,

purified endotoxin-free recombinant protein was filter sterilized and frozen at -70℃. The protein concentration was estimated with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Residual LPS in the Rv0350 preparation was determined using the Limulus amoebocyte lysate (LAL) test (Lonza, Basel, Switzerland), according to the manufacturer's instructions. The purity of Rv0350 was evaluated by Coomassie brilliant blue (CB) staining and Western blot using an anti-histidine antibody. Endotoxin contamination was evaluated by an LAL assay and was less 15 pg/ml (0.1 UE/ml) in Mtb Hsp70 preparation. The protein concentration was estimated with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Preparation and treatment of bone marrow derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were prepared as previously described (Lutz et al., 1999). Briefly, bone marrow cells were cultured with GM-CSF (20ng/ml) containing media, with fresh GM-CSF added on days 3 and 6. After 9 days, non-adherent cells were collected by vigorous aspiration. BMDCs were seeded in 48-well plates at a concentration of 2×10^5/well for cytokine analysis or in 6-well plates at a concentration of 2×10^6 cells/well for

western blot and FACS analysis and treated with Mtb Hsp70. The culture supernatants were collected 12 h after treatment for cytokine analysis and cells were collected 24 h after treatment or at indicated times for Western blot or FACS analysis.

Measurement of cytokines

The concentrations of IL-6, IL-12p40, TNF-α, CXCL-1, CCL-2 and IFN-γ were measured by a commercial ELSIA kit (R&D System, Minneapolis, MN, USA).

Western blot analysis

Prepared cells were lysed in buffer containing 1% Nonidet-P40 supplemented with protease inhibitor (cOmplete Mini EDTA-free, Roche, Mannheim, Germany), phosphatase inhibitor (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich, St. Louis, MO, USA) and 2 mM dithiothreitol. For western blot analysis, lysates were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes by electro-blotting. The membranes were immunoblotted with primary antibodies for phospho- and regular-form of IκB-α (Cell signaling Technology, Beverly, MA,

USA), JNK (Cell signaling Technology), p38 (Santa Cruz biotechnology, TX, USA) and ERK (Santa Cruz biotechnology). After immunoblotting with HRP-conjugated secondary antibodies (Santa Cruz biotechnology, TX, USA), proteins were detected with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Bands were visualized after exposing the blots to a CP-BU new film (Agfa HealthCare, Mortsel, Belgium). Intensities of band were measured using ImageJ version 1.44 (National Institutes of Health, Bethesda, MD, USA).

Mixed leukocyte reaction

$CD4^+$ T-cells were isolated using a MACS column (Miltenyi Biotec, San Jose, California, USA) from spleen of OT-II mice. BMDCs were prepared from wild type C57BL/6, $TRIF^{-/-}$ and $Myd88^{-/-}$ mice. $CD4^+$ T-cells were co-cultured with treated BMDCs stimulated by $OVA_{323-339}$ (Peptron, Daejeon, South Korea) for 24 h at BMDCs:T cell ratios of 1:10. After incubation for 5 days, concentration of IFN-γ in culture supernatant was measured with commercial ELSIA kit (R&D System, Minneapolis, MN, USA).

Analysis of the expression of surface molecules by flow cytometry

Treated BMDCs were resuspended in PBS. After staining with FITC-conjugated anti-I-Ab (BD Biosciences, San Jose, CA, USA), CD80 (BD Biosciences) or CD86 (BD Biosciences) for 15 min at 4℃, cells were washed and resuspended in 0.5% BSA in PBS. The fluorescence was measured by flow cytometry (BD FACSCalibur, BD Biosciences), and the data were analyzed using BD Cell-Quest Pro software (BD Biosciences).

Statistical analysis

The significance of differences in mean values of the groups was evaluated by t-tests, and values are expressed as means ± SD. All statistical calculations were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

4.4 Results

Mtb Hsp70-induced cytokine production of BMDCs was not caused by LPS contamination.

To determine the non-cytotoxic concentration of Mtb Hsp70 on BMDCs, cytotoxic analysis was performed by staining with anti-CD11c, annexin V, and propidium iodide (PI). Mtb Hsp70 displayed no cellular toxicity on BMDCs at a concentration of 10 μg/mL (Figure 1A). These findings indicate that the recombinant protein is not cytotoxic to BMDCs when used at concentrations below 10 μg/mL. After 12 h stimulation, Mtb Hsp70 (5 μg/mL) induced IL-6, TNF-α, and IL-12p40 production in BMDCs (Figure 1B-D). To rule out the effect of LPS contamination, we performed an inhibition assay using polymyxin B (PMB), which inhibits LPS by binding to its functional domain lipid A. Treatment with PMB inhibited LPS-induced production of cytokines, but it did not affect the cytokine production of BMDCs in response to Mtb Hsp70 (Figure 1B–D). These findings indicate that cytokine production by BMDCs in response to our preparation of Mtb Hsp70 was not due to LPS contamination.

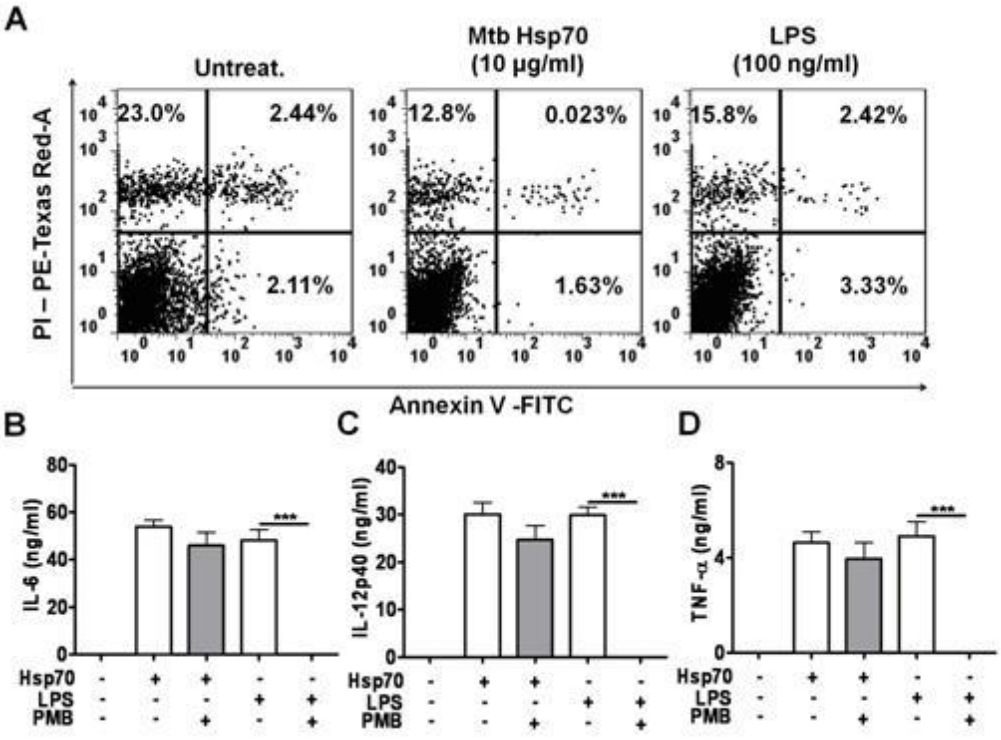

Figure 1 The cytokine production of BMDCs by Mtb Hsp70.

Cytotoxicity of Mtb Hsp70 on dendritic cells analysis (A). BMDCs from WT mice were untreated or stimulated with Mtb Hsp70 (5 μg/m1) or LPS (100 ng/ml) with or without polymyxin B (50 μg/ml) for 12 h. Cell-free supernatants were analyzed for the production of IL-6 (B), TNF-α (C), and IL-12p40 (D) by ELISA. Data are shown as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments (***$p < 0.001$).

Mtb Hsp70 via TLR2 and TLR4 signaling controls cytokine production and activation of NF-κB and MAPKs in BMDCs

To assess the role of TLR2 and TLR4 in DC activation by Mtb Hsp70, we first sought to determine if TLR2 and TLR4 were required for Mtb Hsp70-induced cytokine production in BMDCs. The Mtb Hsp70-induced IL-6, TNF-α, and IL-12p40 production was reduced in TLR4-deficient BMDCs compared with WT cells, while TLR2 deficiency did not influence cytokine production (Figure 2A–C). Remarkably, high-dose Mtb Hsp70 could induce substantial cytokine production, even in TLR4-deficient BMDCs, and this cytokine production was absolutely impaired in TLR2/TLR4-double knockout BMDCs (Figure 2A–C). These results suggest that TLR4 signaling may be the major pathway that leads to Mtb Hsp70-induced cytokines production in BMDCs, and TLR4 may share some redundant functions with TLR2. NF-κB and MAPKs are key molecular factors regulating cytokine and chemokine production in various cell types, including DCs. To clarify the molecular mechanism for Mtb Hsp70-induced cytokine production, we examined NF-κB and MAPK activation in response to Mtb Hsp70, using Western blot analysis.

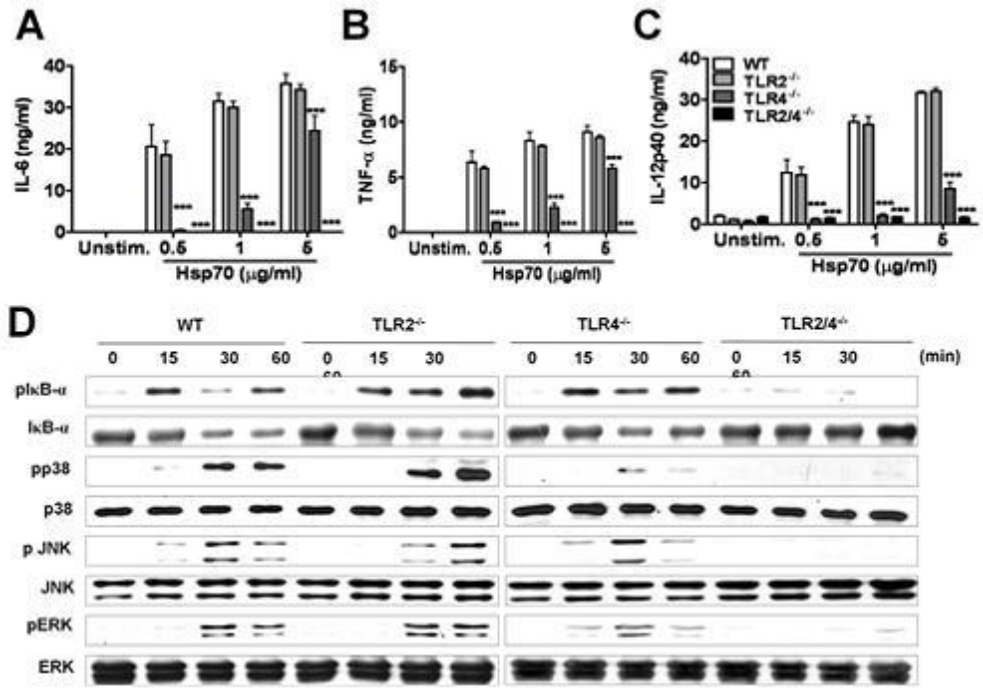

Figure 2 Role of TLR2 and TLR4 in cytokine production and activation of NF-κB and MAPK of Mtb Hsp70 treated BMDCs.

BMDCs from WT, TLR2-, TLR4-, and TLR2/4-double deficient mice were untreated or stimulated with Mtb Hsp70 at indicated doses for 12 h. Culture supernatant was analyzed for the production of IL-6 (A), TNF-α (B), and IL-12p40 (C) by ELISA. BMDCs were also stimulated with Mtb Hsp70 (5 μg/ml) for the indicated times, and whole-cell extracts were immunoblotted with antibodies that detect unphosphorylated and phosphorylated forms of IκB-α, p38, JNK, and ERK (D). Data are shown as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments (***$p < 0.001$).

Mtb Hsp70 induced IκB-α phosphorylation and degradation within 15 min of stimulation in WT BMDCs (Figure 2D). In addition, the phosphorylation of p38, ERK, and JNK was also detected within 30 min of stimulation (Figure 2D). However, this activation of NF-κB and MAPKs was absolutely impaired in TLR2/TLR4 double-knockout BMDCs (Figure 2D). Taken together, both TLR2 and TLR4 signaling play an important role in Mtb Hsp70-mediated cytokine production and NF-κB and MAPK activation in BMDCs.

CD80, CD86, and MHC class II expression levels are upregulated by Mtb Hsp70 via TLR2 and TLR4 signaling and TLR2 and TLR4 are essential for IFN-γ production in $CD4^+$ T cells triggered by Mtb Hsp70-treated BMDCs

Increased expression of surface molecules, such as CD80, CD86, and MHC class II, is a critical marker for DC activation that triggers T cell response. We examined if Mtb Hsp70 treatment upregulated such molecules in BMDCs and if TLR2 or TLR4 signaling was required for the response. Flow cytometric analysis revealed that Mtb Hsp70 treatment increased CD80, CD86, and MHC II expression 24 h after stimulation in BMDCs (Figure 5A and 5B).

This upregulated expression was not affected by TLR2 deficiency, whereas it was significantly reduced in TLR4-deficient BMDCs (Figure 3A). Moreover, the increased expression was abolished in TLR2/TLR4 double-knockout BMDCs (Figure 3A). These findings suggest that TLR4-signaling may be the major signaling pathway for BMDC activation by Mtb Hsp70, although TLR2 may be partially involved in this response. To determine if Mtb Hsp70 could induce differentiation of naïve T cells, BMDCs were treated with OVA in the absence or presence of Mtb Hsp70 (1 or 10 μg/ml) for 1 day, and naive $CD4^+$ T cells from OT-II mice were co-cultured. After 5 days of co-culture, IFN-γ was measured in culture supernatants by ELISA. Both TLR2- and TLR4-deficient BMDCs exhibited partially decreased IFN-γ production induced by Mtb Hsp70 in co-culture supernatants (Figure 3B). However, IFN-γ production was completely depleted in $CD4^+$ T cells co-cultured with TLR2/TLR4 double-deficient BMDCs (Figure 3B). These findings suggest that the ability of Mtb Hsp70-stimulated BMDCs to differentiate naïve T cell into IFN-γ-producing Th1 cells depends on TLR2 and TLR4 signaling.

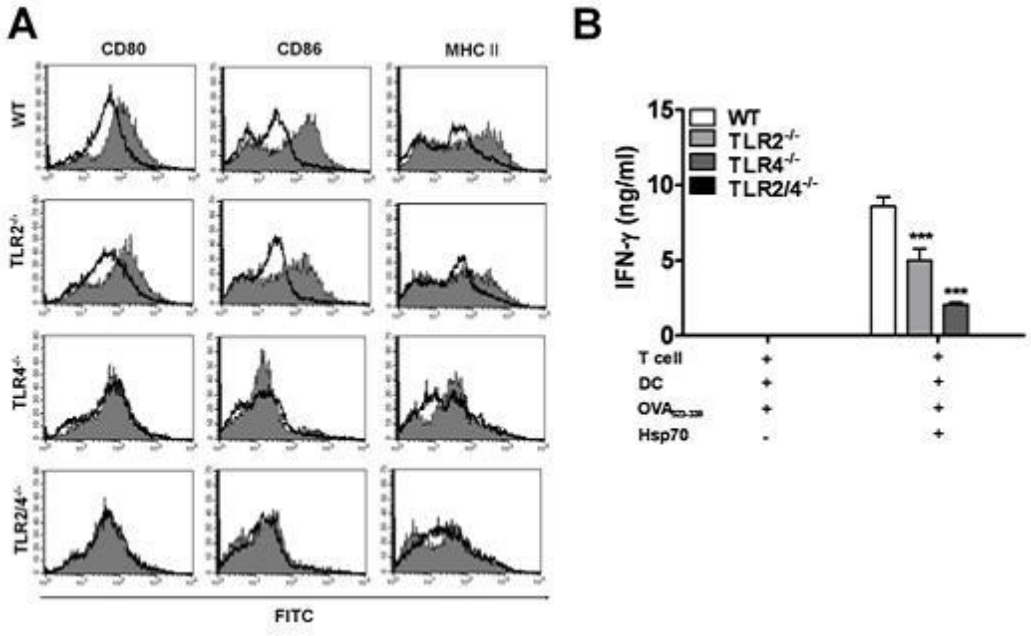

Figure 3 TLR2 and TLR4-mediated up-regulation of cell surface molecules of BMDCs by Mtb Hsp70 and IFN-γ production of $CD4^+$ T cells co-cultured with dendritic cells stimulated by Mtb Hsp70

BMDCs from WT, TLR2-, TLR4-, and TLR2/4 double deficient mice were stimulated with Mtb Hsp70 (5 μg/ml) for 12 h, and the surface expression levels of CD80, CD86, and MHC II were analyzed by flow cytometry (A). Bold lines with white color (untreated), Dotted lines with gray color (Mtb Hsp70-treated). BMDCs from WT, TLR2-, TLR4-, and TLR2/4 double deficient mice were treated with OVA peptide ($OVA_{323-339}$) in the absence or presence of Mtb Hsp70 (5 μg/ml) for 1 day. Then, naive $CD4^+$ T cells from OT-II mice were co-cultured with these

BMDCs for 5 days. The concentration of IFN-γ in culture supernatant was measured by ELISA (B). Data are shown as mean ± SD (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

TRIF and MyD88 regulate cytokines production, activation of transcription factors, and inducding of IFN-γ producing CD4+ T cell proliferation by BMDCs in response to Mtb Hsp70

We next examined the involvement of TRIF and MyD88 in Mtb Hsp70-induced cytokines production in BMDCs. WT BMDCs could produce huge amount of IL-6, IL-12p40, and TNF-α in response to various doses of Mtb Hsp70 (Figure 4A-C). Although TRIF deficiency led to significantly less production of those cytokines in BMDCs, as compared with WT cells, Mtb Hsp70 still induced the production of substantial level of cytokines in TRIF-deficient BMDCs in a dose-dependent manner (Figure 4A-C). In contrast, such cytokines production was mostly impaired in MyD88-deficient BMDCs (Figure 4A-C). These findings suggest that both TRIF and MyD88 are required for optimal production of cytokines by DCs in response to Mtb Hsp70. NF-κB and MAPKs are key molecular factors regulating cytokines and chemokines production in various cell types including DCs. To clarify the molecular mechanism for Mtb Hsp70-induced cytokines production, we examined NF-κB and MAPKs activation in response to Mtb Hsp70 by Western blot analysis. By 15 or 30 (for JNK) min after treatment, TRIF deficiency did not affect IκB-α degradation and the phosphorylation of IκB-α, p38, and JNK in BMDCs in response to Mtb Hsp70, whereas those

phosphorylation became weak in TRIF-deficient BMDCs, as compared with WT cells, after those times (Figure 4D). ERK phosphorylation was also partially impaired in TRIF-deficient cells at entire time points (Figure 4D). In contrast, IκB-α degradation and the phosphorylation of all tested molecules (IκB-α, p38, ERK, and JNK) induced by Mtb Hsp70 remarkably impaired in MyD88-deficient BMDCs at all tested time points (Figure 4D). DCs activation and maturation trigger the differentiation of T cells. To determine whether Mtb Hsp70 induces such differentiation of T cells, BMDCs and CD4+ T cells isolated from spleen of OT-II transgenic mice (OT-II-CD4+ T Cells) were co-cultured at the absence or presence of OVA peptide ($OVA_{323-339}$) or Mtb Hsp70. At 5 days after co-culture, IFN-γ was measured in culture supernatant by ELISA. Single treatment with $OVA_{323-339}$ did not induce IFN-γ production (Figure 4E). At the presence of $OVA_{323-339}$, Mtb Hsp70 induced IFN-γ production in the co-culture of WT BMDCs and OT II-CD4+ T cells, whereas deficiency of TRIF or MyD88 in BMDCs led to complete inhibition of Mtb Hsp70-induced IFN-γ production (Figure 4E).

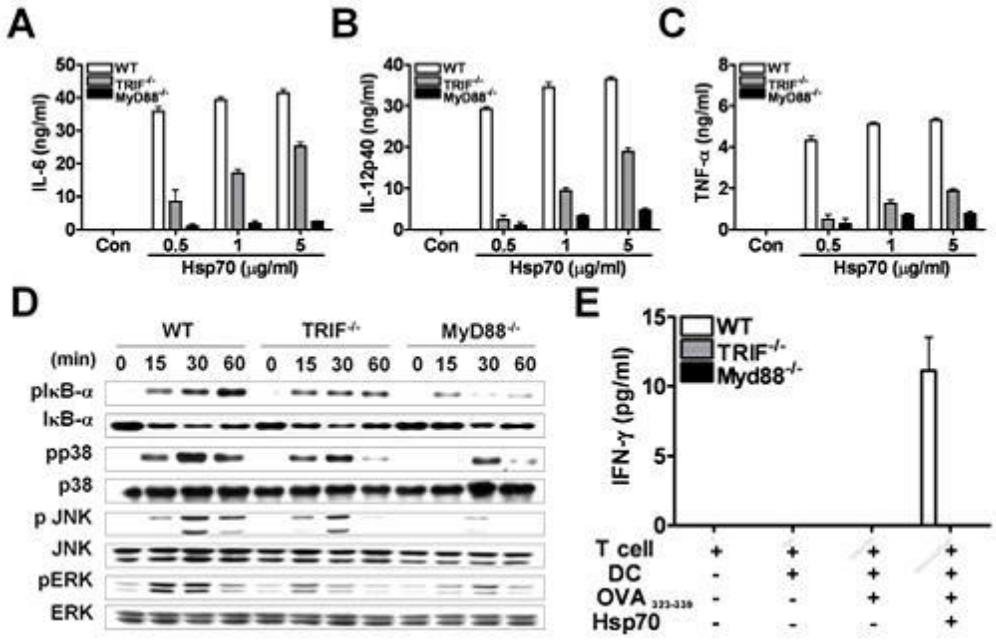

Figure 4 The role of TRIF- and MyD88 in activation of BMDCs by Mtb Hsp70.

WT, TRIF- and MyD88-deficient BMDCs were incubated with Mtb Hsp70 for 12 h. The concentration of IL-6 (A), IL-12p40 (B) and TNF-α (C) in supernatant were measured by ELISA. WT, TRIF-, and MyD88-deficient BMDCs incubated with Mtb Hsp70 (5μg/ml) for indicated times and cellular proteins were extracted. IκB-α degradation and phosphorylation of IκB-α, p38, JNK and ERK of were analyzed using Western blot (A). Relative band intensity of each protein was expressed as a percentage compared to the value of total form of protein or ERK (D). WT, TRIF- and MyD88-deficient BMDCs were pretreated with Mtb Hsp70

(5 μg/ml) with or without OVA peptide ($OVA_{323-339}$) for 24 h, then co-cultured with naïve CD4+ T cells for 5 days. The concentration of IFN-γ in supernatant was measured by ELISA (E).Data are shown as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments (***, $p<0.001$)

Mtb Hsp70 leads to upregulation of CD80, CD86, and MHC class II expression via mostly TRIF-dependent signaling

Increased expression of surface molecules such as CD80, CD86, and MHC class II is a critical marker for DCs activation. We thus examined the role of TRIF and MyD88 on the surface markers expression increased by Mtb Hsp70 in BMDCs (Figure 5). Flow cytometry analysis revealed that Mtb Hsp70 increased the expression of CD80, CD86, and MHC class II at 12 h after stimulation in WT BMDCs. The enhanced expression of CD80, CD86, and MHC class II was almost impaired in TRIF-deficient BMDCs as compared with WT cells (Figure 5). In contrast, MyD88 deficiency exerted only minor effect to reduce the expression of those surface markers (Figure 5).

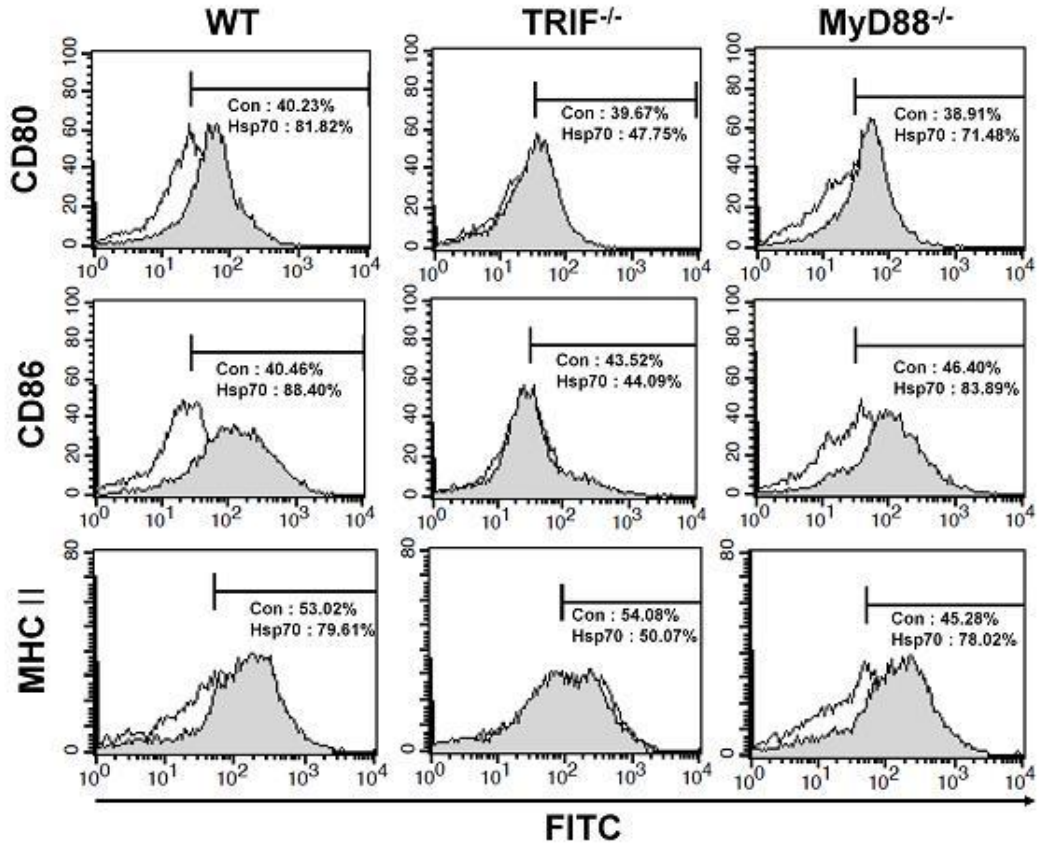

Figure 5 Enhancement of cell surface molecules of BMDCs induced by Mtb Hsp70 via TRIF and MyD88-dependent pathway.

WT, TRIF-and MyD88-deficient BMDCs were stimulated with Mtb Hsp70 (5μg/ml) for 12h and expression of CD80, CD86, and MHC class II was analyzed by flow cytometry.

4.5. Conclusion

In our study, deficiency in TLR2, TLR4, and their adaptor molecules MyD88 and TRIF on DCs disrupted Mtb Hsp70-induced activation of DCs. TLR4 was predominant in cytokine production and activation of NF-κB and MAPKs upon Mtb Hsp70 on DCs. In accordance with our results, Asea *et al.* showed that recombinant human Hsp70 induced maximal cytokine production on DCs through both TLR2 and TLR4 using HEK293 cell transfection experiments (Asea et al., 2002). TLR2 and TLR4 also play a critical role in effect of Mtb Hsp70 on ability of DCs to drive T cell differentiation. Mtb Hsp70-stimulated DCs drove $CD4^+$ T cell from OT-II mice into IFN-γ producing T cells after OVA ligation and both TLR2 and TLR4 were essential to this effect.

TRIF and MyD88, core adaptor proteins in TLRs signaling, seem to be essential for Mtb Hsp70-induced immune responses. MyD88 was indispensable for antigen specific IgG production in mice boosted by and IL-12 production in macrophages in response to Hsp70s originated from various microbes including *M. tuberculosis*, even though they utilized different TLRs to induce such immune responses (Qazi et al., 2007). And IL-6 production induced by Mtb Hsp65 or Hsp70 was absolutely abolished in TRIF- or TRAM-deficient macrophages (Bulut et al., 2005). However, it is unclear which one is dominant pathway for Mtb

Hsp70-mediated immune responses between TRIF and MyD88, as there is no report of direct comparison. In this study, we revealed that both TRIF and MyD88 are involved in optimal production of cytokines by DCs in response to Mtb Hsp70. Strikingly, although cytokine production by Mtb Hsp70 was totally abolished in MyD88-deficient DCs, the expression of MHC II, CD80, and CD86 of those DCs was intact. These results can be explained by recent reports demonstrating that LPS treatment induced expression of MHC II, CD80, and CD86 in DCs via TRIF not MyD88 signaling (Hoebe et al., 2003; Kamon et al., 2006). Although TRIF, and MyD88 differently affect on the activation and maturation of DCs, differentiation of T cells into IFN-γ producing T cells by Mtb Hsp70-treated DCs were completely abolished when co-cultured with TRIF- or MyD88-deficient DCs.

These results can be explained by the necessity of both adhesion molecules expression and cytokines production by DCs in T cell activation. In antigen presenting procedure by DCs, engagement of T cell receptor with the MHC:peptide complex and co-stimulatory signal that originated from the interaction of receptor on T cells such as CD28 with ligands such as CD80/86 on DC are necessary (Bluestone, 1995). The absence of further signal by co-stimulatory molecules lead to a T cell state called "anergy" (Gimmi et al., 1993; Quaratino et al., 2000). But in addition to co-stimulatory signals, cytokines

produced by DCs are also important for T cell activation. IL-12 is one of the representative cytokine to differentiate naive T cells into IFN-γ producing effector T cells (Langenkamp et al., 2000; Moser and Murphy, 2000). Even though MyD88-deficient DCs have express similar amount of co-stimulatory molecules and MHC class II by Mtb Hsp70 stimulation, they could not differentiate T cells due to loss of cytokines production ability. It also considered that T cell activation might be not achieved at co-culture with TRIF-deficient DCs due to impairment of up-regulation of CD80, CD86, and MHC class II in response to Mtb Hsp70. Taken together, our findings suggest that TLR2, TLR4, TRIF, and MyD88 are critical factors for DCs activation induced by Mtb Hsp70.

DCs are the most efficient antigen presenting cells (Diebold, 2008) and direct differentiation of naive $CD4^+$ T cells into Th1 or Th2 effector by co-stimulatory molecules and cytokines (Kapsenberg, 2003; Langenkamp et al., 2000; Liu, 2001). And their activation and maturation is related to mechanism of many adjuvant (De Becker et al., 2000; De Smedt et al., 1996; Fujii et al., 2003; Shah et al., 2003). Therefore, modulation of immune response of dendritic cells can be the effective therapeutic targets for many diseases. In this study, we revealed the mechanism of TLR2 and TLR4 signaling pathway-mediated DCs activation by Mtb Hsp70.

CHAPTER V

Synergistic effect of muramyl dipeptide with *Mycobacterium tuberculosis*-derived Hsp70 on activation of BMDCs

5.1. Abstract

Heat shock protein 70 from *Mycobacterium tuberculosis* (Mtb Hsp70) activates dendritic cells via TLRs signaling pathway. In previous studies, Muramyl dipeptide (MDP) enhances the immune activation induced by ligands of TLRs. In this study, we evaluated effect of MDP on activation of bone marrow derived dendritic cells (BMDCs).

MDP treatment with Mtb Hsp70 dramatically increased production of IL-6, IL12p40 and TNF-α in BMDCs compared with Mtb Hsp70 alone whereas these effects were abolished in Nod2-deficient BMDCs. Phosphorylation of IκB-α and ERK and impairment of phagocytosis, which is an indicator of DC maturation were enhanced by MDP co-treatment with Mtb hsp70 in BMDCs. In addition, ability of Mtb Hsp70-stimulated BMDCs to induce IFN-γ productions of T cells was increased by MDP co-treatment.

Our study showed the synergistic effects of MDP with Mtb Hsp70 on DCs activation. The use of MDP with Mtb Hsp70 to induce immune activation may provide an effective strategy for vaccination to treat cancer and protect against pathogens.

5.2. Introduction

Muramyl dipeptide (MDP) is an immunoreactive derivative of peptidoglycan from all Gram-negative and Gram-positive bacteria (Traub et al., 2006) and recognized by Nod2, a cytosolic pattern recognition receptor (PRR) in host innate immune cells (Chen et al., 2009; Girardin et al., 2003). After recognition by Nod2, MDP directly recruit receptor interacting protein 2 (RIP2), a caspase recruitment domain (CARD)-containing serine/threonine kinase, and are associated with it through CARD-CARD interaction. Subsequently, this signaling leads to the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), resulting in the production of pro-inflammatory cytokines and chemokines (Chen et al., 2009; Inohara et al., 2000; Kobayashi et al., 2002). In addition, MDP enhanced the antibody production in ovalumin-immunized mice (Leclerc et al., 1978) and it has been known to be responsible for function of Freund's complete adjuvant (Ellouz et al., 1974).

It has been reported that MDP has synergistic action with other immune activating molecules including lipopolysaccharide (LPS) and Pam3Cys, well known ligands for Toll-like receptor (TLR) 4 and TLR2 respectively. MDP enhanced production of TNF-α and IL-10 in Pam3Cys-stimulated murine peritoneal macrophages (Netea et al., 2005) and production of TNF-α and IL-6 in

human whole blood, monocytes and DCs stimulated with LPS (Fritz et al., 2005; Wang et al., 2001a).

Dendritic cells are antigen presenting cells (Diebold, 2008) and direct differentiation of naive $CD4^+$ T cells by co-stimulatory molecules and cytokines (Kapsenberg, 2003; Langenkamp et al., 2000; Liu, 2001). And they are main target of immune activators such many adjuvant (De Becker et al., 2000; De Smedt et al., 1996; Fujii et al., 2003; Shah et al., 2003). Development of effective way to activate of dendritic cells may help to find novel strategies to enhance the effect of vaccination and immunotherapy for cancer and allergic disease.

In previous study, I observed that purified Hsp70 from *M. tuberculosis* activate murine dendritic cells via TLR signaling associated mechanism. In the present study, we evaluated effect of MDP on activation of BMDCs induced by Mtb Hsp70.

5.3. Materials and Methods

Animals

Wild-type C57BL/6 and BALB/c mouse were purchased from KOATECH (Pyeongtaek, Kyeonggi-do, Korea). Nod2-deficient mice on C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The animals were maintained at Konyang University and fed a commercial mouse diet and provided with water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of Konyang University.

Purificed recombinant Mtb hsp70

To produce recombinant Mtb Hsp70 protein, the corresponding gene was amplified by PCR using Mtb H37Rv ATCC27294 genomic DNA as a template and the following primers: forward, 5´-GGGCCCCATATGGCTCGTGCGGTCGGGATC-3´, and reverse, 5´-GGGCCCAAGCTTCTTGGCCTCCCGGCCGTCGTC-3´. After cutting with *Nde*I and *Hind*III, PCR products were inserted into the pET22b (+) vector (Novagen, Madison, WI, USA). The recombinant plasmids were transformed into

E. coli BL21 cells by heat-shock for 1 min at 42℃. The overexpressed Mtb Hsp70 was prepared with slight modifications as previously described (Franken et al., 2000). The recombinant Mtb Hsp70 was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography in accordance with the manufacturer's instructions (Qiagen, Chatsworth, CA, USA). Purified endotoxin-free recombinant protein was filter sterilized and frozen at -70℃ until use.

Preparation and treatment of bone marrow derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were prepared as previously described (Lutz et al. 1999). Briefly, bone marrow cells from WT and Nod2-deficient mice were cultured with RPMI-1640 media containing GM-CSF (20 ng/ml) with additional fresh GM-CSF containing media added on days 3 and 6. After 9 days, non-adherent cells were collected by vigorous aspiration and seeded in 48-well plates at a concentration of 2×10^5/well for cytokine analysis or in 6-well plates at a concentration of 2×10^6 cells/well for western blot or endocytic activity analysis. Then cells were treated with Mtb Hsp70 (0.05 μg/ml), MDP (1 μg/ml, Ac-(6-O-stearoyl)-muramyl-Ala-D-Glu-NH_2, Bachem, Hauptstrasse, Switzerland) alone or Mtb Hsp70 with MDP for 12 h and culture supernatants were collected for cytokine analysis.

Measurement of cytokines

The concentrations of IL-6, IL-12p40, TNF-α, CXCL-1, CCL-2 and IFN-γ were measured by a commercial ELSIA kit (R&D System, Minneapolis, MN, USA).

Western blot analysis

Prepared cells were lysed in buffer containing 1% Nonidet-P40 supplemented with protease inhibitor (cOmplete Mini EDTA-free, Roche, Mannheim, Germany), phosphatase inhibitor (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich, St. Louis, MO, USA) and 2 mM dithiothreitol. For western blot analysis, lysates were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes by electro-blotting. The membranes were immunoblotted with primary antibodies for phospho- and regular-form of IκB-α (Cell signaling Technology, Beverly, MA, USA), JNK (Cell signaling Technology), p38 (Santa Cruz biotechnology, TX, USA) and ERK (Santa Cruz biotechnology). After immunoblotting with HRP-conjugated secondary antibodies (Santa Cruz biotechnology, TX, USA), proteins were detected with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Bands were visualized after exposing the

blots to a CP-BU new film (Agfa HealthCare, Mortsel, Belgium). Intensities of band were measured using ImageJ version 1.44 (National Institutes of Health, Bethesda, MD, USA).

Mixed leukocyte reaction

$CD4^+$ T-cells were isolated using a MACS column (Miltenyi Biotec, San Jose, California, USA) from spleen of OT-II mice. BMDCs were prepared from wild type C57BL/6, $TRIF^{-/-}$ and $Myd88^{-/-}$ mice. $CD4^+$ T-cells were co-cultured with treated BMDCs stimulated by $OVA_{323-339}$ (Peptron, Daejeon, South Korea) for 24 h at BMDCs:T cell ratios of 1:10. After incubation for 5 days, concentration of IFN-γ in culture supernatant was measured with commercial ELSIA kit (R&D System, Minneapolis, MN, USA).

Quantification of endocytic activity of BMDCs

BMDCs were incubated with Hsp70 (0.05 μg/ml), MDP (1 μg/ml) alone or Mtb Hsp70 with MDP for 24 h, and then 1 mg/ml FITC-conjugated dextran was pulsed at 37℃ for 45min. Cells were washed with cold PBS and resuspended in 0.5% BSA in PBS. The fluorescence was measured by flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA) and the data were analyzed

using BD Cell-Quest Pro software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

The significance of differences in mean values of the groups was evaluated by t-tests, and values are expressed as means ± SD. All statistical calculations were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

5.4. Results

MDP synergized with Mtb Hsp70 to produce proinflammatory cytokines in BMDCs via Nod2.

I determined effect of MDP on this cytokine production of BMDCs as an *in vitro* assay. Cells were treated with Mtb Hsp70 (0.05 μg/ml), MDP (1 μg/ml) alone or Mtb Hsp70 (0.05 μg/ml) with MDP (1 μg/ml) for 12h. Mtb Hsp70 induced production IL-6, TNF-α and IL-12p40 in BMDCs, whereas 1 μg/ml of MDP did not (Figure 1A-C). However, MDP treatment with Mtb Hsp70 dramatically increased cytokine production in BMDCs compared to Mtb Hsp70 treatment (Figure 1A-C). To see whether this increased production of cytokines by MDP with Mtb hsp70 in BMDCs is mediated by Nod2, a specific receptor for MDP, we repeated this experiment in Nod2-deficient BMDCs. The ability of MDP to incease Mtb Hsp70-induced cytokine production was abolished in Nod2-deficient BMDCs (Figure 1D-F). These findings suggest that MDP enhance the Mtb Hsp70-induced cytokine production in BMDCs via Nod2 dependent pathway.

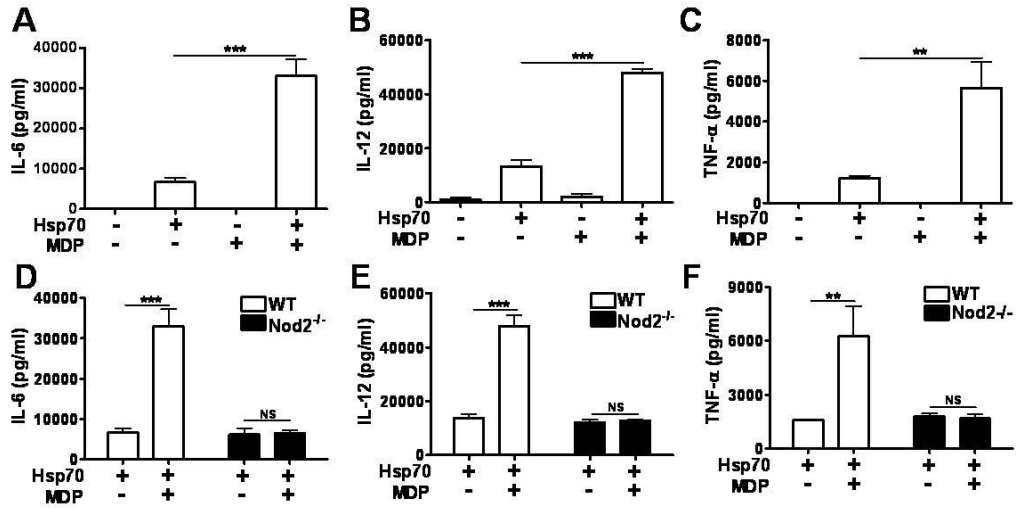

Figure 1 Enhancement of Mtb Hsp70-induced cytokine production of BMDCs by MDP via Nod2.

(A-F) BMDCs from WT and Nod2-deficient mice were treated with Mtb Hsp70 (0.05μg/ml), MDP (1 μg/ml) alone or Mtb Hsp70 (0.05μg/ml) with MDP (1 μg/ml) for 12h. The concentration of (A and D) IL-6, (B and E) IL-12p40 and (C and F) TNF-α in supernatant were measured by ELISA. Data are shown as mean ± SD of triplicate samples from one experiment that is representative of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

MDP synergized with Mtb Hsp70 to activation of NF-κB and ERK in BMDCs.

Activation of NF-κB and MAPKs are key molecular factors regulating cytokine and chemokine production in various cell types, including DCs. To define molecular mechanism of synergistic effect of MDP with Mtb Hsp70 on cytokine production of BMDCs, we determined activation of NF-κB and MAPK by MDP with Mtb Hsp70 in BMDCs using Western blot analysis. Both Mtb Hsp70 and MDP alone induced weak phosphorylation of IκB-α by 60 min and 30 min respectively and ERK by 30 min in BMDCs (Figure 2). However, MDP with Mtb Hsp70 induced strong phosphorylation of IκB-α and ERK by 30 min in BMDCs compared with Mtb Hsp70 or MDP alone (Figure 2). None of all treatments induced phosphorylation of JNK and p38. These findings suggest that synergistic effect of MDP on cytokine production in Mtb Hsp70-stimulated BMDCs is mediated by activation of NF-κB and ERK.

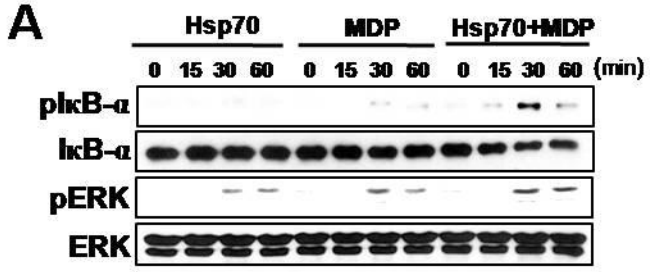

Figure 2 Increasement of Mtb Hsp70-induced activation of NF-κB and ERK of BMDCs by MDP.

BMDCs from WT mice were treated with Mtb Hsp70 (0.05μg/ml), MDP (1 μg/ml) alone or Mtb Hsp70 (0.05μg/ml) with MDP (1 μg/ml) and cellular proteins were extracted at the indicated time points. Degradation of IκB-α and the phosphorylation of IκB-α and ERK were examined by Western blotting. Primary antibody against total ERK was used to verify equal loading. The results are from one representative experiment of two independent experiments.

MDP synergized with Mtb Hsp70 to impair endocytic ability of BMDCs.

Because DC maturation is associated with marked down-regulation of endocytic capacity (Sallusto et al., 1995), we determined the effect of MDP treatment with Mtb Hsp70 on endocytotic activity of BMDCs. After 12 h stimulation byh Mtb Hsp70, MDP alone or MDP with Mtb Hsp70, BMDCs were incubated with FITC conjugated dextran for 45 min and dextran uptake ability were measured by flow cytometry. The ratio of FITC-positive cells of Mtb Hsp70 and MDP-treated BMDCs was 32.04% and 29.94% respectively, whereas intact BMDCs showed 39.50 % of FITC-positive cells (Fig. 3A-C). However, 23.94 % of BMDCs treated by MDP with Mtb Hsp70 was FITC-positive cells (Fig. 3D). These results suggest that MDP synergize with Mtb Hsp70 to mature BMDCs.

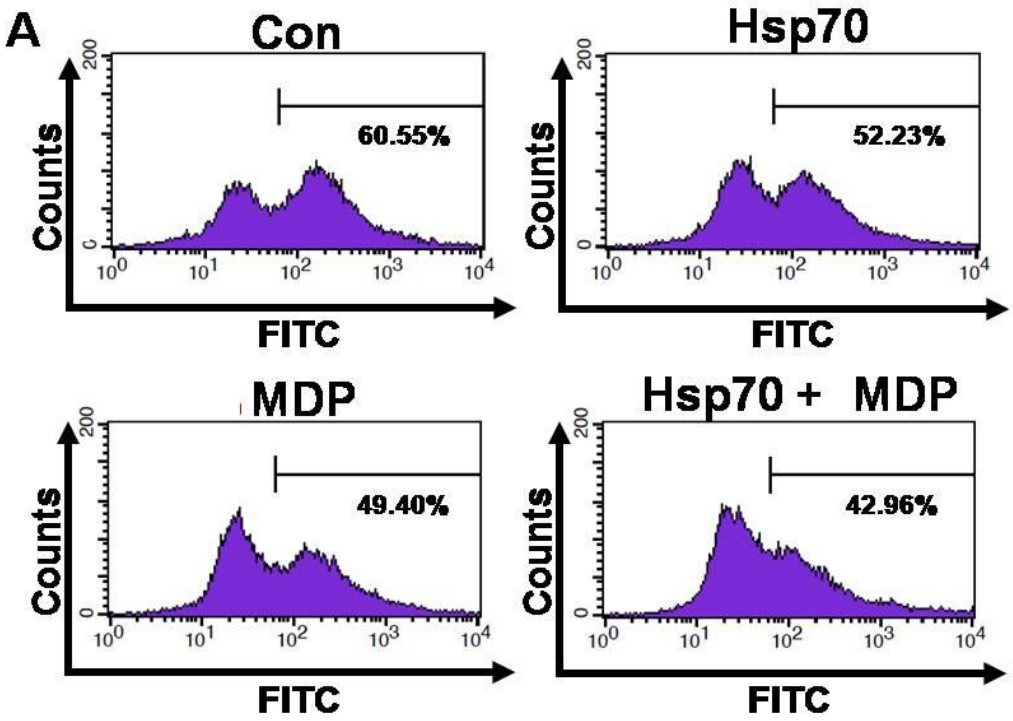

Figure 3 Effect of MDP on reduction of endocytic ability of BMDCs by Mtb Hsp70.

BMDCs from WT mice were treated with Mtb Hsp70 (0.05μg/ml), MDP (1 μg/ml) alone or Mtb Hsp70 (0.05μg/ml) with MDP (1 μg/ml) for 24h and endocytic ability of BMDCs were determined by evaluating level of dextran-FITC uptake using flow cytometry analysis. The results are from one representative experiment of two independent experiments.

MDP synergized with Mtb Hsp70 for BMDCs to generate IFN-γ producing $CD4^+$ T cells.

As a special antigen presenting cell, activated DCs can differentiate naïve T cells into effector T cells. I evaluated the effect of MDP on ability of Mtb Hsp70-stimulated BMDCs to differentiate $CD4^+$ T cells into IFN-γ producing $CD4^+$ T cells. Mtb Hsp70 stimulation of OVA-pulsed BMDCs activated naïve T cell to produce IFN-γ, whereas unstimulation or MDP stimulation showed basal level IFN-γ production (Figure 4). However, MDP stimulation of BMDCs with Mtb Hsp70 increased production of IFN-γ in $CD4^+$ T cells. These findings suggest that MDP synergize with Mtb Hsp70 for DCs to differentiate T cell into IFN-γ-producing $CD4^+$ T cells.

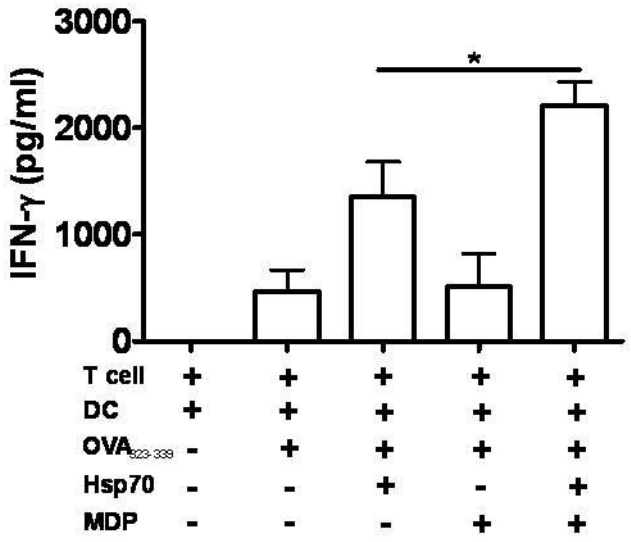

Figure 4 Effect of MDP on ability of Mtb Hsp70-treated BMDCs to generate generate IFN-γ producing $CD4^+$ T cells.

BMDCs from WT mice were treated with OVA peptide ($OVA_{323-339}$) in the absence or presence of Mtb Hsp70 (0.05 μg/ml), MDP (1 μg/ml) alone or Mtb Hsp70 (0.05 μg/ml) with MDP (1 μg/ml) for 1 day. Then, $CD4^+$ T cells from OT-II mice were co-cultured with these BMDCs for 5 days. The concentration of IFN-γ in culture supernatant was measured by ELISA. Data are shown as mean ± SD of triplicate samples from one experiment that is representative of two independent experiments (*$p < 0.05$).

5.5. Conclusion

Bacterial cell wall components including peptidoglycan and LPS (Petrovsky and Aguilar, 2004) and bacterial toxins of *Vibrio cholerae* (Cholera toxin) and *Escherichia coli* (Heat-labile enterotoxin) have been known to enhance immune response against co-administrated antigens and considered as useful adjuvant for vaccination (Elson, 1992; McGhee et al., 1992; Walker, 1994). Recently, many studies evaluated synergistic effect of these immune modulating molecules to improve adjuvant efficacy (McCluskie et al., 2013; Rivera et al., 2003). Although low concentration of MDP alone showed little or no change in production of IL-8, co-treatment of MDP with other bacterial immune stimulating components synergistically increased the production of IL-8 in THP-1 cells (Uehara et al., 2005). In line with these studies, we showed enhanced production of proinflammatory cytokine and chemokines and activation of NF-κB and ERK in BMDCs stimulated by MDP with Mtb Hsp70 compared to those with Mtb Hsp70.

Although we did not define precise mechanism of synergistic effects of MDP on immune activation in Mtb Hsp70-stimulated DCs, the impairment of synergistic effects of MDP in Nod2-deficient DCs suggested that Nod2 signaling has an important role on these effects. Previous studies showed that Nod2

signaling enhanced TLR response in immune cells and intestinal epithelial cells (Hiemstra et al., 2012; Selvanantham et al., 2013) and Mtb Hsp70-induced immune activation in macrophages and DCs has been known to be mediated by TLR2 or TLR4, although still controversial (Asea et al., 2002; Bulut et al., 2005). Taken together, synergistic effect of MDP in Mtb Hsp70-stimulated DCs can be explained by Nod2-mediated enhancement of TLR responses. In contrast, Watanabe et al. (Watanabe et al., 2004; Watanabe et al., 2005) reported that Nod2 signaling is negative regulator of TLR signaling in macrophages and Th1 response. In addition to synergistic effects of MDP on activation and maturation of Mtb Hsp70-stimulated DCs, MDP treatment enhanced ability of Mtb Hsp70-situmulated DCs to differentiate naïve T cells into IFN-γ producing $CD4^+$ T cells *in vitro*. However, treatment of neither Mtb Hsp70 nor Mtb Hsp70 with MDP differentiated T cell into IL-4 producing Th2 cells or IL-17 producing Th17 cells (Data not shown). In accordance with our results, Mtb Hsp70 has been suggested as Th1-polarizing adjuvant, because of its ability to induce IL-12 production in human dendritic cells (Wang et al., 2002).

In this study, we showed the synergistic effects of MDP with Mtb Hsp70 on dendritic cell activation. The use of MDP with Mtb Hsp70 to induce immune activation may provide an effective strategy for vaccination to treat cancer and

protect against pathogens. Further study will be necessary to define how MDP-induced Nod2 signaling enhances Mtb Hsp70-induced subsequent signaling through TLR2 or TLR4.

General Conclusion

Pattern-recognition receptors initiate immune response to bacteria or virus and are classified as a member of innate immune system. Recently, there have been many reports about the effects of activation of these receptors on allergic asthma. It is necessary to study the role of PRRs signaling in allergic asthma.

TRIF and RIP2 are adaptor molecules mediating the TLRs and Nod signaling respectively. In this study, the role of TRIF and RIP2 in development of allergic airway inflammation was determined using animal model in chapter I and II. Although there have been many evidence about the possibility that TRIF and RIP2 have an effect on allergic asthma, deficienty of TRIF or RIP2 dose not caused the difference in severity of allergic airway inflammation.

The effect of *Mycobacterium tuberculosis* derived heat shock protein 70 (Mtb Hsp70) was evaulated using allergic airway inflammation mice model. In addition, the role of TLR2, TLR4, TRIF and MyD88 in effect of Mtb Hsp70 was studied using bone marrow-derived dendritic cells.

In chapter III, administration of Mtb Hsp70 attenuated the allergic airway inflammation in mice model. Experiment using TLR2/4 double-deficient mice revealed that TLR2 and 4 signaling are associated to effects of Mtb Hsp70 on development of allergic airway inflammation.

The role of TLR2 and TLR4 in immune activation by Mtb Hsp70 is not determined precisely. So, I used the dendritic cells to study the molecular mechanism related to the effect of Mtb Hsp70 in chapter IV. Mtb Hsp70 induced the produce the cytokine, express the surface molecules, activation of transciption factors and ability to generate IFN-γ producing T cells. These effects of Mtb Hsp70 depend on the TLR2, TLR4, TRIF and MyD88 of dendritic cells. And MDP enhanced the activation of dendiritc cells by Mtb Hsp70.

In conclusion, it was observed that TRIF and RIP2 did not have critical role in development of allergic airway inflammation in mice model. And Mtb Hsp70 attenuated the development of allergic airway inflammation and these effects are mediated by TLR2 and TLR4 signaling. Study using dendritic cells showed that TLR2, TLR4, TRIF and MyD88 signaling pathway are related to the effect of Mtb Hsp70 and MDP increased the effects of Mtb Hsp70. The result of this study can help to understand the role of PRRs on allergic asthma and suggest that Mtb Hsp70 can be the candidate for the allergic asthma treatment.

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국문초록

알러지성 기도 염증 발생에서 TRIF과 RIP2의 역할 및

결핵균 유래 열충격 단백질70의 억제효과

서울대학교 대학원

수의학과

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알러지성 천식은 기도 폐색을 통한 호흡 곤란을 주요 증상으로 하는 호흡기계의 만성 염증성 질환으로 전세계적으로 3억명 이상의 환자가 있는 것으로 알려져 있다. 천식에 대해서는 그동안 대증적인 치료 방법이 사용되어 왔으나 약의 부작용 및 재발 등을 비롯하여 근원적인 치료가 어려운 한계로 인해 많은 사회적인 비용을 발생시켜 왔다. 이에 알러지성 천식에 대한 기전 연구를 통해 그 원인을

치료하고자 하는 노력이 진행되어왔으며 알러젠에 대한 Th2 type의 면역 반응이 관련되어 천식의 반응에 관련되어 있다는 것이 알려지게 되었다. 이러한 알러지 반응에서의 Th2 면역반응을 억제하여 알러지성 질병을 개선하고자 하는 노력이 지속되어 왔다.

패턴 인식 수용체는 미생물 유래의 물질을 인식하여 면역 반응을 유발하는 선천적인 면역 시스템이며 이들 패턴인식수용체가 알러지성 천식 발생의 관련성이 있다는 것이 알려졌다. 패턴 인식 수용체를 자극함으로써 효과적으로 알러지성 천식을 치료하고자 하는 연구가 있어왔다.

이에 본 연구에서는 패턴인식 수용체의 신호기전이 알러지성 천식에 미치는 영향을 연구하기 위하여 알러지성 천식의 주요한 증상 중 하나이며 천식 연구에 많이 활용되고 있는 알러지성 기도 염증 마우스 모델을 이용하였다. 또한 TLR의 리간드 이며 면역활성 효과를 가진 결핵균 유래 열충격 단백질 70 이 알러지성 기도 염증의 발생에 미치는 동물 모델을 통해 확인하고 결핵균 유래 열충격 단백질 70의 면역 활성 효과에 대한 톨 유사 수용체의 신호기전의 역할을 연구하기 위하여 마우스 골수 유래의 수지상 세포를 이용하였다.

첫번째 장에서는 톨 유사 수용체중 TLR3와 TLR4의 신호 기전에 관련된 TRIF 단백질 알러지성 기도 염증의 발생에 미치는

영향을 연구하였다. TRIF이 결여된 마우스에서 OVA를 알러젠으로 이용하여 알러지성 기도 염증 마우스에서 유도하고 기도내 염증반응 및 Th2 사이토카인의 생성과 알러젠 특이적인 항체 생성을 정상 마우스와 비교하였다. 그결과 TRIF 단백질 결여 마우스에서 발생한 알러지성 기도 염증 및 Th2 면역 반응 관련 지표들은 정상 마우스와 큰 차이를 보이지 않았다.

두번째 장에서는 패턴 인식 수용체인 Nod1과 Nod2의 신호전달에 관여하고 있는 RIP2가 알러지성 기도 염증에 미치는 영향을 평가하였다. RIP2 가 결여된 마우스에서 OVA를 알러젠으로 이용하여 알러지성 기도 염증 마우스에서 유도하고 관련 지표를 알러지성 기도 염증이 유발된 정상 마우스의. 그것과 비교한 유의적인 차이를 확인할 수 없었다.

세번째 장에서는 톨 유사 수용체의 리간드로서 면역 활성 효과를 가진 것으로 알려진 결핵균 유래의 열충격단백질 70를 제작하여 집먼지 진드기와 OVA를 알러젠으로 활용한 알러지성 기도 염증 모델에 투여하고 그 영향을 평가하였다. 그 결과 결핵균 유래 열충격단백질 70를 알러젠에 감작되는 시기에 함께 투여할 경우 알러지성 기도 염증 발생과 관련된 지표들이 감소하는 것을 확인하였다. 기존에 TLR2와 TLR4가 열충격단백질 70에 의한

면역활성효과에 관여한다는 것이 알려져 있어 TLR2와 TLR4가 모두 결여된 마우스에서 알러지성 기도 염증을 유발시키고 위와 동일하게 알러젠 감작시기에 열충격단백질을 주입하였을 때는 알러지성 기도 염증 발생 완화 효과를 확인할 수 없었다.

네번째 장에서는 알러지성 기도 염증의 완화 효과가 확인된 결핵균유래 열충격단백질 70의 면역 활성 기전에 관련된 패턴인식수용체의 신호기전 연구를 실시하였다. 결핵균 유래 열충격단백질 70는 TLR2와 TLR4를 모두 이용하여 수지상세포를 활성화하고 톨 유사 수용체 신호기전에 중요한 단백질인 TRIF과 MyD88 모두 결핵균 유래 열충격 단백질 70의 수지상 세포 활성화 효과에 필수적인 요소임을 확인하였다.

다섯번째 장에서는 기존에 알려진 MDP에 의한 TLR2와 TLR4 리간드의 면역 활성 능력 증가 효과가 결핵균 유래의 열충격 단백질 70에서도 확인하였다. 이에 결핵균 유래의 열충격 단백질에 의한 수지상 세포의 활성화가 MDP를 함께 처리하였을때 더욱 증가된다는 것을 확인하였고 이는 MDP와 결핵균 유래의 열충격 단백질을 함께 사용하는 것이 효과를 더욱 증강 시켜줄 수 있는 방법으로 활용될 수 있다는 가능성을 제시해 주었다.

본 연구에서는 패턴인식수용체 신호기전에 관련된 TRIF과 RIP2가

알러지성 기도 염증의 발생에는 큰 영향을 미치지 못한다는 것을 동물 모델을 통해 확인하였으며 TLR2와 TLR4의 활성화 효과를 가진 결핵균 유래의 열충격 단백질 70이 알러지성 기도 염증 발생을 감소시키는 효과를 가지고 있다는 것을 확인할 수 있었다. 이 후 TLR2와 TLR4과 신호 기전이 결핵균 유래의 열충격단백질 70의 효과의 관련성에 대한 기전 연구를 위해 수지상 세포를 이용였고 TLR2와 TLR4 신호기전 모두 결핵균 유래의 열충격 단백질 70에 의한 수지상 세포 활성화에 관련이 있으며 이러한 결핵균 유래 열충격 단백질 70의 수지상 세포 활성화 효과는 MDP에 의해 증가된다는 것을 발견하였다. 본 연구를 통해 알러지성 천식 발생에 미치는 패턴인식수용체의 영향에 대한 이해를 높이는데 활용되고 이는 새로운 천식 치료제 개발에 활용될 것 수 있을 것으로 기대한다.

주요어 : 패턴인식수용체, 톨 유사 수용체, 노드 유사 수용체, 알러지성 기도 염증, 수지상세포, 결핵균 유래 열충격단백질

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