TOLL-LIKE RECEPTORS ARE REGULATED BY AN AUTOPHAGY PROTEIN MAP1S & TLR-INDUCED INNATE RESISTANCE AGAINST FLU

A Dissertation

by

YIFAN ZHANG

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Chair of Committee,	Dekai Zhang
Committee Members,	Leyuan Liu
	Jianhua Yang
	Yi Xu
	Yubin Zhou
Head of Program,	Warren Zimmer

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ABSTRACT

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). The recognition leads to the activation of TLR signaling pathway, which initiates innate immune responses and facilitates the activation of adaptive immune responses. To control the immune response, the regulation of TLR activation is critical but is not well understood. Recent evidence has suggested that autophagy could regulate TLR signaling, but the underlying mechanism is not clear. Autophagy is a process of degrading intracellular components through lysosomal machinery to maintain homeostasis under stress conditions, and MAP1S is a recently characterized autophagy-related protein. We found that MAP1S deficiency in macrophages impaired cell membrane TLR activation and TLR mediated phagocytosis of bacteria. MAP1S interacts directly with MyD88 to regulate TLR signaling pathway. Furthermore, we demonstrate that, upon TLR activation, MyD88 is recruited to autophagy processing by co-localizing with LC3. Thus we reveal that an autophagy-related molecule MAP1S regulates TLR signaling.

To take advantage of TLR-mediated innate immune responses to prevent infectious diseases, we set out to boost innate immunity against influenza. TLR9 agonist CpG-ODN in combination with TLR2 agonist Pam2CSK4 can induce innate resistance against lethal influenza A virus infection; however, the mechanisms are unclear. We found that in lung epithelial cell lines, pretreatment with CpG-ODN but not Pam2CSK4, inhibited influenza A virus infection. Surprisingly, CpG-ODN induced antiviral effect is

TLR9-MyD88 independent. Also, other intracellular DNA sensors including STING, DDX58, and DNA-PKcs are not required for CpG-ODN induced antiviral effect. Intriguingly, we found that CpG-ODN induced antiviral activity occurred at the early stage of influenza A virus life cycle. Together, our findings demonstrate that CpG-ODN inhibit influenza A viral infection in lung epithelial cells in a TLR9-MyD88 independent manner.

Thus, we identified an autophagy protein MAP1S to regulate TLR signaling pathway. We also found that boosting innate immunity with TLR agonists can prevent infectious diseases, and TLR9 ligand CpG-ODN can induce a robust antiviral activity in lung epithelial cells. Therefore, this strategy may eventually help develop a novel therapeutic agent against influenza.

DEDICATION

I dedicate this thesis to my parents. You are the reason of what I become today. Thanks for your support and eternal love. In hopes, I can give back to you and be the perfect son.

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Finally, thanks to my mother and father for their support and to my wife for her love.

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The work in Chapter II was conducted together with a former lab member Ming Shi and was published in 2014. Part of work in Chapter III was performed under the guidance of Dr. Magnus Höök. All other experiments included in this thesis were performed by the student-Yifan Zhang.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Immunity and Toll-like Receptors

Overview of immune system

The immune system is a complex interactive network of organs, tissues, cells and chemicals which has evolved to protect the host from infections. Functionally, the immune system can recognize and eliminate a limitless variety of foreign invaders, trigger a series of immune responses to battle infection and maintain homeostasis (Medina, 2016; Parkin and Cohen, 2001). The immune system also mobilizes immune responses to toxins and allergens. The fundamental principle of the immune system is to discriminate between self (host's cells and proteins) and non-self (foreign molecules). A healthy state of the host requires a tight control and regulation of the immune system. Failure to tolerate self or nonresponse to non-self will cause immune disorders such as autoimmune diseases and immunodeficient diseases.

A pathogenic microorganism must circumvent a series of coordinated immune events to establish an infection and cause diseases. These immune responses involve the participation of various structural, cellular and molecular components that can be divided into two categories: innate immunity and adaptive immunity (Figure 1). Innate immunity (also called "non-specific immunity") provides the first line of defensive mechanisms. It

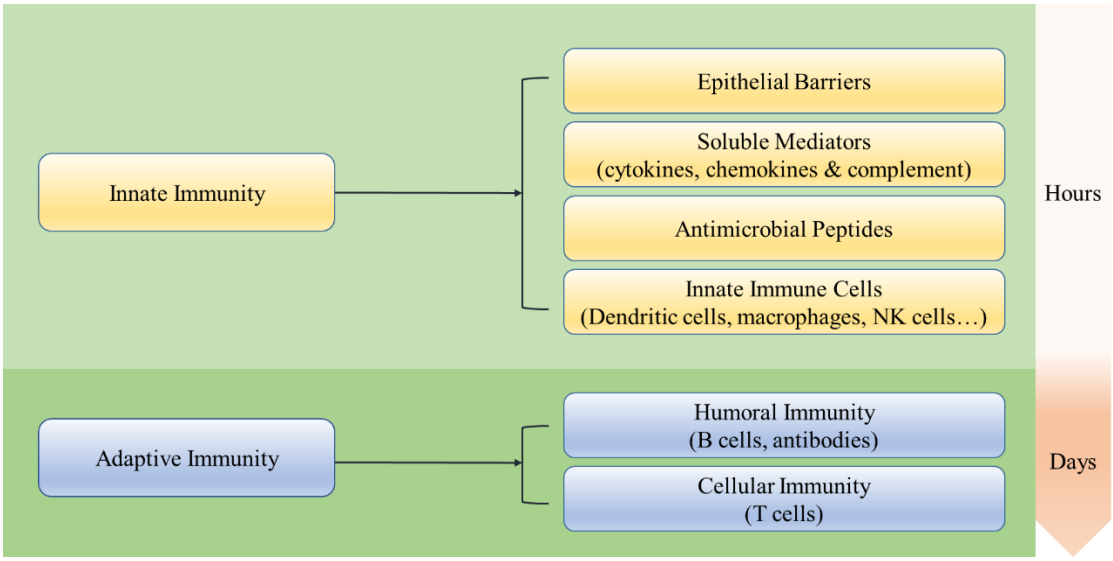

Figure 1. Immunity overview

Immunity consists of innate immunity and adaptive immunity. Innate immunity provides an immediate but nonspecific response to invading pathogens. The major components of innate immunity are epithelial barriers, cytokines, chemokines, antimicrobial peptides and innate immune cells. The adaptive immune response is slower but antigen-specific. Adaptive immunity includes cellular and humoral immunity.

mounts rapid but non-specific immune responses against pathogens. Moreover, the innate immune system plays a crucial role in the initiation and subsequent activation of highly specific but relatively slow adaptive immunity(Janeway CA Jr, 2001). Adaptive immunity (also called “acquired immunity”) includes humoral immunity (B cells and antibodies) and cellular immunity (T cells). B cells (B lymphocytes) mature in the bone marrow and are activated in secondary lymphoid organs such as spleen and lymph nodes. When B cells are activated, they secrete antibodies specific to the antigens. T cells have various types and play a central role in cell-mediated immune responses. The functions of T cells include: direct elimination of infected cells, attack on foreign invaders, augment of B cells, and production of cytokines. Another characteristic of adaptive immunity is the immunological memory which elicits a rapid and robust response on the second infection of a pathogen and provides the host a long lasting protection.

Since the initiation of adaptive immunity takes several days, the innate immune responses are vital in the first days immediately following exposure to pathogens. Moreover, in order to activate adaptive immunity, activation of innate immunity appears to be essential (Paul, 2011).

Innate immune system

The innate immune system is comprised of diverse defensive mechanisms against infection in vertebrates. The skin and the mucosal surfaces provide mechanical barriers to prevent the entry of pathogens. Skin is capable to prevent the penetration of most microorganisms although some pathogens can cause skin infection. Epithelial cells of

mucous membrane on alimentary and respiratory tract secrete fluids called mucus to trap pathogens. The cilia of the respiratory tract help to remove those trapped pathogens out of host body. Nonpathogenic commensal bacteria (known as normal flora) help maintain host homeostasis and prevent pathogens to reduce the probability of infection. In addition, antimicrobial peptides which are secreted by those surface barriers, have broad spectrum antimicrobial activity against pathogens such as bacteria, viruses and fungi. However, if the surface barriers are damaged such as a wound or some organisms have evolved escaping mechanisms, the pathogens are able to invade the host body. A variety of innate immune components including innate immune cells and their secreted soluble factors contribute to innate immune clearance of the pathogens that have entered the host.

Lysozyme is one of the soluble factors found in tears, saliva mucus as well as in the cytoplasmic granules of macrophages and polymorphonuclear neutrophils. It acts as glycoside hydrolases and attacks bacteria by cleaving the peptidoglycan layer of the bacterial cell wall (McKenzie and White, 1991). Deficiency in lysozyme in airway secretion may cause bronchopulmonary dysplasia (Revenis and Kaliner, 1992). On the other hand, excessive production of lysozyme is toxic to blood and kidney.

The complement system is comprised of a group of serum proteins that circulate in the blood. When stimulated, the complement proteins are converted from an inactive state to an active state by a series of proteolytic cleavage. Activation of complement system triggered by the classical, lectin or alternative pathways results in the following immune functions: opsonization and facilitating phagocytosis of pathogens by phagocytes; production of anaphylatoxins that attracts innate immune cells to the infection

site; damaging the membranes of pathogenic organisms for killing (Sarma and Ward, 2011).

Central players in the innate immunity are the innate immune cells which include macrophages, neutrophils, dendritic cells, Natural killer cells (NK cells), mast cells, eosinophils and basophils (Figure 2). Microorganisms that penetrate the surface barriers are targeted by innate immune cells to active an innate immune response. The major functions of the innate immune cells are recognizing pathogens to secret biologically active molecules such as cytokines to affect the behavior of other cells and chemokines to attract other immune cells, ingesting and destroying microbes directly, and facilitating the activation of adaptive immune cells by presenting antigens (Akira, 2009; Guermonprez et al., 2002; Janeway and Medzhitov, 2002; Lacy, 2015).

Macrophages, a type of phagocytes, are differentiated from monocytes. Macrophages are distributed in almost all tissues. Some macrophages that reside in specific tissues are given various names such as alveolar macrophages in the lung, Kupffer cells in the liver, histiocytes in the connective tissues, osteoclasts in the bone tissues, and mesangial cells in the kidney (Davies et al., 2013; Haldar and Murphy, 2014; Perdiguero et al., 2015). Other macrophages are circulating within tissues or patrolling by amoeboid movement such as peritoneal macrophages. Two types of macrophages (M1 and M2) have been defined with distinct roles in inflammatory responses (Italiani and Boraschi, 2014; Martinez and Gordon, 2014; Mills, 2012). Macrophages engulf and digest invading pathogens through a process called phagocytosis for direct clearance.

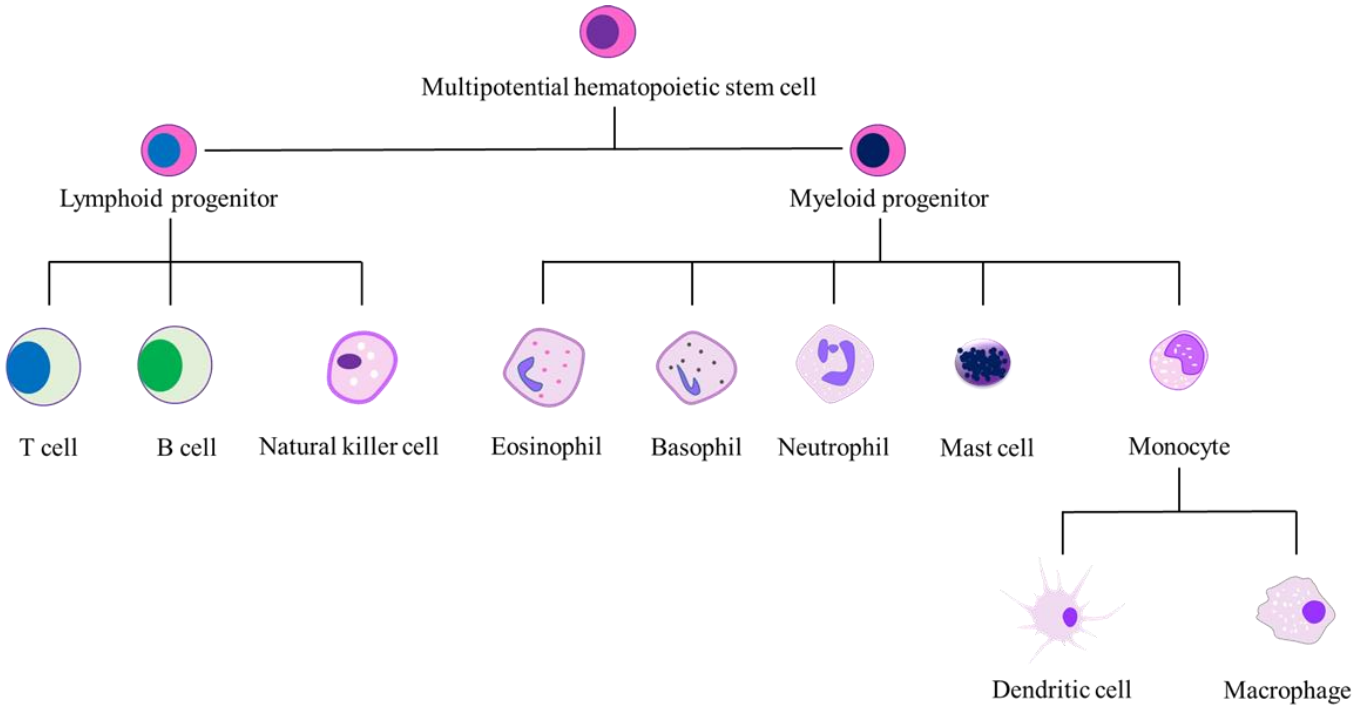

Figure 2. Cells of immune system

Multipotential hematopoietic stem cells give rise to myeloid and lymphoid progenitor cells. Lymphoid progenitor cells differentiate into T cells, B cells and NK cells. Myeloid progenitor cells differentiate into eosinophils, basophils, neutrophils, mast cells and monocytes. Monocytes are able to further differentiate into DCs and macrophages.

After digesting the pathogens, macrophages will present antigens from the pathogens to T cells through MHC I/II molecules. In addition, the interaction between costimulatory molecules on macrophages (B7-1/2) and T cells (CD28) help to facilitate the activation of adaptive immunity (Martinez-Pomares and Gordon, 2007; Orlikowsky et al., 2003; Unanue, 1984). At the site of infection, macrophages are activated to produce a variety of inflammatory cytokines such as IL-1β, IL-6 and TNFα, and chemokines such as MCP-1 and IL-8 which will recruit other immune cells (Stow et al., 2009). The role of macrophages in the regulation of immune responses is complex. Pro-inflammatory cytokines secreted by macrophages such as IL-6, IL-18 and TNFα could promote the activation of T cells, B cells and NK cells, while anti-inflammatory cytokines secreted by macrophages such as IL-10 could inhibit the activation of immune cells (Arango Duque and Descoteaux, 2014; Ding et al., 1993; Fiorentino et al., 1991; Shi and Pamer, 2011). Macrophages have also been shown to manipulate cancer cells (Pollard, 2004; Richards et al., 2013), and play a role in wound healing and tissue regeneration (Godwin et al., 2013; He and Marneros, 2013; Koh and DiPietro, 2011; Leibovich and Ross, 1975).

Neutrophils are another type of phagocytes derived from myeloid precursors in the bone marrow. They are the most abundant type of circulating leukocytes in humans (50% to 70%). Neutrophils are short-lived cells comparing with other immune cells such as macrophage and only can live 1 to 3 days, but their daily production can reach up to 2.5 x 10^{11} cells (Cartwright et al., 1964; Moore et al., 1979). Neutrophils are a type of granulocytes which form distinct subsets of granules within the cytoplasm that contains pro-inflammatory proteins and enzymes to kill microbes and to digest tissues (Borregaard,

2010). Their nucleus is segmented and multi-lobulated. Together with eosinophil and basophil, they are called polymorphonuclear cells (PMNs). Neutrophils are generally among the first leukocytes to be recruited to the infection site. To reach the inflammatory site, the neutrophils need to cross the vascular wall and extravasate to the tissues. This process requires several steps. Neutrophils are loosely attached to the surface of endothelium and activated by various chemoattractants such as chemokines (e.g. IL-8 and MIP-1β) which trigger signals inducing conformational changes in the integrin molecules on the neutrophils membrane. This process increases the neutrophils' affinity with endothelium and the interactions between integrins and cell adhesion molecules (CAMs) enables the cell attached firmly. Subsequently, the neutrophils migrate transendothelially through the vessel wall into the inflammatory tissues. Once neutrophils reach the infection site, they eliminate the microbes by multiple mechanisms. After phagocytosis of microorganisms, neutrophils undergo a process called respiratory burst (or oxidative burst) rapidly releasing reactive oxygen species (ROS) by activating NADH and NADPH oxidase (Clark, 1999; Dahlgren and Karlsson, 1999). NADPH oxidase can reduce oxygen to generate superoxide anion, hydrogen peroxide and other toxic species, which have strong antimicrobial activity. Neutrophils also utilize myeloperoxidase (MPO) in the granules to produce hypochlorous acid from hydrogen peroxide and chloride anion which has a direct antimicrobial effect. Neutrophils also have a diverse group of antibacterial peptides such as defensins, lactoferrin, cathelicidin, and lysozyme (Falloon and Gallin, 1986; Hager et al., 2010; Miles et al., 2009). Besides, neutrophils can release a combination of processed histones, proteins and enzymes (neutrophil elastase and

antimicrobial peptides) to form a structure called neutrophil extracellular traps (NETs). The process of forming NETs is called NETosis. NETs have been shown to immobilize bacteria and fungi and directly kill those microorganisms (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012).

Dendritic cells (DCs), another member of phagocytes, are present in major organs such as skin, lung, heart and spleen. Dendritic cells are a type of professional antigen presenting cells (APCs). They are essential in the recognition of a universe of antigens and control of innate and adaptive responses. Dendritic cells capture and process antigens to be recognized by T cells through MHC molecules (Figure 3). Two major subsets of dendritic cells have been identified: conventional DC (cDC) and plasmacytoid DC (pDC). They differ in their phenotype and functions (Liu et al., 2009). pDCs are a small subset of DCs that are fully developed in the bone marrow and they circulate mainly in the blood and lymphoid tissues (Shortman and Naik, 2007). pDCs express pattern recognition receptors such as TLR7 and TLR9 and produce large amounts of type I IFN upon virus infection or nucleic acids stimulation (Gilliet et al., 2008; Ito et al., 2005; Perussia et al., 1985). pDCs express low levels of MHC II molecules and costimulatory molecules. They are not sufficient to stimulate naïve T cells unless activated as cDC-like cells (Liu, 2005). Precursors of cDCs are called pre-cDCs which derive from bone marrow and migrate to peripheral lymphoid organs where they differentiate into cDCs. cDCs are known to initiate and orchestrate adaptive immune responses by activating primary T cells (Steinman and Witmer, 1978). cDCs are found in both lymphoid and peripheral tissues and they have a high MHC class II expression (LeibundGut-Landmann et al., 2004; Young et al., 2008).

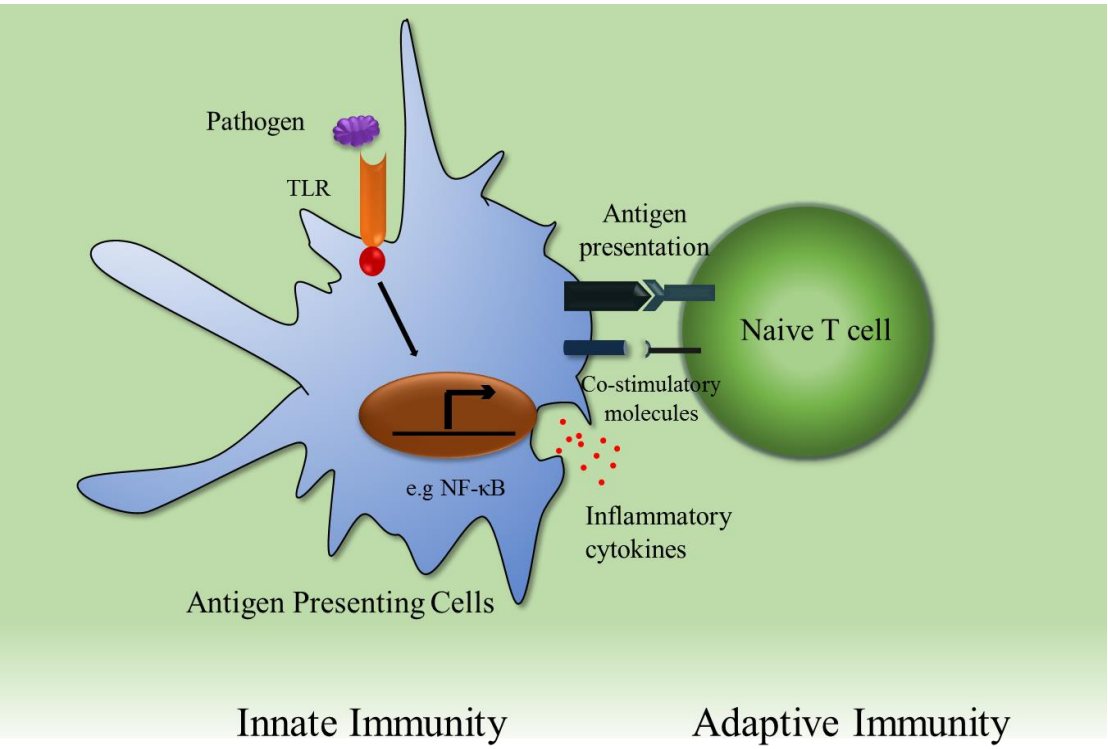

Figure 3. Innate immunity facilitates activation of adaptive immunity

Recognition of pathogens by PRRs such as TLRs on antigen presenting cells triggers downstream signaling pathways leading to NF-κB activation, resulting in the production of inflammatory cytokines and chemokines. Antigens are processed and loaded onto MHC class I/II molecules, which activate adaptive immune cells. APCs also express co-stimulatory molecules to facilitate activation of adaptive immune cells.

cDCs are found immature with accumulated MHC class II in endosomes and lysosomes. When immature cDCs take up pathogens, they become mature, with dramatically increased expression of cell membrane MHC class II and co-stimulatory molecules and decreased endocytic activity. Peptides derived from antigens process by cDCs are loaded on MHC class II and presented to CD4+ T cells.

Toll-like receptors

Immunologists used to believe that the adaptive immune system (with T cells and B cells) alone was able to recognize stimuli and mount immune responses. However, an unneglectable fact is that a foreign antigen alone is not sufficient to induce the adaptive immune responses without the addition of "nasty" adjuvants such as mineral oil, alum and killed mycobacteria. It raised the question why the lymphocytes failed to recognize "non-self" antigens alone. This phenomenon that immunologists did not intend to admit was termed as "immunologist's dirty little secret." Back to 1980s, Charles A. Janeway, Jr. (1943-2003), a professor of immunology at Yale and Howard Hughes Medical Institute, proposed that activation of the adaptive immunity would require an additional signal from the "adjuvants." He suggested that the additional signal was from non-lymphocyte cells, prior to the adaptive immunity. The "adjuvants" or other substances such as lipopolysaccharide could trigger the additional signal rapidly, and this process was not involved with receptor rearranging. He believed that on the innate arm, there must be a system recognizing certain characteristics or patterns rather than specific antigen determinants, and such receptors should be distributed non-clonally on antigen presenting

cells (Janeway, 1989). He proposed the theory of pattern recognition receptors (PRRs) on APCs could discriminate self from infectious non-self.

Charles' hypothesis shed light on the studies of innate immune recognition. Within less than two decades, multiple pattern recognition receptor families had been identified (Table 1). They were found to recognize pathogens associated molecular patterns (PAMPs) which are conserved microbial patterns derived from bacteria, viruses, fungi and parasites, in exactly the same way as Charles proposed. Five families of PRRs have been classified so far including Toll-like receptors (TLRs), nucleotide-binding domain, leucine-rich repeat (LRR) – containing (NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and the AIM2-like receptors (ALRs) (Brubaker et al., 2015). These PRRs are located either on the cell surface or intracellularly detecting conserved microbial patterns such as lipoproteins, LPS, flagellin and nucleic acids. Upon recognition of PAMPs, PRRs initiate downstream signaling pathways, which lead to the production of pro-inflammatory cytokines, chemokines, and interferons that are critical for innate and adaptive immune responses (Lander et al., 2001).

Toll-like receptors are the first characterized member of PRRs family. TLRs owe their names to a fruit fly *Drosophila melanogaster* gene "Toll", which was initially identified to be only involved in the differentiation of dorsoventral axis of the embryo (Hashimoto et al., 1988). Several years later after Charles' hypothesis, the antifungal peptide Drosomycin in fruit fly appeared to be regulated by Toll. Jules A. Hoffman and colleagues found that Toll mutants were highly susceptible to fungi infection due to a lack expression of Drosomycin (Lemaitre et al., 1996). The cytoplasmic domains of Toll were

Family	Members	Location	Shared domains	Ligands	Origin of Ligands	Reference
TLR (1-10)in human (1-9) and (11-13) in mouse	TLR1	Cell membrane	LRR, TIR	Triacyl lipoprotein	Bacteria	(Alexopoulou et al., 2001; Aliprantis et al., 1999; Hayashi et al., 2001; Heil et al., 2004; Hemmi et al., 2000; Mathur et al., 2012; Oldenburg et al., 2012; Poltorak et al., 1998; Song et al., 2015; Yarovinsky et al., 2005)
	TLR2	Cell membrane		Lipoprotein	Bacteria, viruses	
	TLR3	Endosome		dsRNA	Virus	
	TLR4	Cell membrane		LPS	Bacteria	
	TLR5	Cell membrane		Flagellin	Bacteria	
	TLR6	Cell membrane		Diacyl lipoprotein	Bacteria, viruses	
	TLR7	Endosome		ssRNA	Bacteria, viruses	
	TLR8	Endosome		ssRNA	Bacteria, viruses	
	TLR9	Endosome		CpG-DNA	Bacteria, synthetic	
	TLR10	Endosome		Unknown	Unknown	
	TLR11	Cell membrane		Profilin-like proteins; flagellin	Protozoa; bacteria	
	TLR13	Endosome		rRNA, ssRNA	Bacteria, viruses	
CLR	Dectin-1	Cell membrane	C-type lectin	β-Glucan	Fungi	(Goodridge et al., 2009; Ishikawa et al., 2013; Yamasaki et al., 2008)
	Dectin-2	Cell membrane		β-Glucan	Fungi	
	MINCLE	Cell membrane		SAP130	Fungi	
RLR	RIG-I	Cytoplasm	DExD/H helicase	dsRNA	Viruses	(Gitlin et al., 2006; Hornung et al., 2006; Li et al., 2009)
	MDA5	Cytoplasm		dsRNA	Viruses	
	LGP2	Cytoplasm		dsRNA	Viruses	
NLR	NOD1	Cytoplasm	LRR, NACHT	iE-DAP	Bacteria	(Chamaillard et al., 2003; Girardin et al., 2003; Inohara et al., 2003)
	NOD2	Cytoplasm		MDP	Bacteria	
ALR	AIM2	Cytoplasm	PYRIN, HIN200	dsDNA		(Hornung et al., 2009; Unterholzner et al., 2010)
	IFI16	Cytoplasm, nucleus		dsDNA	Viruses	

Table 1. Pattern recognition receptor families

found to be highly conserved with the mammalian IL1 receptor. Toll signaling pathway also shows similarity with NF-κB signaling pathway downstream of the IL-1 receptor (Belvin and Anderson, 1996). These suggest that Toll pathway plays a role in regulating fruit fly immune responses. Shortly after discovering the function of Toll in fruit fly immunity, Charles and Medzhitov identified the human homolog of the drosophila Toll (Medzhitov et al., 1997). Subsequently, more TLRs, as well as their ligands were discovered (Chuang and Ulevitch, 2001; Chuang and Ulevitch, 2000; Du et al., 2000; Rock et al., 1998; Takeuchi et al., 1999).

TLRs are a family of type I transmembrane receptors containing three domains: N-terminal ectodomain, a transmembrane domain and C-terminal cytoplasmic domain (Figure 4) (Bell et al., 2003). The N-terminal ectodomain is either extracellular or in the endosome characterized by a horseshoe-like structure. It has a motif in the inner concave surface known as the leucine-rich repeat (LRR) which is responsible for recognition of pathogens molecules (Jin et al., 2007; Kobe and Kajava, 2001; Liu et al., 2008; Park et al., 2009). The C-terminal cytoplasmic domain is also known as Toll IL-1 receptor (TIR) domain because it shares homology with IL-1 receptor family (O'Neill and Bowie, 2007). After binding with ligands, TLRs dimerize and recruit adaptor proteins to TIR domain to activate downstream signaling pathways.

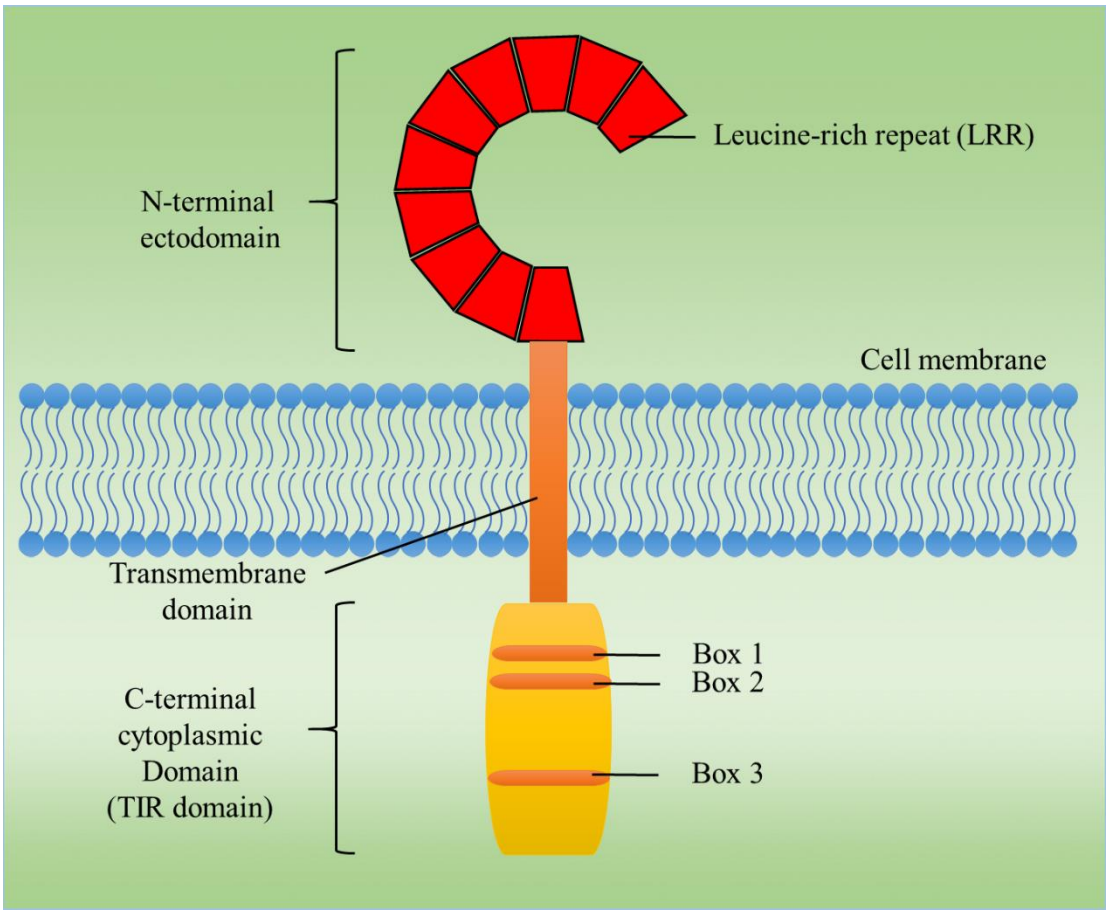

Figure 4. Schematic diagram of TLR structure

TLRs are type I integral membrane glycoproteins. The structure of TLRs consists of three domains: N-terminal ectodomain (ECD), single transmembrane domain and C-terminal cytoplasmic domain (TIR domain). TLR ECD is built from leucine-rich repeats and recognizes PAMPs. TIR domain has 3 conserved boxes and is used for interaction with downstream adaptor proteins.

In human, 10 TLRs have been identified (Figure 5, Table 1). TLR1, TLR2, TLR4 TLR5 and TLR6 are localized on cell membrane, whereas TLR3, TLR7, TLR8 and TLR9 were localized within endosomes. TLR2 forms heterodimer with TLR1 or TLR6 to recognize bacterial lipoproteins, TLR3 recognizes dsRNA and poly (I: C), TLR4 together with co-receptor MD2 recognizes lipopolysaccharides (LPS), TLR5 recognizes flagellin, TLR7 and TLR8 recognizes ssRNA, and TLR9 recognizes unmethylated CpG motifs in microbial DNA. The ligand for TLR10 has not been identified so far, and the role of TLR10 has been suggested to be anti-inflammatory (Jiang et al., 2016; Oosting et al., 2014).

One or two of four TIR domain-containing adaptor proteins (MyD88, TRIF, TIRAP/Mal and TRAM) are recruited to TLRs upon ligands stimulation. Depending on the adaptor protein being recruited, TLR signaling could be divided into MyD88-dependent signaling pathway and TRIF-dependent signaling pathway. All TLRs except TLR3 signal through MyD88 and TLR3 signals through TRIF. TLR4 triggers both MyD88-dependent and TRIF-dependent signaling pathways.

MyD88 (Myeloid differentiation primary response gene 88) is indispensable for the signaling of all TLRs except TLR3. MyD88 is recruited to TIR domain of TLRs. TLR2 and TLR4 require TIRAP/Mal to facilitate recruitment of MyD88 (Fitzgerald et al., 2001). MyD88 interacts with a serine/threonine kinase IL-1R-associated kinase IRAK4 and activates IRAK1 and IRAK2 (Kawagoe et al., 2008). The IRAKs complex then dissociates from MyD88 and interact with an E3 ubiquitin protein ligase TNFR-associated factor 6 (TRAF6). TRAF6 catalyzes itself and activates the complex of TGF-β-activated kinase 1

(TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3. The complex phosphorylates IκB kinase (IKK) - β, MAP kinase kinase 3 (MKK3), MKK4, MKK6 or MKK7. The IKK complex phosphorylates and degrades IκB-α which allows translocation of NF-κB to the nucleus, thus further activate the expression of pro-inflammatory cytokine and chemokine genes such as IL-1, IL-6, IL-8 and TNFα. MKK3 or MKK6 activates p-38/CREB and MKK4 or MKK7 activates JNK/AP-1, respectively.

TLR3 recruits TRIF upon stimulation, while TLR4 requires TRAM to facilitate recruitment of TRIF (Yamamoto et al., 2003b). TRIF interacts with another E3 ubiquitin ligase TRAF3 which activates TANK-binding kinases 1 (TBK 1) and IKK-ε (Hacker et al., 2006; Yamamoto et al., 2003a). TBK1 and IKK-ε further phosphorylate IFR3 and IRF which are responsible for expression of type I interferons expression and interferon-inducible genes.

TLRs and immunity

TLRs are widely expressed in epithelial and innate immune cells. TLRs triggers innate immune responses upon recognition of PAMPs from pathogens or DAMPs (Damage-associated molecular pattern) from the host, and lead to the production of cytokines and chemokines, which recruit immune cells such as macrophages and neutrophils to the inflammation site. During virus infection, activation of endosomal TLRs result in the production of type I interferons and activate IFN-inducible genes. Stimulation of TLRs in phagocytes increased phagocytic activity and intracellular bacterial killing (Doyle et al., 2004; Ribes et al., 2010). Stimulation of epithelial cells TLRs could produce

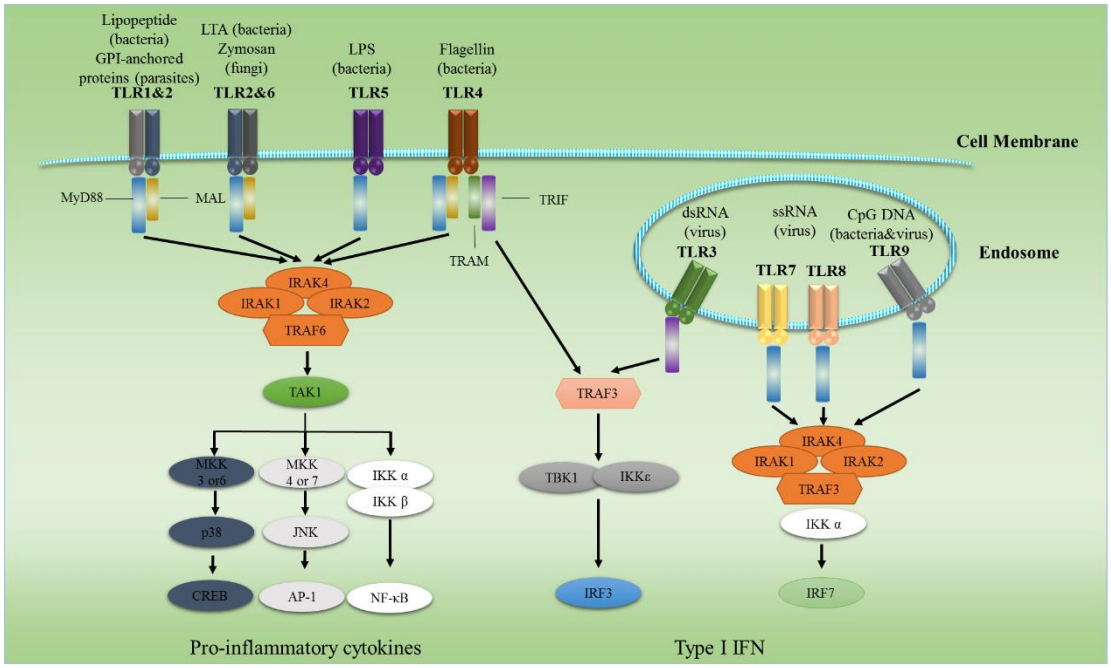

Figure 5. Toll-like receptors and TLRs mediated signaling

TLRs recognize PAMPs derived from pathogens. TLR signaling is triggered by ligand-induced dimerization. All TLRs except TLR3 utilize MyD88 as the adaptor protein, whereas TLR3 and TLR4 utilize TRIF. Engagement of adaptor proteins activates IRAKs and TRAFs, which lead to the activation of MAPKs JNK and p38, NF-κB, or IRFs. TLR signaling results in the induction of pro-inflammatory cytokines or IFNs.

antimicrobial peptides in response to bacteria or LPS challenge (Ayabe et al., 2000; Birchler et al., 2001; Lehrer and Ganz, 2002). Peripheral immature DCs have strong endocytic activity to uptake pathogens. Microbial ligands activate TLRs on DCs to facilitate their maturation (Hertz et al., 2001; Michelsen et al., 2001). Activation of TLRs on DCs also induces expression of co-stimulatory molecules such as B7.1/B7.2 as well as inflammatory cytokines such as IL-12 (Akira et al., 2001). IL-12 could direct T_H cells differentiation into T_H1 effector cells. Mature DCs express high MHC molecules. They migrate to draining lymph nodes where they present the antigens to naïve T cells, thus facilitating the activation of adaptive immunity (Banchereau and Steinman, 1998; Steinman, 2001).

CHAPTER II

CHARACTERIZATION OF AUTOPHAGY PROTEIN MAP1S IN THE REGULATION OF TLR SIGNALING AND PHAGOCYTOSIS*

Introduction

Autophagy

Autophagy (from the Greek words: auto means "self" and phagy means "to eat") is a conserved catabolic process of self-degradation of intracellular component through the lysosome machinery. Under harsh conditions such as nutrient deprivation, organelles, cytoplasm and cellular proteins are engulfed by a double-membrane vesicle called autophagosome, which is then delivered to the lysosome for degradation. This process promotes the recycling and salvage of nutrients to maintain cell homeostasis (Glick et al., 2010; He and Klionsky, 2009; Kaur and Debnath, 2015). Multiple types of autophagy, including macroautophagy, microautophagy, and chaperone-mediated autophagy have been characterized. In this study, the term "autophagy" refers to macroautophagy unless otherwise specified.

The membrane dynamics during autophagy are highly conserved (Figure 6). The process of autophagosome formation and subsequent fusion with lysosome are tightly regulated by autophagy-related genes (ATGs). In autophagy, initiation starts when

* A part of the work presented in this chapter has been published in The Journal of Biological Chemistry (Shi et al., 2016)

cytoplasmic components are sequestered by isolation membrane (or phagophore). The membrane is mainly from the endoplasmic reticulum (ER), and also perhaps from other sources such as mitochondria, nuclear membrane and plasma membrane (Mari et al., 2011; Militello and Colombo, 2011; Tooze and Yoshimori, 2010). The initiation site is also known as phagophore assembly site (PAS) (Kofinger et al., 2015; Suzuki and Ohsumi, 2003). Formation of PAS is mediated by the ULK1 complex (ULK1-ATG13-FIP200-ATG101). Further nucleation requires the activation of autophagy-specific class III PI3K complex (Beclin1-ATG14L-VPS14-VPS34). Maturation of autophagosome requires the complex of ATG-ATG12-ATG16L and PE-conjugated LC3. The complete autophagosome then fuses with the lysosome, leading to the formation of autolysosome in which the cargos are degraded (Mizushima, 2007; Xie and Klionsky, 2007).

Autophagy in immunity

Autophagy plays important roles not only in the cellular metabolic and homeostatic function but also in immunity. However, the connection between autophagy and immunity seems to be complex and is not fully elucidated. Autophagy appears to orchestrate immunity in diverse regulatory systems (Figure 7).

Autophagy facilitates direct elimination of intracellular microorganisms. LC3 associated phagocytosis (LAP) is a process in which LC3 is recruited to the phagosomal membrane after phagocytosis of pathogens. Pathogens engage PRRs to trigger LAP which requires members of autophagic machinery such as class III PI3K complex, ATG5 and ATG7 (Martinez et al., 2015; Romao and Munz, 2014). LAP effectively promotes the

clearance of pathogens including bacteria, viruses, mycobacteria and fungi (Gong et al., 2011; Lerena and Colombo, 2011; Sanjuan et al., 2007). Another mechanism is through selective autophagy, also termed as xenophagy, which engulfs the pathogens into autophagosome and delivers the pathogens to the lysosome for subsequent killing. Xenophagy has been shown to target invasive bacteria in both phagocytic and non-phagocytic cells (Nakagawa et al., 2004).

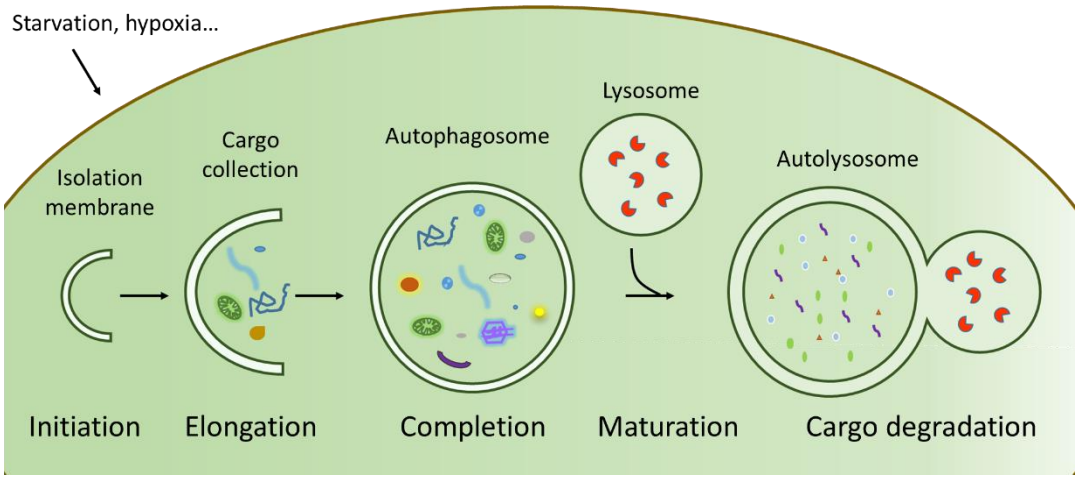

Figure 6. Process of autophagy

Autophagy is initiated when cytoplasmic organelles are sequestered by an isolation membrane which further forms an autophagosome. The autophagosome then fuses with the lysosome to form autolysosome where the internal material is degraded.

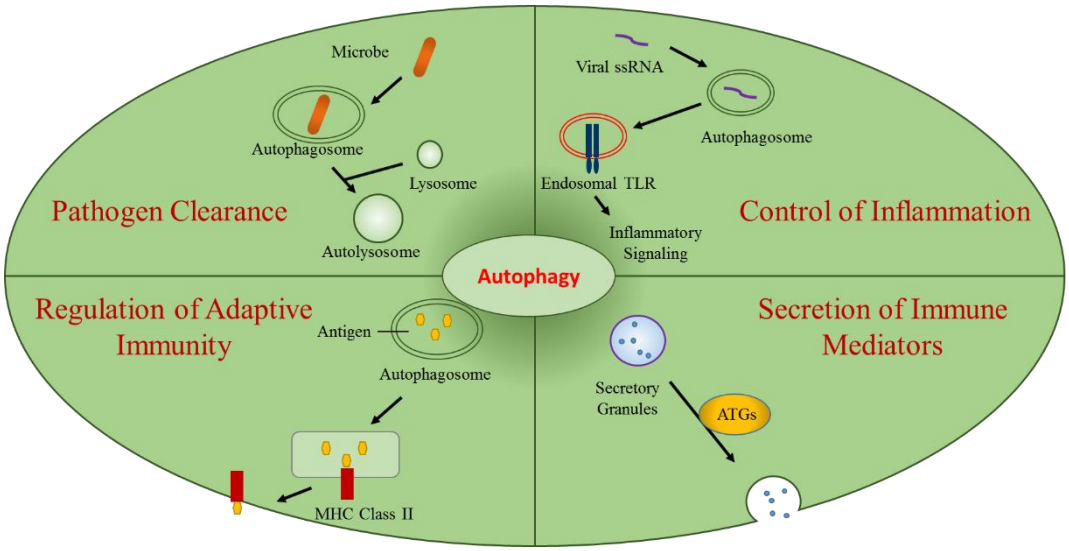

Figure 7. Autophagy in immunity

The major functions of autophagy in immunity. Pathogens in the cytoplasm can be eliminated by autophagic machineries such as LAP and xenophagy. Autophagy can deliver PAMPs to endosomal TLRs and activate inflammatory signaling. Autophagy can enhance the antigen presentation by MHC class II molecules. Secretion of immune mediators from pre-stored granules is regulated by autophagy-related proteins.

Autophagy broadly controls inflammatory signaling with various mechanisms. Autophagy machinery is required for the recognition of cytosolic viral replication intermediates by TLR7 and IFNα production by pDCs (Lee et al., 2007). ATG5-ATG12 negatively regulates RLR-mediated induction of type I IFN production. ATG9A negatively regulates the activation of STING which is required type I IFN production in response to double-stranded DNA (Saitoh et al., 2009). In autophagy-deficient cells, p62 accumulation interacts with TRAF6 to promote the activation of NF-κB and pro-inflammatory signaling (Moscat and Diaz-Meco, 2009; Moscat et al., 2006; Sanz et al., 2000). It is reported that autophagy negatively regulates inflammasome activation. Depletion of the autophagic proteins such as beclin 1 and LC3B enhances activation of caspase-1 and production of IL-1β and IL-18 through interfering with mitochondrial DNA (Nakahira et al., 2011). Production of IL-1β and IL-18 is increased in ATG16L1-/- and ATG7-/- mouse macrophages after TLR4 stimulation (Saitoh et al., 2008). Activation of autophagy limits the IL-1β secretion by eliminating ubiquitinated inflammasome (Shi et al., 2012). Secretion of IL-1α is increased ATG5 deficient macrophages in a calpain-dependent pathway (Castillo et al., 2012). Selective autophagy of the adaptor protein BCL-10 modulates TCR to activation of NF-κB signaling (Paul et al., 2012).

Autophagy delivers intracellular proteins to the lysosome for degradation and contributes to endogenous antigen presentation by MHC class II molecules (Jagannath et al., 2009; Jin et al., 2014; Schmid et al., 2007). Autophagy is also necessary for MHC class II-dependent processing and presentation of extracellular antigens (Lee et al., 2010). LAP and NOD2 have been shown to enhance autophagic antigen presentation (Cooney et

al., 2010). In addition, autophagy affects the secretion of immune mediators. Autophagy manipulates ER to Golgi apparatus secretory pathway to affect the secretion of IL-6 and IL-8 (Narita et al., 2011).

Perturbations in autophagy are suggested to occur in inflammatory and autoimmune diseases. Genome-wide association studies have linked autophagic gene polymorphisms to Systemic Lupus Erythematosus (SLE) (Gateva et al., 2009; Han et al., 2009; International Consortium for Systemic Lupus Erythematosus et al., 2008). Defects in autophagy protein function contribute to the pathogenesis of Crohn's disease, a chronic inflammatory disorder of the small intestine. For example, autophagy protein ATG16L1 and IRGM polymorphisms confer a strong risk of developing Crohn's disease (Hampe et al., 2007; Lees et al., 2011; Murthy et al., 2014; Plantinga et al., 2011; Rioux et al., 2007).

Autophagy and TLRs

TLRs are innate immune sensors for recognizing pathogens-derived PAMPs. Recent evidence has linked TLR signaling to the induction of autophagy (Figure 8). It has been shown that stimulation of TLR signaling activates autophagy processes. For instance, activation of TLR4 by LPS induces the formation of LC3 associated autophagosomes and promotes the clearance of mycobacteria in macrophages (Xu et al., 2007). This process is dependent on TRIF but not MyD88 and then signals through RIP1 and p38 MAPK. Stimulation of TLR7 with Imiquimod or ssRNA induces autophagy in a MyD88 dependent manner (Delgado et al., 2008). Beclin 1 is shown to interact with MyD88 and TRIF to facilitate the induction of autophagy after TLR engagement (Shi and Kehrl, 2008).

Other TLR ligands including zymosan, pam3csk4, poly (I: C), flagellin and MALP-2 are also able to induce autophagy or LC3 associated autophagic structures. TLR signaling also mediates autophagic elimination of microbe (Figure 9). TLR2 recognizes zymosan to induce LAP by recruiting autophagic protein LC3, ATG5 and ATG7 to phagosome to eliminate fungi (Sanjuan et al., 2007). TLR2 signaling is also shown to trigger autophagic clearance of Listeria *monocytogenes* in a MyD88-ERK dependent manner (Anand et al., 2011). TLR4 and TLR7 engagement reduce mycobacteria viability in macrophages through autophagic machinery (Gutierrez et al., 2004; Xu et al., 2013).

Our understanding of how autophagy regulates TLR induced signaling is limited. Autophagy delivers cytosolic single-stranded viral RNA to endosomal TLR7 to stimulate type I IFN response (Lee et al., 2007). Autophagy protein SQSTM1 and HDAC6 support MyD88 aggregation and suppress TLR4-induced activation of p38 and JNK signaling (Into et al., 2010). Another autophagy-related protein NDP52 mediate autophagic degradation of TRIF and TRAF6 to suppress TLR3/4 induced activation of IRF3 and NF-κB signaling (Inomata et al., 2012). However, how other autophagy-related protein regulate TLR signaling is unclear.

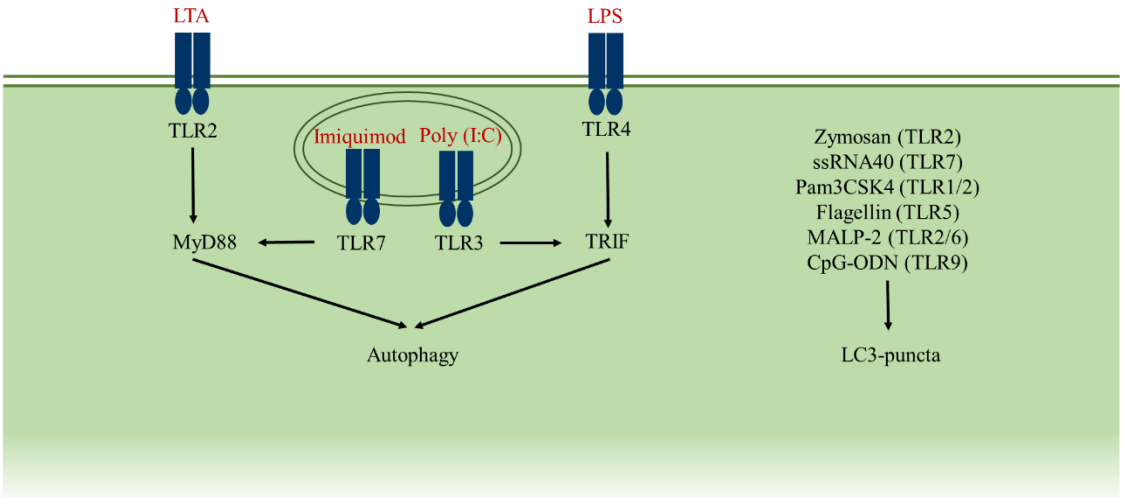

Figure 8. TLRs induce autophagy

Autophagy can be induced directly by TLR agonists. Activation of TLR2 and TLR7 induce autophagy in a MyD88-dependent pathway. Activation of TLR3 and TLR5 induce autophagy in a TRIF-dependent pathway. In addition, various TLR ligands can induce LC3-associated structure.

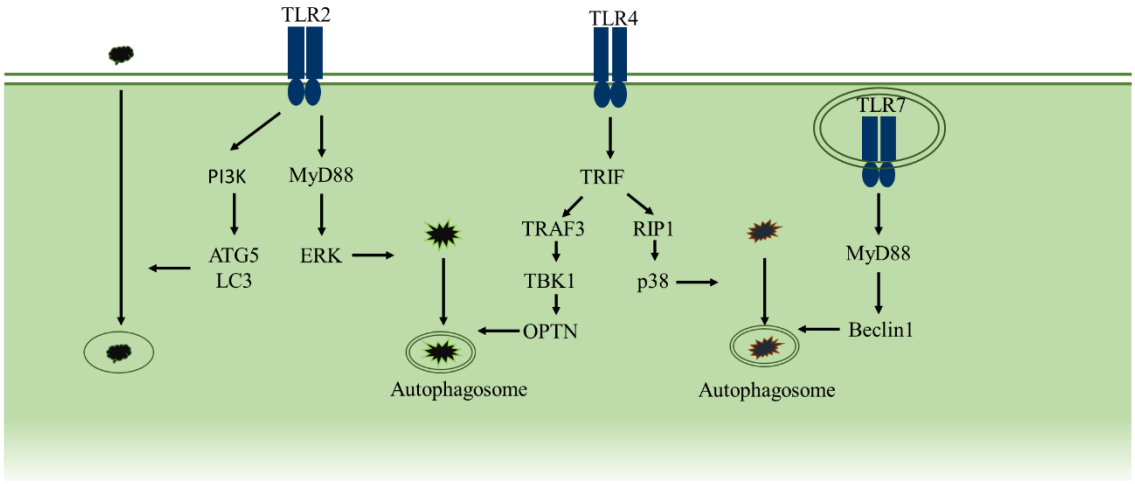

Figure 9. TLR signaling mediates autophagic elimination of microbes

TLR2 signaling recruit ATG5 and LC3 to LAP to facilitate clearance of zymosan. Activation of TLR2 also promotes autophagic clearance of Listeria *monocytogenes* in a MyD88-ERK dependent manner. TLR4 and TLR7 induced autophagy reduce mycobacteria viability through TRIF-RIP1-p38 and MyD88-Beclin1 signaling, respectively. LPS induces selective xenophagy to eliminate Salmonella in a TRIF-TRAF3-TBK1-OPTN pathway.

Microtubule-associated protein 1S (MAP1S)

MAP1S (previously termed as C19ORF5) belongs to the microtubule-associated protein 1family. It is ubiquitously expressed in a wide range of tissues and organs. MAP1S binds to actin and stabilizes microtubules (Orban-Nemeth et al., 2005). MAP1S also binds to double stranded DNA through its microtubule binding domain (Liu et al., 2005). It is suggested that MAP1S plays a role in microtubule dynamics, mitochondria activity, mitotic abnormality and tumor development (Dallol et al., 2007; Jiang et al., 2015; Shi et al., 2014; Xie et al., 2011b). Recent evidence has shown that MAP1S is an autophagy-related protein. MAP1S affect autophagosomal biogenesis and degradation (Liu et al., 2012; Xie et al., 2011a). MAP1S interacts with LC3 and regulates autophagy flux. Here, by utilizing MAP1S KO macrophages that derived from MAP1S deficient mice, we will characterize the role of autophagy protein MAP1S in the regulation of TLR signaling.

Phagocytosis

Phagocytosis is a process by which phagocytes uptake extracellular particles within a plasma-membrane envelope known as phagosome. Phagocytosis is an ancient innate defensive mechanism against invading pathogens (Gordon, 2016; Greenberg and Grinstein, 2002; Jutras and Desjardins, 2005). A variety of innate immune cells are classified as phagocytes including macrophages, dendritic cells and neutrophils (Figure 10A). Phagocytosis facilitates direct clearance of microorganisms. Microbes such as bacteria are engulfed into phagosome, which is then fused with the lysosome containing digestive enzymes (Figure 10B).

Phagocytosis is a receptor-mediated process (Flannagan et al., 2012). Numerous types of receptors such as PRRs, opsonic receptors, scavenger receptors and apoptotic corpse receptors have been shown to contribute to phagocytosis (Freeman and Grinstein, 2014). Phagocytosis of bacteria is often accompanied by TLR induced inflammatory signaling. Previous reports showed that TLRs regulate phagocytosis by activating MyD88-p38 MAPK pathway to accelerate phagosome maturation (Doyle et al., 2004). Meanwhile, TLRs also regulate phagocytosis independent of MyD88 (Kong and Ge, 2008). In addition, TLR2 is recruited to phagosomes during phagocytosis in macrophages (Underhill et al., 1999). Activation of TLR signaling increases phagocytosis of bacteria (Redlich et al., 2013). Activation of TLR2 and TLR9 signaling enhances the phagocytosis of *E. coli* and *S. aureus* (Doyle et al., 2004). A possible mechanism is through altering gene transcription programs associated with phagocytosis. Here, we will investigate the role of MAP1S in phagocytosis of bacteria.

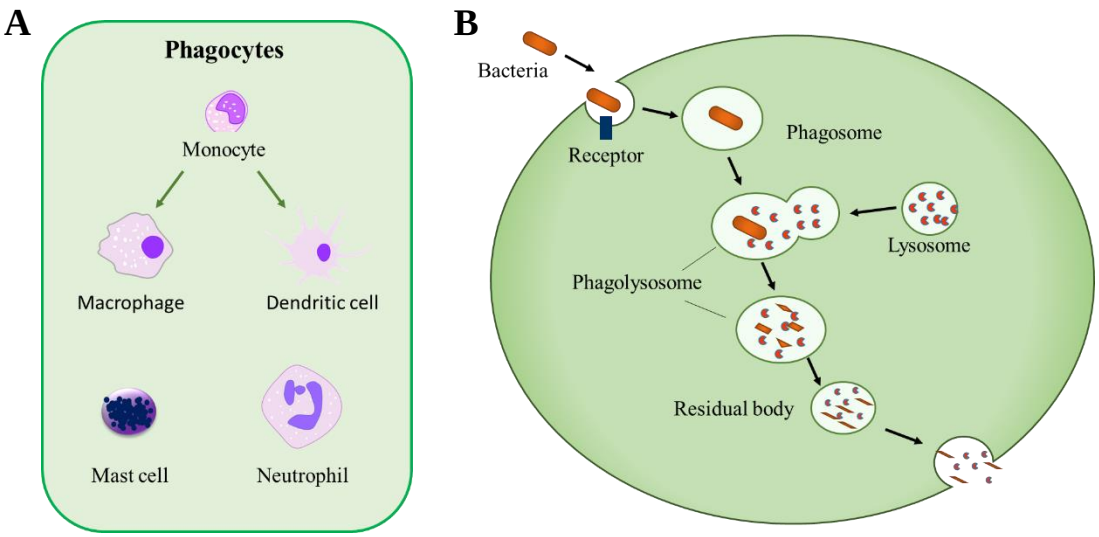

Figure 10. Phagocytes and phagocytosis

(A) Phagocytes include macrophages, dendritic cells, neutrophils and mast cells. (B) The process of phagocytosis of bacteria. Phagocytosis is a receptor-mediated process. Upon recognition, phagocytes engulf bacteria in a vacuole known as phagosome. Lysosome in the cytoplasm fuses with phagosome, releasing digestive enzymes into the phagosome to form the phagolysosome. Within the phagolysosome, microorganisms are killed and degraded. The digested contents are eliminated from phagocytes by exocytosis.

Materials and Methods

Cells and reagent

Raw264.7 and primary macrophages were maintained in DMEM supplemented with 10% FBS (HyClone). Peritoneal macrophages (PMs) were isolated from peritoneal lavage 5 days after intraperitoneal stimulation with 2 ml of 3% thioglycollate medium (BD Biosciences). Bone marrow-derived macrophages (BMDMs) were derived from bone marrows cells that were cultured in the presence of 10 ng/ml macrophage colony-stimulating factor (R&D System) for 6 to 8 days. Bacteria strains *Salmonella typhimurium* SR11, *Staphylococcus aureus* Newman and *Escherichia coli*-GFP were cultured in LB medium. MAP1S antibody was purchased from Precision Antibody. IκB-α, phosphor-p38, and phosphor-Akt antibodies were purchased from Cell Signaling Technology. MyD88 antibody was purchased from Imgenex. LC3 and p62 antibodies were purchased from MBL. LPS, ODN2216, and LTA were purchased from Invivogen. TRIzol for RNA isolation and Superscript II reverse transcriptase for cDNA synthesis were from Invitrogen.

Mice

MAP1S knockout mice in the C57BL/6 background were kindly provided by Dr. Leyuan Liu (Xie et al., 2011b). All mice were maintained at Texas A&M University Health Science Center in Houston, and all the experiments were performed by following the protocols approved by IACUC.

Phagocytosis assay

Peritoneal macrophages or BMDMs were infected with *S. typhimurium* SR11 at MOI 10 to 1 and then the plate was centrifuged at 1000 rpm for 2min. The bacteria were allowed for phagocytosis for 30 min at 37 ℃. The cells were wash three times with PBS Then DMEM with 100 μg/ml gentamicin was added and incubated for 60 min. Then the cells were lysed in sterile water for 10min. Cell lysates containing phagocytosed bacteria were serially diluted and plated on LB plate. Bacteria colonies were counted the next day.

Cell sorting and FACS analysis

Splenocytes generated from C57BL/6 mice were stained with combinations of fluorescence-conjugated monoclonal antibodies (BD Biosciences), including B cells, CD19+; CD4 T cells, CD3+CD4+; NK cells, NK1.1+; macrophages, CD11b+F4/80+; dendritic cells, CD11c+CD11b+. Then, the splenocytes were sorted by FACSAria (BD Biosciences). BMDMs from WT and MAP1S KO mice were infected with GFP-*E. coli* (MOI 100: 1) for different time points. Then the cells were washed, fixed and analyzed by Flow Cytometry at the core facility at Brown Foundation Institute of Molecular Medicine (IMM), UTHSC.

Immunofluorescence staining

Macrophages (2 x 10^5 cells/well) were seeded in a 24-well plate containing coverslips and incubated overnight. At room temperature, the cells were washed and fixed in 4% paraformaldehyde for 10min and permeabilized with 0.5% Triton X-100 for 15min,

and then the cells were blocked with 5% BSA in PBS for 1h. The cells were then incubated with primary antibody at 4℃ overnight. Then the cells were washed with PBST for three times and incubated with fluorescence-conjugated secondary antibody for 1h at room temperature. After extensive washing, the cells were mounted with Aqua mount solution and images were analyzed with confocal microscopy (Zeiss).

Immunoprecipitation and Western blot

Cell lysates were incubated with protein G-Sepharose and indicated antibodies at 4℃ overnight. Then the protein complexes were used for Western blot. Proteins were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% fat-free milk in TBST at room temperature for 30min and incubated with primary antibody at 4℃ overnight or 2 hours at room temperature. Then the membrane was washed in TBST for 3 times and incubated with HRP-conjugated secondary antibody for 1 hour. After extensive washing, the membrane was developed with ECL plus immunoblotting detection reagents (Amersham Biosciences).

ELISA

BMDMs from WT and MAP1S KO macrophages were stimulated with different TLR ligands or heat-killed bacteria, with the concentrations as indicated in the result section for 24 hours or 36 hours. Cell supernatant were collected and analyzed with respective ELISA kits following manufacturer instructions (R&D Systems). Briefly, ELISA plate was coated with capture antibody diluted in 100 ul coating buffer. The plate

was sealed and incubated at 4℃ overnight. On the next day, the plate was washed 3 times with washing buffer and then added 200 ul blocking buffer. The plate was incubated at room temperature for 1 hour. Then the plate was washed 3 times and 100 ul sample or standard was added to each well. The plate was incubated at room temperature for 2 hours. Then the plate was washed 5 times and 100 ul working detector was added to each well. The plate was incubated for 1 hour. Then the plate was washed 7 times and 100 ul substrate solution was added to each well. The plate was incubated for 30 minutes. Finally, 50 ul stop solution was added to each well and OD 450 nm was measured.

Cell viability assay

Cell counting kit-8 (Dojindo) was used to determine cell viability. Briefly, WT and MAP1S KO macrophages (2 x 10^4 cells/well) were seeded in a 96-well plate 24 hours before treatment. Then the cells were treated with indicated heat-killed bacteria or LTA for 24 hours. Then 10 μl of CCK-8 solution was added to each well of the plate and incubated at 37℃ until the desired color develops. OD 450 nm was then measured.

Results

MAP1S regulates cell surface TLR signaling

We examined the expression of MAP1S in different types of immune cells within the spleen. We found that MAP1S level in macrophages was relatively high comparing with other cell types including DCs, NK cells, T cells and B cells (Figure 11). TLRs expressed on macrophages recognize invading microorganisms and facilitate macrophage activation. This process initiates immune responses and is vital during infection. Autophagy has been suggested to be linked to TLRs (Into et al., 2012), but the molecular mechanisms are unclear. So we wonder whether autophagy protein MAP1S can regulate TLR signaling.

Bone marrow derived macrophages isolated from C57BL/6 WT and MAP1S KO mice were challenged with heat-killed *S. typhimurium* (SR11) or TLR agonists: OSPA- TLR1/2, LTA- TLR2, poly (I: C)- TLR3, LPS- TLR4, flagellin- TLR5 and CpG ODN 2216- TLR9 for 36 hours. The IL-6 and TNF-α level in the supernatant were evaluated by ELISA. We found that treatment with heat-killed bacteria and stimulation of cell surface TLR2 and TLR4 signaling secreted significantly less IL-6 and TNF-α in MAP1S KO macrophages than in WT macrophages (Figure 12A and Figure 13A). Activation of endosomal TLR3 and TLR9 signaling showed comparable cytokine secretion or type I IFN expression in both WT and MAP1S KO macrophages (Figure 12A, Figure 13A and B). Flagellin failed to induce a response, which is consistent with the previous report that flagellin had no stimulatory effect on macrophages (Kwon et al., 2011).

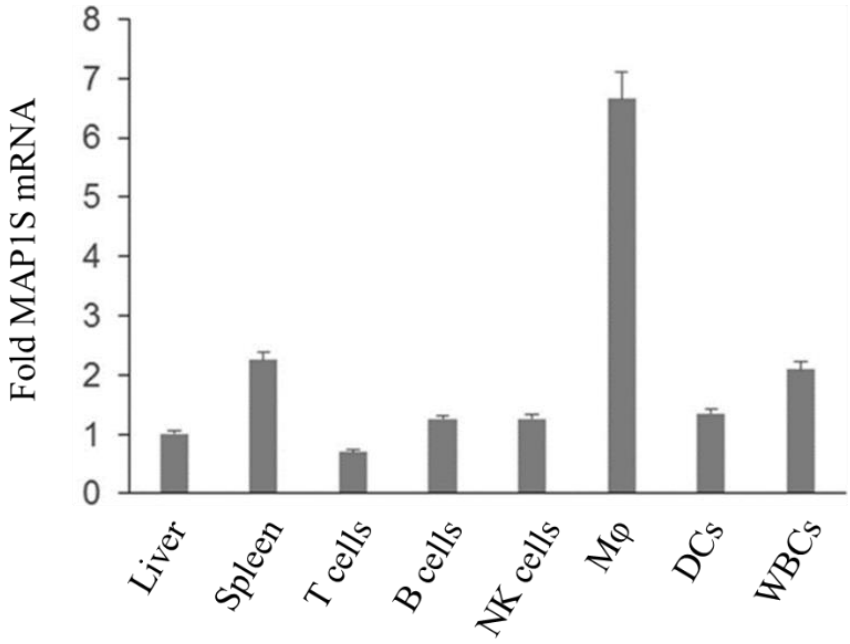

Figure 11. MAP1S is highly expressed in macrophages

Various types of immune cells were analyzed for MAP1S mRNA level by real-time RT-qPCR. Fold change is shown comparing with the expression level in the mouse liver. Single cell suspensions of splenocytes were sorted by FACS. All data represent mean ± SD.

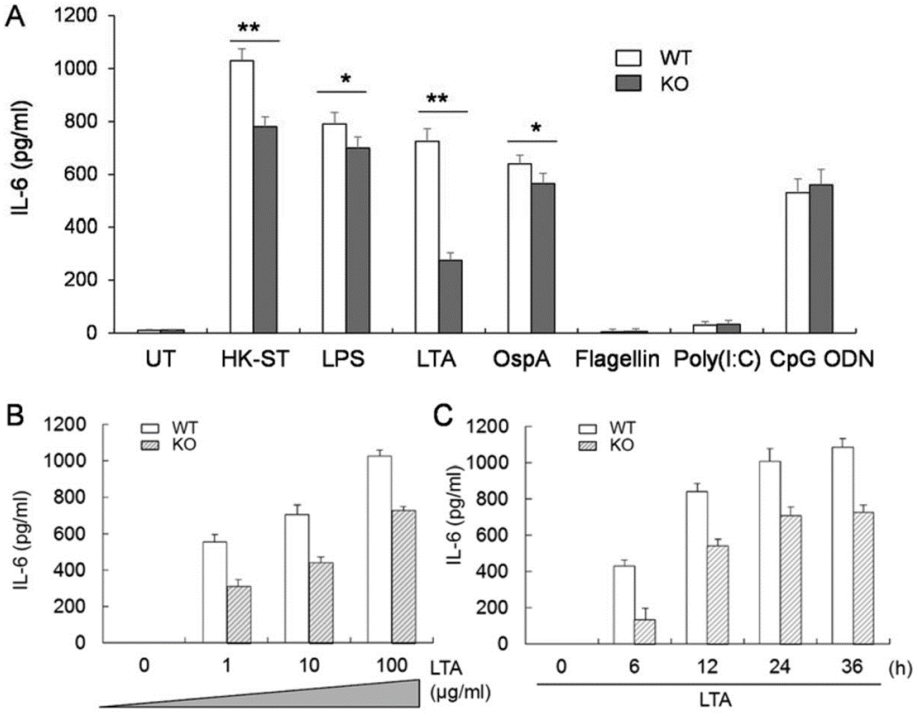

Figure 12. Decreased IL-6 production in MAP1S deficient macrophages after cell surface TLR activation

BMDMs generated from WT and MAP1S KO mice were treated with heat-killed *S.typhimurium* (HK-ST, 10^5 CFU/ml), LPS (100 ng/ml), LTA (10 μg/ml), OspA (50 ng/ml), Flagellin (100 ng/ml), Poly (I:C) (100 ng/ml), ODN2216 (10 μg/ml) or untreated (A), or with indicated doses of LTA (B) or with LTA (10 μg/ml) for indicated time (C). IL-6 in the cell supernatant was measured 36 hours after stimulation by ELISA. All data represent mean ± SD. *p<0.05, **p<0.01. Unpaired *t*-test was used.

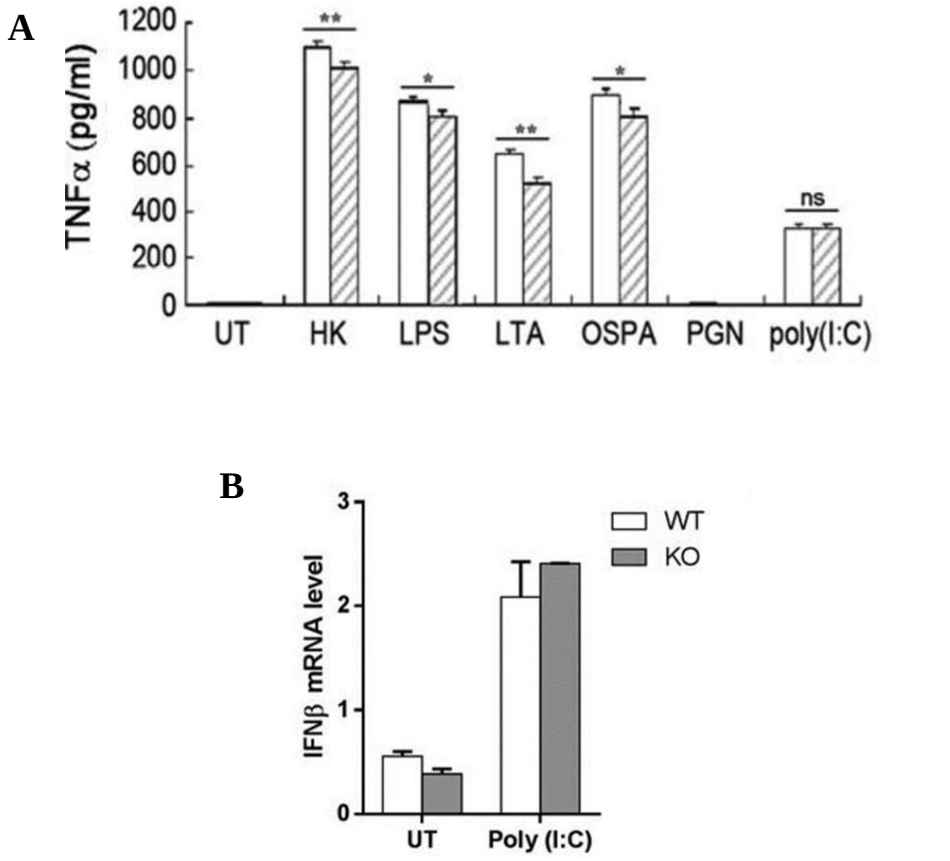

Figure 13. TNFα production decreased in MAP1S deficient macrophages after cell surface TLR ligands stimulation and type I interferon was unaffected

(A) BMDMs generated from WT and MAP1S KO macrophages were treated with LTA (10 μg/ml) for 24 hours. TNFα in the cell supernatant was measured by ELISA. (B) BMDMs were treated with Poly (I: C) (1 μg/ml) for 4 hours, and IFNβ mRNA level was measured by real-time RT-qPCR. All data represent mean ± SD. *$p<0.05$, **$p<0.01$, ns-non-significant. Unpaired *t*-test was used.

We noticed that stimulation of TLR2 ligand LTA exhibited the most dramatic difference in cytokine secretion between WT and MAP1S KO macrophages. We further confirmed that upon LTA stimulation, IL-6 secretion in MAP1S KO BMDMs was significantly less than in WT BMDMs, both in time-dependent and dose-dependent fashion (Figure 12B and C). TLR1/2, TLR2/6, and TLR4 localize on the cell membrane, whereas TLR3 and TLR9 localize in the endosome. Stimulation with cell membrane TLR ligands caused a significant impaired signaling in MAP1S KO macrophages, while stimulation with endosomal TLR ligand showed no difference. These data suggest that autophagy protein MAP1S only affects cell surface TLRs activation.

MAP1S controls the phagocytosis of bacteria in macrophages

Macrophages are a type of phagocytic cells. They directly eliminate bacteria by phagocytosis during infection. Phagocytosis is a receptor-mediated process, and activation of TLRs has been shown to facilitate this process (Doyle et al., 2004; Ribes et al., 2010). Moreover, it is suggested that autophagy proteins might contribute to phagocytosis (Bonilla et al., 2013; Sanjuan et al., 2007). To investigate whether MAP1S affect phagocytosis of bacteria, we performed traditional phagocytosis assay by infecting primary peritoneal macrophages from WT and MAP1S KO mice with *S. typhimurium*. We found that phagocytosis of bacteria was impaired in MAP1S KO macrophages compared with WT macrophages (Figure 14A, B and C). We also infected WT and MAP1S KO macrophages with Texas-red stained bacteria and analyzed the phagocytosed bacteria with confocal microscopy. MAP1S KO macrophages phagocytosed fewer bacteria than

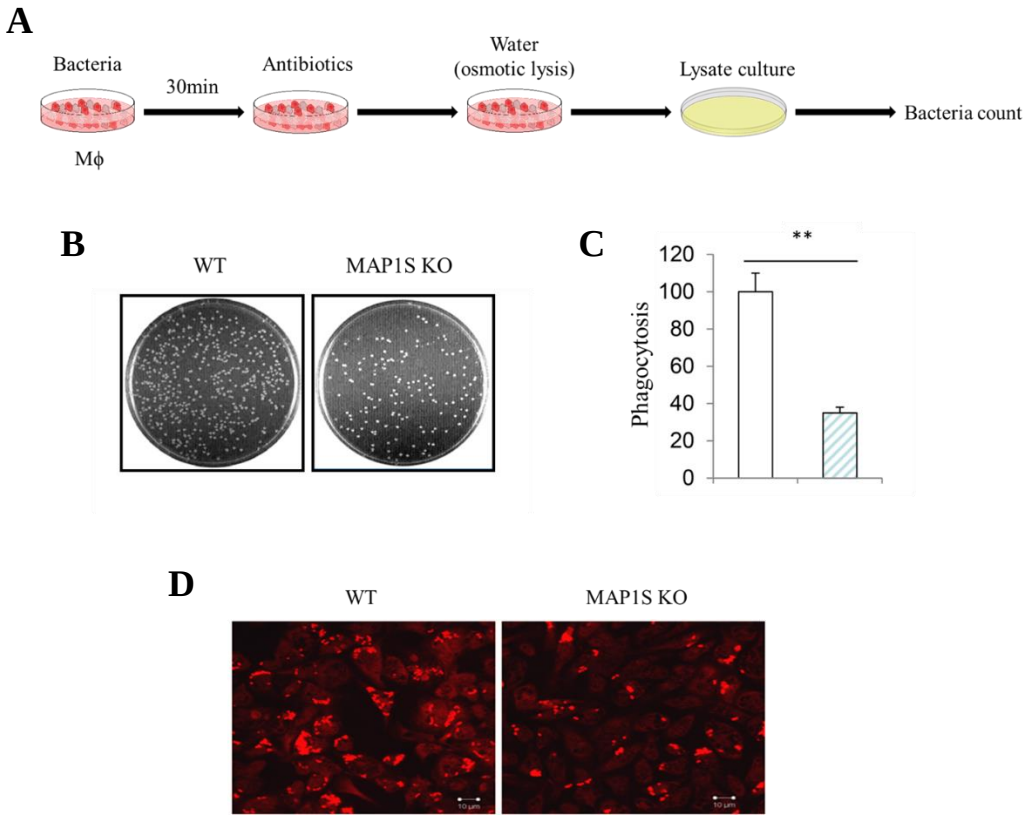

Figure 14. Impaired phagocytosis of bacteria in MAP1S KO macrophages

(A) Schematic diagram of plate count phagocytosis assay. Peritoneal macrophages from WT and MAP1S KO mice were infected with SR11 (MOI 10: 1), (B) representative images and (C) quantification of phagocytosis rate comparing with WT cells were shown. (D) Representative images of phagocytosis of Texas-red stained SR11 in peritoneal macrophages from WT and MAP1S KO mice. (E) Peritoneal macrophages from WT and MAP1S KO mice were infected with GFP-E.*coli* (MOI 100: 1) for different time intervals. The number of phagocytosed bacteria was determined under confocal microscopy by counting GFP-E.*coli* in 200 cells. (F) BMDMs from WT and MAP1S KO macrophages were infected with GFP-E.*coli* (MOI 100: 1) for different time intervals and flow cytometry was performed to determine the phagocytosis of bacteria. All data represent mean ± SEM. **p<0.01. Unpaired *t*-test was used.

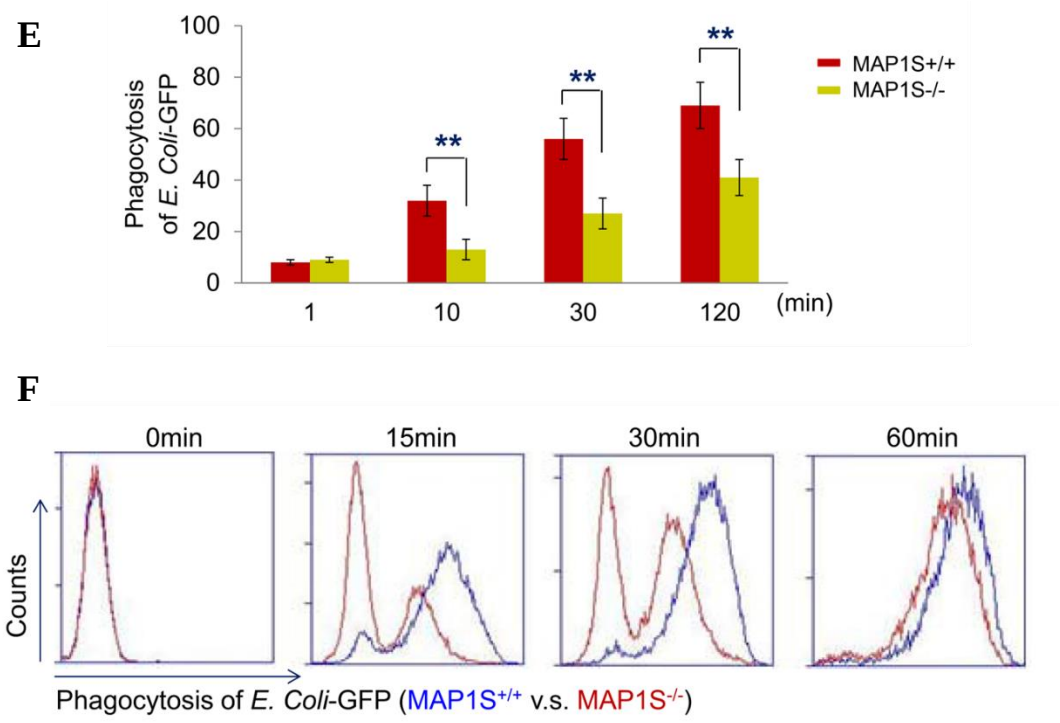

Figure 14. Continued

WT cells (Figure 14D). We further confirmed the results by assessing phagocytosis of an *E. coli* strain expressing GFP with confocal microscopy and flow cytometry (Figure 14E and F). MAP1S is reported to be important for mitochondrial stress which can affect cell viability during starvation (Xie et al., 2011a; Xie et al., 2011b). However, in our study, the cell viability showed no significant difference between WT and MAP1S KO macrophages with or without TLR ligand stimulation (Figure 15). Taken together, these data suggest that MAP1S plays a role in immune events in macrophages by regulating cell surface TLR signaling and controlling bacterial phagocytosis.

MAP1S interacts with MyD88

MAP1S is expressed intracellularly in the cells including macrophages (Xie et al., 2011a). To determine the mechanism of how intracellular protein MAP1S regulates cell surface TLR activation, we reasoned that it might be associated with components of the downstream signaling pathway. Upon TLR activation, one or more adaptor proteins are recruited to TIR domain of TLRs. In co-immunoprecipitation experiments, we found that MAP1S directly associated with MyD88 in RAW264.7 cells upon LTA stimulation (Figure 16A, B and C). Furthermore, LTA-induced upregulation of MAP1S was in parallel with the increasing expression level of MyD88 (Figure 16D). Collectively, these data indicate that MAP1S regulates TLR signaling and controls bacterial phagocytosis by interacting with adaptor protein MyD88.

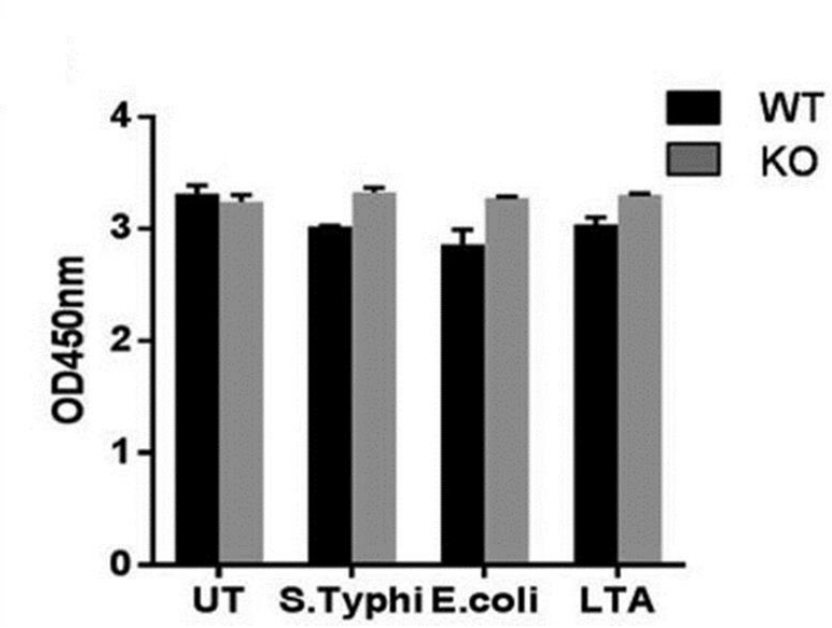

Figure 15. MAP1S deficiency does not affect macrophage cell viability

BMDMs were from WT and MAP1S KO mice were treated with heat-killed ST (10^5 CFU/ml), heat-killed *E.coli* (10^5 CFU/ml) and LTA (10 μg/ml) for 24 hours. Then cell viability assay was performed. The data represent mean ± SD.

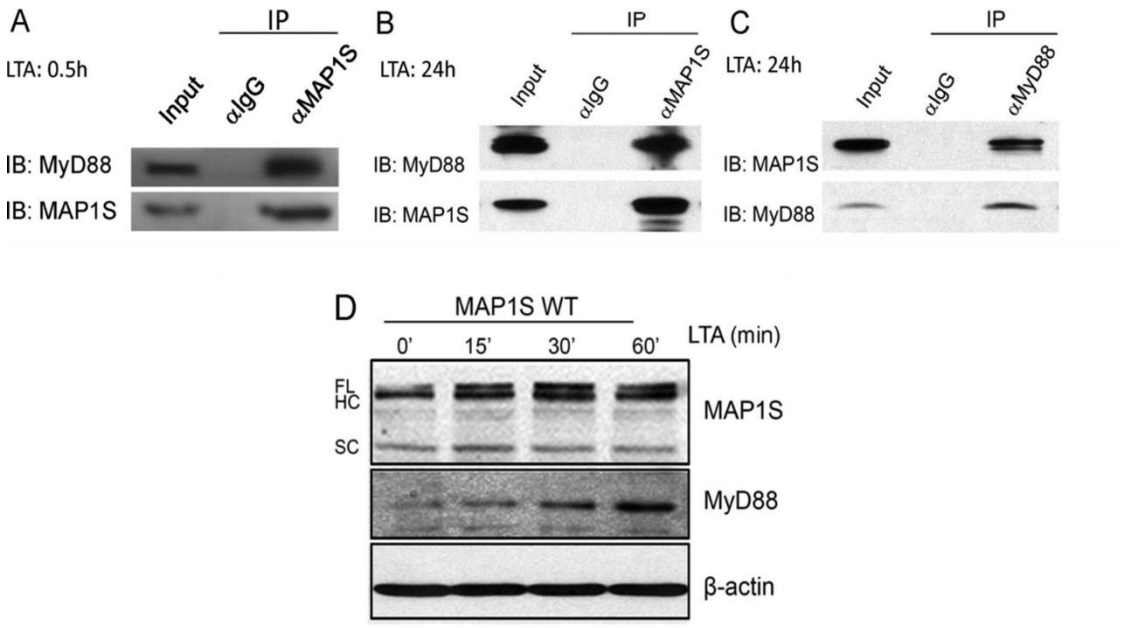

Figure 16. MAP1S interacts with MyD88

Raw264.7 cells were stimulated with LTA for 0.5 hour or 24 hours. Cell lysates were immunoprecipitated with control IgG, MAP1S antibody (A and B), or MyD88 antibody (C), followed by Western blot detecting MyD88 and MAP1S, respectively. (D) Western blot demonstrating the accumulation of MAP1S and MyD88 in WT macrophages after LTA stimulation. FL, full-length; HC, heavy chain; SC, short chain.

Next, we evaluated the activation of downstream signaling pathways. Stimulation of TLR2 signaling generally would lead to the activation of NF-κB, JNK or p-38. We found no difference in IκBα degradation between WT and MAP1S KO primary macrophages (Figure 17). In contrast, the phosphorylation level of p38 dramatically decreased in MAP1S KO macrophages (Figure 17). The results are consistent with previous reports that inhibition of p38 decreased the expression of cytokines and impaired phagocytosis by inhibiting phagosome maturation (Blander and Medzhitov, 2004, 2006). It has been reported that activation of P38 MAPK and Akt are negatively correlated. Overexpression of Akt abrogates p38 MAPK phosphorylation. Downregulation of Akt activation contributes to enhanced p38 MAPK activation (Liao and Hung, 2003; Rane et al., 2010). Indeed, after LTA stimulation, p38 MAPK was activated in MAP1S WT macrophages with no activation of Akt, while in MAP1S KO macrophages, activation of p38 MAPK decreased with significant upregulation of phosphorylated Akt. Therefore, these data indicate that MAP1S is involved in TLR signaling through regulation of p38 MAPK pathway but not NF-κB pathway.

MyD88 is recruited to TLR-induced punctate structure by MAP1S

TLR signaling has been shown not only to regulate autophagy, but also to directly induce autophagy and autophagy protein associated punctate structures (Delgado and Deretic, 2009; Delgado et al., 2008; Into et al., 2012; Xu et al., 2007). We stimulated WT and MAP1S KO macrophages with LTA and evaluated the accumulation of MyD88 and autophagosome marker LC3. Intriguingly, LTA-induced MyD88 accumulation with

punctate structure in a MAP1S-dependent manner (Figure 18A). Remarkably, we also found that MyD88 co-localized with LC3 after LTA stimulation in a MAP1S dependent mode (Figure 18B). It is consistent with the previous report that TLR2 signaling induced autophagy and punctate structure is MyD88 dependent (Shi and Kehrl, 2008). Taken together, the results indicate that MAP1S facilitates the recruitment and accumulation of MyD88 into autophagy process in response to TLR activation.

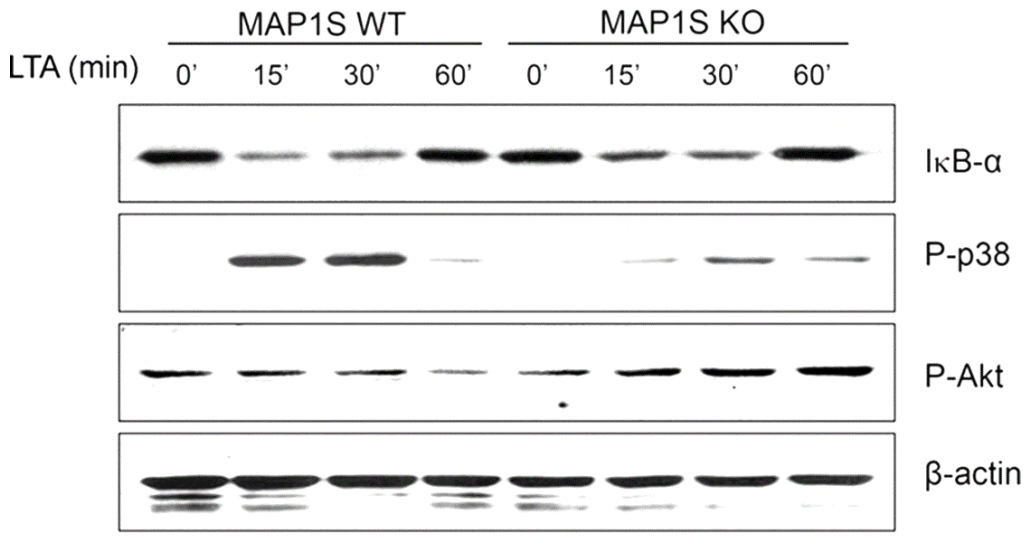

Figure 17. MAP1S regulates p38 but not NF-κB activation in the TLR signaling pathway

BMDMs from WT and MAP1S KO mice were treated with LTA (10 μg/ml) and analyzed the protein level change of IκB-α, phosphor-p38 and phosphor-Akt by Western blot.

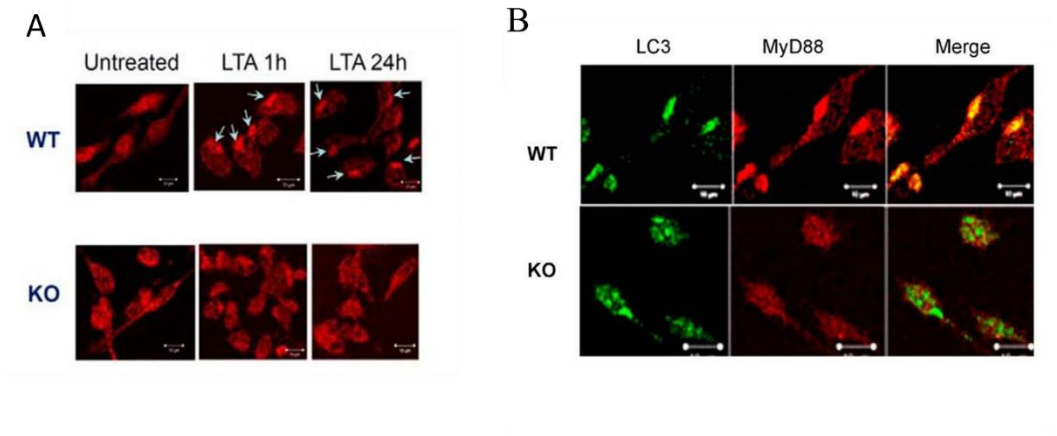

Figure 18. MyD88 is recruited into the processing of TLR-induced punctate structure by MAP1S

(A) Representative images of immunofluorescence staining of MyD88 in WT and MAP1S KO peritoneal macrophages after LTA (10 μg/ml) stimulation for 24 hours. (B) BMDMs from WT and MAP1S KO mice were stimulated with LTA for 24 hours and co-localization of MyD88 (red) and LC3 (green) was analyzed by confocal microscopy. Scale bar = 10 μm.

Discussion

TLRs detect invading pathogens, trigger innate immune responses and facilitate antigen presentation to activate adaptive immunity. Autophagy is a cellular process of degrading its own components through the lysosomal machinery to maintain cell homeostasis under harmful conditions (Glick et al., 2010; Mizushima, 2007). Autophagy is also an ancient mechanism for clearance of intracellular pathogens. More evidence has suggested that autophagy functions in diverse aspects of immunity (Deretic et al., 2013; Levine and Deretic, 2007; Schmid and Munz, 2007). TLR signaling has been shown to activate autophagic process in macrophages which is dependent on MyD88 and TRIF (Delgado et al., 2008; Xu et al., 2007). TLR-induced immune responses are also regulated by autophagy, but the mechanisms are not fully understood.

Our study demonstrates that an autophagy protein MAP1S regulates cell surface TLR activation by directly interacting with adaptor protein MyD88. MAP1S is a novel characterized autophagy protein that regulates autophagosome biogenesis and degradation and interacts with LC3 (Xie et al., 2011a; Xie et al., 2011b). Surprisingly, secretion of cytokines was significantly decreased in MAP1S KO macrophages upon TLR agonists stimulation. We found that MAP1S only affected cell surface TLR activation, but not the intracellular TLR3 and TLR9 signaling. To investigate how an intracellular protein could regulate cell membrane receptors activation, we examined TLR downstream molecules and found that MAP1S could interact with adaptor protein MyD88, which is recruited to TIR domain after TLR dimerization. A function of microtubule-associated proteins is to

facilitate cargo transportation in the cytoplasm. The result suggests that MAP1S is likely to aid the recruitment of MyD88 to TIR domain of TLRs. It provides evidence that an autophagy protein regulates TLR signaling. One interesting question is how MAP1S interacts with MyD88 and facilitates its recruitment after TLR activation. We also noticed that LTA-induced parallel accumulation of MAP1S and MyD88. This result indicates that TLR signaling might also induce MAP1S translational upregulation or protein stability in the cytosol, and in turn MAP1S aids TLR activation.

Although LTA stimulation exhibited a dramatic difference in cytokine secretion between WT and MAP1S KO macrophages, the difference in LPS and OspA treated group was relatively mild. The underlying mechanism is unclear. One possibility is that MAP1S might also facilitate protein trafficking in TLR2 signaling. Of note, MAP1S only affects p38 MAPK signaling, but not NF-κB signaling after TLR2 activation. This indicates that MAP1S might not only interact with MyD88 but also downstream molecules in TLR2-MyD88-p38 MAPK pathway. Given the data that Akt signaling and p38 MAPK signaling might be inversely related to previous reports, another possibility is that deficiency in MAP1S upregulates Akt signaling, which in turn inhibits p38 MAPK signaling.

TLR signaling is important in mediating phagocytosis in macrophages. Our results also underscore the contribution of MAP1S to phagocytosis of bacteria. MAP1S deficiency dramatically impaired the efficiency of phagocytosis of both gram-positive and gram-negative bacteria. Together with the data that MAP1S affects p38 MAPK signaling, the results are consistent with previous reports showing that p38 MAPK modulates phagosome maturation in macrophages (Blander and Medzhitov, 2004; Doyle et al.,

2004). The hypothesis is that MAP1S deficiency decreases TLR induced p38 MAPK signaling which affects phagosome maturation, thus downregulates phagocytosis of bacteria in macrophages. Autophagy proteins have been shown to be recruited to phagosome during maturation (e.g., LC3- associated phagocytosis) (Martinez et al., 2011; Martinez et al., 2015). MAP1S also interacts with LC3. Another possible mechanism is that since MAP1S affects autophagosome biogenesis, it might also affect phagosome biogenesis.

Activation of TLR signaling could induce autophagy or autophagy protein associated structure. This process is reported to be dependent on MyD88 (Shi and Kehrl, 2008).We found that MAP1S facilitated MyD88 accumulation in the cytoplasm. Moreover, after LTA stimulation, MyD88 co-localized with LC3 in WT macrophages, but not in MAP1S KO macrophages. It indicates that MAP1S recruits MyD88 and promotes autophagic process in response to TLR activation.

Increasing evidence has shown the connections between TLR signaling and autophagy (Arroyo et al., 2013; Into et al., 2012; Kim et al., 2015). Studies have shown autophagy could be induced by TLR ligands and knocking out autophagy associated proteins could affect TLR signaling. However, the mechanisms of how autophagy controls TLR activation and downstream signaling are unclear. Here, our study shows that an autophagy-associated protein, MAP1S, could regulate cell surface TLR activation in macrophages. Deficiency in MAP1S decreased cytokine secretion after TLR ligands stimulation likely through the p38 MAPK signaling pathway. MAP1S deficient

macrophages showed an impaired efficiency of phagocytosis. MAP1S interacts with MyD88 and TLR induced MyD88 recruitment is facilitated by MAP1S (Figure 19).

Our work opens avenues for understanding the connections between autophagy and TLR signaling. Our study also provides a rationale of how autophagy protein regulates TLR signaling.

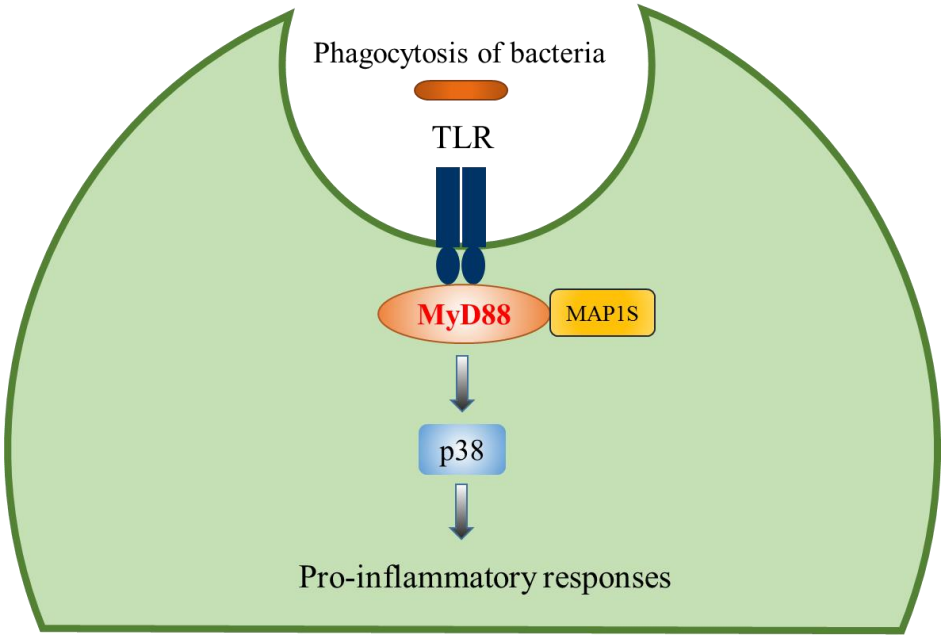

Figure 19. Schematic diagram of the regulation of TLR signaling and phagocytosis by MAP1S

MAP1S interacts with MyD88 to regulate cell surface TLR activation and control TLR mediated phagocytosis of bacteria. MAP1S regulates downstream p38 MAPK signaling to control pro-inflammatory responses.

CHAPTER III

CPG-ODN INDUCED INNATE RESISTANCE AGAINST FLU

Introduction

Influenza overview

Influenza (also called Flu) is a contagious respiratory disease caused by influenza virus. The influenza virus mainly infects respiratory systems such as nose, throat, and lungs, resulting in mild to severe symptoms such as fever, cough, sore throat, headaches, stuffy nose, fatigue and sometimes even death. Most researchers think that influenza is spread mainly by droplets containing virus particles made by infected people when coughing, sneezing or talking (Bridges et al., 2003; Nicas et al., 2005). Those aerosolized droplets (<5- 10 μm) reach host lower respiratory tract with high efficiency. The onset of symptoms begins in 1 to 4 days after exposing to influenza virus. People infected with influenza are susceptible to complications including bacteria pneumonia, ear and sinus infections. Influenza infection can also worsen long term medical conditions such as asthma, heart failure, and diabetes. Old people, young children, pregnant women and people with chronic medical conditions described above are at high risk of developing more severe flu-associated complications when infected. It is strongly recommended to get a flu vaccination each year to prevent influenza infection, as well as staying away from people who are already infected.

Although usually influenza is not a deadly disease, it can be catastrophic. During the 1918 - 1919 flu pandemics (also known as Spanish flu), an estimated 50 million people died with an approximately 20% to 40% population being infected worldwide (Johnson and Mueller, 2002). 10% to 20% of infected people died. It was one of the deadliest disasters described as "the greatest medical holocaust in history". The 1957 - 1958 Asian flu pandemic outbreak originated in China and then spread worldwide. The estimated death was 1 million to 4 million. The 1968 -1969 Hong Kong flu pandemic killed approximately 1 million people. The elderly were especially vulnerable and had the greatest death rates.

Humankind is still facing the threat of an influenza pandemic nowadays with the emergence of highly pathogenic avian influenza (HPAI) strains. Widespread circulation of H5N1 has been monitored since its appearance, since it not only causes devastating influenza pandemics in avian populations but also has the potential of transmission to humans and other mammalian species, which makes the pandemic threat unpredictable. Epidemic outbreaks each year because of a process called "antigenic drift" which results in a new strain of virus with slight genetic changes occurring in both influenza A and influenza B viruses. In fact, studies have shown that the pandemic flu is caused by another process called "antigenic shift" resulting in a dramatically new subtype against which most people have no immunity (Gething et al., 1980; Treanor, 2004). Influenza outbreaks cause huge healthcare burden and economic loss.

Influenza A virus and replication

Influenza viruses belong to the family of Orthomyxoviridae. Three types of influenza viruses have been identified to circulate in human populations: type A, B and C. Type B and C influenza almost exclusively infect human, whereas type A influenza infect a wide variety of animal species including swine, human and birds (Ito and Kawaoka, 2000). All the major flu pandemics are caused by influenza A virus which is believed to be originated from avian: 1918-1919 Spanish flu (H1N1), 1957- 1958 Asian flu (H2N2), 1968- 1969 Hong Kong flu (H3N2) and the bird flu (H5N1).

The influenza virion is a spherical enveloped particle which consists of an outer lipid membrane and an inner viral core. The lipid bilayer is derived from host plasma membrane and contains 80% hemagglutinin, 17% neuraminidase and a small amount of matrix 2 protein. Hemagglutinin and neuraminidase are the determinants of the subtype of influenza virus and also important in inducing host immune responses. Beneath the lipid bilayer is matrix protein 1 that supports the envelope rigidity. The viral core contains viral RNA segments associated with RNA polymerase proteins and non-structural proteins (Szewczyk et al., 2014). The genome of influenza A virus contains eight segments of negative-sense single-stranded RNA, encoding 11 viral genes: hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix 1 (M1), matrix 2 (M2), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase basic protein 1-F2 (PB1-F2), non-structural protein 1 (NSP1), and non-structural protein 2(NS2/NEP) (Bouvier and Palese, 2008; Szewczyk et al., 2014). Influenza A virus only replicates in living cells. The viruses undergo replication in the

following stages: virus adsorption and entry into the host cell, viral RNA import into nucleus, transcription and replication of the viral genome, assembly of new viral particles, and exiting host cells. The newly released viruses then infect other surrounding cells.

Innate immune responses to influenza virus infection and therapeutic implications

After entering the host respiratory system, the influenza virus is recognized by innate immune PRRs including TLRs, RIG-I and NOD-like receptors expressed in cells such as epithelial cells, macrophages, and pDCs. Activation of TLR3 in influenza virus-infected lung epithelial cells induces the production of pro-inflammatory cytokines (Le Goffic et al., 2007). TLR7 recognizes influenza virion ssRNA genome and activates NF-κB or IRF7 signaling (Diebold et al., 2004; Lund et al., 2004). RIG-I recognizes the 5'-triphosphate viral ssRNA in the cytosol of influenza virus infected cDCs, macrophages and epithelial cells (Baum et al., 2010; Hornung et al., 2006; Kato et al., 2005; Pichlmair et al., 2006). Activation of PRRs during influenza infection induces expression of pro-inflammatory cytokines, type I and type III interferons and interferon-stimulated genes (ISGs), which induce antiviral resistance and promote adaptive immune responses.

The more specific and effective adaptive immunity produces a slower response than that of the innate immunity (Figure 20). When given a lethal dose of influenza virus infection, the host may not be able to generate an effective adaptive immune response in time for protection. The innate immunity is antigen-unspecific, however, provides the host immediate protection against influenza virus infection. An ideal strategy is to boost the innate immune system to induce innate resistance against influenza virus infection.

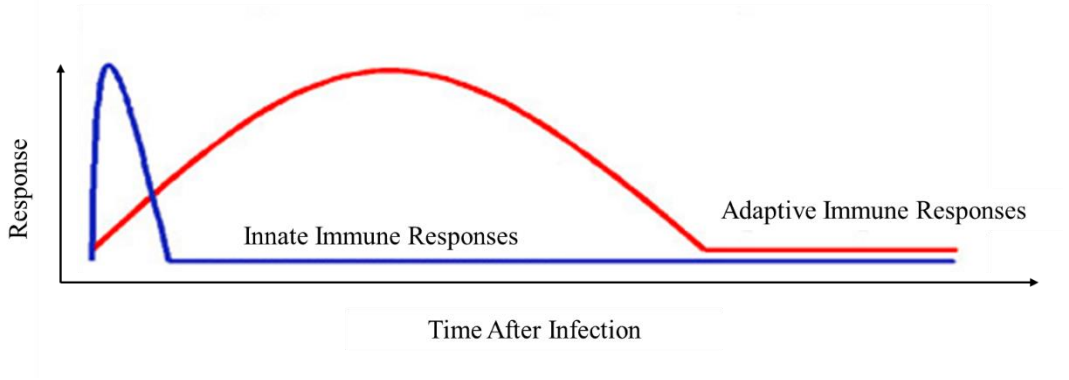

Figure 20. Immune responses after influenza virus infection

Innate immune responses are triggered immediately after influenza virus infection. The innate immune responses include activation of pro-inflammatory signaling, production of cytokines, chemokines, and interferons, expression of ISGs and recruitment of innate immune cells. It takes days to weeks for the establishment of a specific adaptive immune response.

Indeed, previous studies have demonstrated that pretreatment with a combination of aerosolized TLR2/6 and TLR9 ligands (Pam2CSK4 and CpG-ODN, respectively) could induce a high level of innate resistance against both bacteria and influenza virus infection in mice (Duggan et al., 2011; Evans et al., 2010a; Tuvim et al., 2012). Of note, Pam2CSK4 and CpG-ODN synergistically protect mice against lethal influenza A virus infection. The mechanisms are unknown, but it has been suggested that the lung epithelium rather than recruited leukocytes contributes to induced innate resistance (Evans et al., 2011). Here, we investigate the induced innate resistance against influenza A virus infection by one of the components CpG-ODN *in vitro*.

CpG oligodeoxynucleotides

CpG oligodeoxynucleotides (CpG-ODN) are short synthetic single-stranded oligodeoxynucleotides (ODN) that contain unmethylated CpG motifs (C: cytosine triphosphate deoxynucleotide; p: phosphodiester link or phosphorothioate (PS) link; G: guanine triphosphate deoxynucleotide) recognized by TLR9. CpG motifs are relatively common in the genomes of bacteria and DNA viruses. The immune-stimulatory activity of an ODN is determined by multiple characteristics. GTCGTT is the optimal CpG motif for the human while in mouse the optimal motif is GACGTT (Hartmann and Krieg, 2000; Krieg et al., 1995; Rankin et al., 2001). An optimal CpG-ODN sequence usually contains several CpG motifs (two intervening bases between each motif), TpC dinucleotide on the 5’ end, pyrimidine repeats on the 3’ side and modified PS backbone (Ballas et al., 1996;

Hartmann and Krieg, 2000; Hartmann et al., 2000; Pisetsky and Reich, 1998; Roberts et al., 2005).

Three major classes of CpG ODN have been classified based on structural and biological characteristics: Class A, Class B and Class C. Class A ODN contain a central palindromic phosphodiester region and poly-G motifs at the 5' or 3' end which can form highly ordered structures. They induce strong activation of pDCs and expression of IFNα (Hartmann et al., 2003; Kerkmann et al., 2005). Class B ODN contain a full phosphorothioate backbone and are strong stimulators of B cells. Class C ODN combine both features of Class A and Class B ODN containing a palindromic region and fully phosphorothioate backbone. They activate both pDCs and B cells and induce IFNα secretion (Marshall et al., 2003).

Previous reports showed that prior administration of inactivated avian H5N1 influenza virus with a modified CpG-ODN could protect chickens effectively (Fu et al., 2013). In addition, CpG-ODN enhances immune responses to influenza virus vaccine (Lopez et al., 2006; Mallick et al., 2011; Singh et al., 2016a; Singh et al., 2016b).

Materials and Methods

Cell lines and reagents

A549 cell line ((Adenocarcinomic human alveolar basal epithelial cells) was purchased from ATCC, and MLE15 cell line (Immortalized mouse lung epithelial cells) was kindly provided by Dr. Michael J Tuvim, University of Texas MD Anderson Cancer Center. A549 cells were maintained in F-12K medium (ATCC), whereas MLE15 cells were maintained in RPMI-1640 medium (Lonza). Both media were supplemented with 5% FBS and 100IU/ml penicillin-streptomycin. PAM2CSK4 and ODN M362 were kindly provided by Pulmotect, Inc. ODN2216, ODN2006, ODN2395 and their respective ODN controls were purchased from Invivogen. Influenza A H3N2 antibody was purchased from Takara. Influenza A H3N2/HK/8/68 was a gift from Dr. Brian Edward Gilbert, Baylor College of Medicine.

Mouse model

The experiments with influenza infection were performed at BL2 animal facility by following the protocols approved by TAMHSC Institutional Animal Care and Use Committee (IACUC). In the *in vivo* experiment, the C57BL/6 mice were pretreated with aerosolized 2 μM ODN M362 and 8 μM Pam2CSK4 for 15 min with Aerotech II nebulizer flowing at 10 L/min air from an Easy Air compressor with drying coil (PM15F, Precision Medial). After 24 hours, the mice were infected with influenza virus aerosol (DMEM with

0.05% gelatin solution) calculated to deposit approximately 119 $TCID_{50}$ per mouse for 20 min. Morbidity and mortality were monitored for approximately 2 weeks.

In vitro antiviral assays

Cells ($1x10^5$/well) were seeded into a 24-well plate 24 hours before treatment in complete growth medium. Then, the cells were pretreated with 5 μM ODN M362 for 4 hours in serum-free medium, and then H3N2 influenza virus was added (at ratio 1: 1) for another 24 hours. Real-time RT-qPCR or immunohistochemistry (IHC) were used to analyze the antiviral activities.

Real-time qPCR and semi-quantitative PCR

Total RNA was isolated from cells and supernatant with RNA isolation kit by following the instruction (OMEGA), and then the RNA was used to generate cDNA by reverse transcription (Quanta bio). Real-time qPCR was performed on Bio-rad IQ5 system by using SYBR green (Bioline) (Table 2). For semi-quantitative PCR, half of the 50 μl reaction products were electrophoresed on an agarose gel after 25 cycles, while the other half was electrophoresed after 35 cycles.

Immunofluorescence staining

A549 cells ($1x10^5$/well) were seeded on coverslips in 24-well plate. After influenza virus infection, cells were washed with PBS and fixed with 4% paraformaldehyde. The cells were permeabilized with 0.5% Triton-X100 for 10min and blocked with 1% BSA for

1 hour. The cells were incubated with H3N2 antibody at 4℃ overnight. Then the cells were incubated with anti-mouse FITC for 1 hour following DAPI staining for 5 min. Cells were washed with PBST for 3 times after each step. The coverslip was mounted on slide with Aqua Mount and analyzed by DeltaVision Elite imaging system (GE).

Cell immunohistochemistry

Cells were fixed on the coverslip in 4% paraformaldehyde for 15 min and treated with 0.3% H_2O_2 until bubbling stopped. Cells were incubated with blocking solution (Vectastain ABC kit) for 20 min. Then the cells were incubated with H3N2 antibody for 45 min. Then the cells were incubated with biotinylated secondary antibody for 30 min. The cells were then incubated with HRP-conjugated avidin-biotin complex for 30 min. Next, the substrate solution (Vector novared) was added until suitable staining developed. Then the cells were counterstained with hematoxylin (Sigma) for 2 min and washed with water until blue color developed. Cells were washed with PBST for 3 times after each step. Air dry coverslip at room temperature for 10min and mount in Aqua Mount.

ELISA

Interferon lambda secretion was measured in influenza virus-infected cell supernatants. Briefly, a 96-well microplate was coated with capture antibody at room temperature overnight. The plate was washed with PBS the next day and blocked with reagent diluent for 1 hour. Cell supernatant was collected, added to the plate and incubated for 2 hours. Then detection antibody (R&D systems) was added and incubated for 2 hours

at room temperature. Aspiration/washing was performed after each step as the instruction. Then Streptavidin-HRP was added for 20 minutes. Stop solution was added and then measure absorbance at OD450nm using a microplate reader (SpectraMax M).

CRISPR/Cas9

To generate MyD88 and STING-deficient A549 cells, CRISPR/Cas9 was performed as described (Sanjana et al., 2014; Shalem et al., 2014). Three pairs of oligonucleotides for sgRNA were designed for each gene (Table 3). Plasmids used were kindly provided by Dr. Yubin Zhou, Institutes of Bioscience and Technology, Texas A&M University. Lentiviral CRISPR plasmid was digested and dephosphorylate with BsmBl (Fermentas) for 30 min at 37℃. The Gel with digested plasmid was purified using QIAquick Gel Extraction Kit and eluted in the elution buffer. Then each pair of oligos was phosphorylated and annealed as described (Sanjana et al., 2014; Shalem et al., 2014). Annealed oligos were diluted at 1:200 dilution and ligated with digested plasmid. Then the plasmid was transformed into and amplified in Stbl3 bacteria. All three plasmids were transfected into A549 cells with Lipofectamine 2000 (Life technology). After transfection, puromycin was added for selection. Then the cells were serially diluted into 96 well plate for the single clone. After selection and expansion of single clones, the gene expression level was tested with Western blot to confirm efficiency.

Western blot

The expression of MyD88 and STING proteins in A549 cells were detected by Western blot analysis. Briefly, 40 μg of cell lysate proteins were used for each sample. Proteins were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 1%BSA in TBST for 1hr at room temperature. Primary antibody (Cell signaling) diluted (1: 1000) in 1%BSA was added and incubated at 4℃ overnight. The membranes were washed 3 times for 5min each with TBST. The HRP-secondary antibody was then added and incubated at room temperature for 1 hour, followed by detection with ECL. The image was taken by FluorChem M System (Bucher Biotec).

Cell proliferation and cytotoxicity assay

Cell counting kit-8 (Dojindo) was used to determine cell proliferation and cytotoxicity. Briefly, 5000 cells were seeded in a 96-well plate for 24 hours. After treatment, 10 μl of CCK-8 solution was added into each well of the plate and incubated for in 37℃ incubator for 1-4 hours until desired color develops. Then, measure the absorbance at 450 nm using a microplate reader (SpectraMax M).

Luciferase assay

MLE15 cells were seeded in a 48-well plate 24 hours before transfection so that the cell confluence would reach 70% to 90% on the next day. On the following day, 0.05 NF-κB luciferase reporter plasmid was delivered into the cells with Lipofectamine 2000

transfection reagent as instructed. On the third day, the cells were stimulated with Pam2CSK4, or ODN M362 for 8 hours. Then the cells were lysed and luciferase reagent (Promega) was added. The luminescence was measured by FLUOstar Optima microplate reader.

Gene	Sequence
IAV forward	GGACTGCAGCGTAGACGCTT
IAV reverse	CATCCTGTTGTATATGAGGCCCAT
human GAPDH forward	CGGATTTGGTCGTATTGGG
human GAPDH reverse	CGCTCCTGGAAGATGGTGAT
mouse GAPDH forward	GTTGTCTCCTGCGACTTCA
mouse GAPDH reverse	GGTGGTCCAGGGTTTCTTA

Table 2. Real-time qPCR primer

Gene	Sequence
MyD88 1F	CACCGGTTCTTGAACGTGCGGACAC
MyD88 1R	AAACGTGTCCGCACGTTCAAGAACC
MyD88 2F	CACCGCTGCTCTCAACATGCGAGTG
MyD88 2R	AAACCACTCGCATGTTGAGAGCAGC
MyD88 3F	CACCGCTCGAGCAGTCGGCCTACAG
MyD88 3R	AAACCTGTAGGCCGACTGCTCGAGC
STING 1F	CACCGAGAGCACACTCTCCGGTACC
STING 1R	AAACGGTACCGGAGAGTGTGCTCTC
STING 2F	CACCGAAGGGCGGGCCGACCGCATT
STING 2R	AAACAATGCGGTCGGCCCGCCCTTC
STING 3F	CACCGAGGGCGGGCCGACCGCATTT
STING 3R	AAACAAATGCGGTCGGCCCGCCCTC

Table 3. CRISPR/Cas9 sgRNA oligo sequences

Results

CpG-ODN and Pam2CSK4 protect mice against lethal influenza A virus infection

Laboratory mouse strain C57BL/6 is highly susceptible to Influenza A virus (IAV) H3N2/HK/8/68 infections. The IAV pathogenicity and severity of IAV-induced disease depend on the challenge dose administered. Mice receiving a lethal dose of aerosolized influenza virus develop severe symptoms in several days characterized by lethargy, anorexia, and huddling. The infection results in massive weight loss and ultimately death. In the lethal influenza infection mouse model, pre-treatment with the combination of aerosolized TLR9 agonist (ODN M362) and TLR2/6 agonist (Pam2CSK4) protected mice from death (Figure 21A), while the single component alone resulted in no protection (Tuvim et al., 2012). Besides, weight loss in mice pretreated with ODN and Pam2 was significantly less than that of untreated mice (Figure 21B). Similar studies have been done in bacteria-induced pneumonia mouse model. Pretreatment with ODN and Pam2 in the lungs induced resistance to pneumonia associated with rapid pathogen killing. (Duggan et al., 2011; Evans et al., 2010a; Evans et al., 2011; Evans et al., 2010b; Tuvim et al., 2009). In these models, serial reductions in leukocyte lineages by antibodies or toxins did not impair the inducible protection while disruption of TLR signaling in the lung epithelium abrogated the resistance (Cleaver et al., 2014). Given the fact that influenza virus mainly targets the respiratory epithelium, it is possible that TLR agonists ODN and Pam2 may directly reduce the pathogenicity of influenza virus in lung epithelial cells.

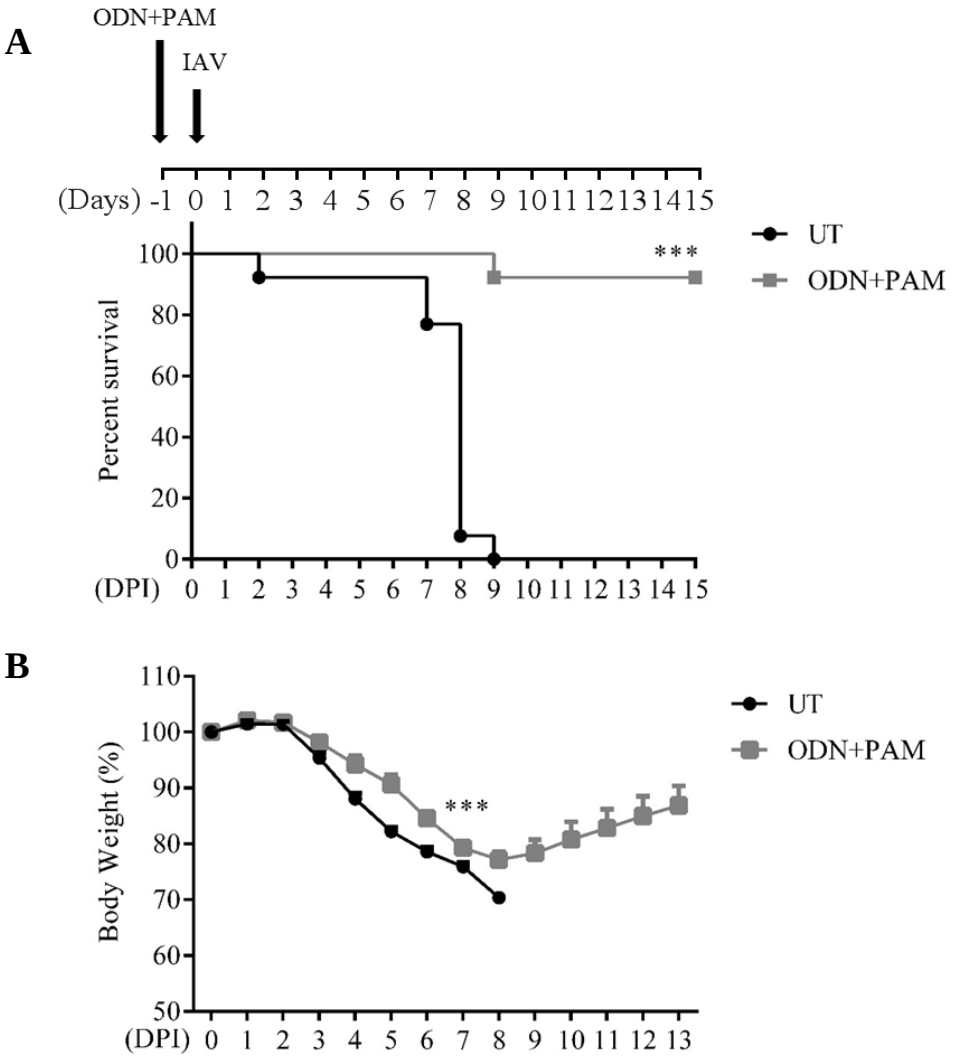

Figure 21. CpG-ODN and PAM2CSK4 synergistically protect mice from infection with lethal dose of flu virus

C57BL/6 mice were inhaled with aerosolized influenza virus (119 TCID50 per mouse) 24 hours after pretreatment with aerosolized ODN M362 and Pam2CSK4 (concentration). (A) Survival proportions of untreated and pretreated mice (log-rank test, n=15/group) and (B) Body weight change (multiple *t*-test, n=15/group) were monitored daily. All data represent mean ± SEM; ***, $p<0.001$.

Lung epithelial cells play a critical role in CpG-ODN/Pam2CSK4-induced protection against influenza virus infection and CpG-ODN is the major component

To investigate if CpG-ODN and PAM2CSK4 could exert a protective effect in alveolar epithelial cells, we established an *in vitro* model to mimic the *in vivo* studies utilizing human alveolar basal epithelial cell line A549 and mouse immortalized lung epithelial cell line MLE. Both of these two cell lines express TLR2 and TLR9 (Figure 22A, B and data not shown). Cells were pretreated with the combination of CpG-ODN and Pam2CSK4 ("ODN-Pam2") for 4 hours and then infected with influenza virus for an additional 24 hours (Figure 23A). As expected, ODN-Pam2 induced an antiviral effect in both cell lines, in which both virus particles (Figure 23B) and virus RNA level (Figure 23C) were significantly reduced. Surprisingly, CpG-ODN appeared to be the major player in the protective effect *in vitro* while Pam2CSK4 played a very limited role (Figure 23B, C), although Pam2CSK4 induced a much stronger NF-κB signaling compared with CpG-ODN M362 in MLE15 cells (Figure 24), as well as cytokine/chemokine expression (Tuvim et al., 2012). Given the synergistic protective effect by CpG-ODN and Pam2CSK4, it is possible that CpG-ODN induced antiviral activity in the lung epithelial cells might be one of the mechanisms of the protection effect *in vivo*.

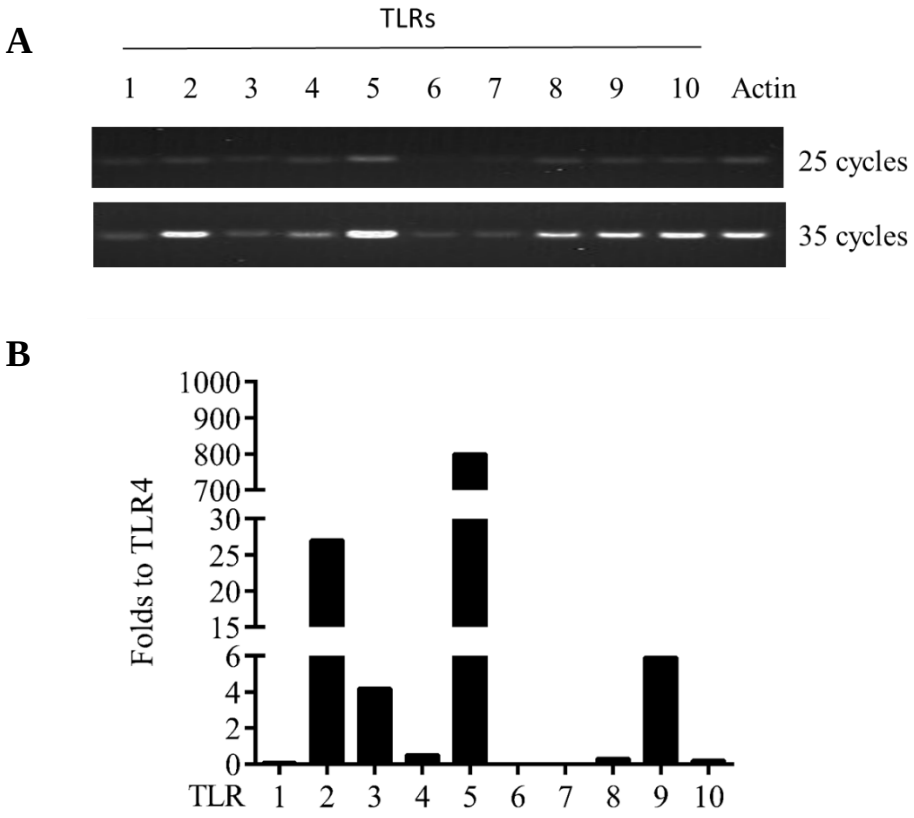

Figure 22. TLR expression in human lung epithelial A549 cells

(A) Semi-quantitative RT-PCR was used to determine TLR1-10 expression in A549 cells. PCR products from PCR 25 cycles and 35 cycles were electrophoresed on agarose gel. (B) Real-time RT-qPCR of TLR1-10 expression in A549 cells.

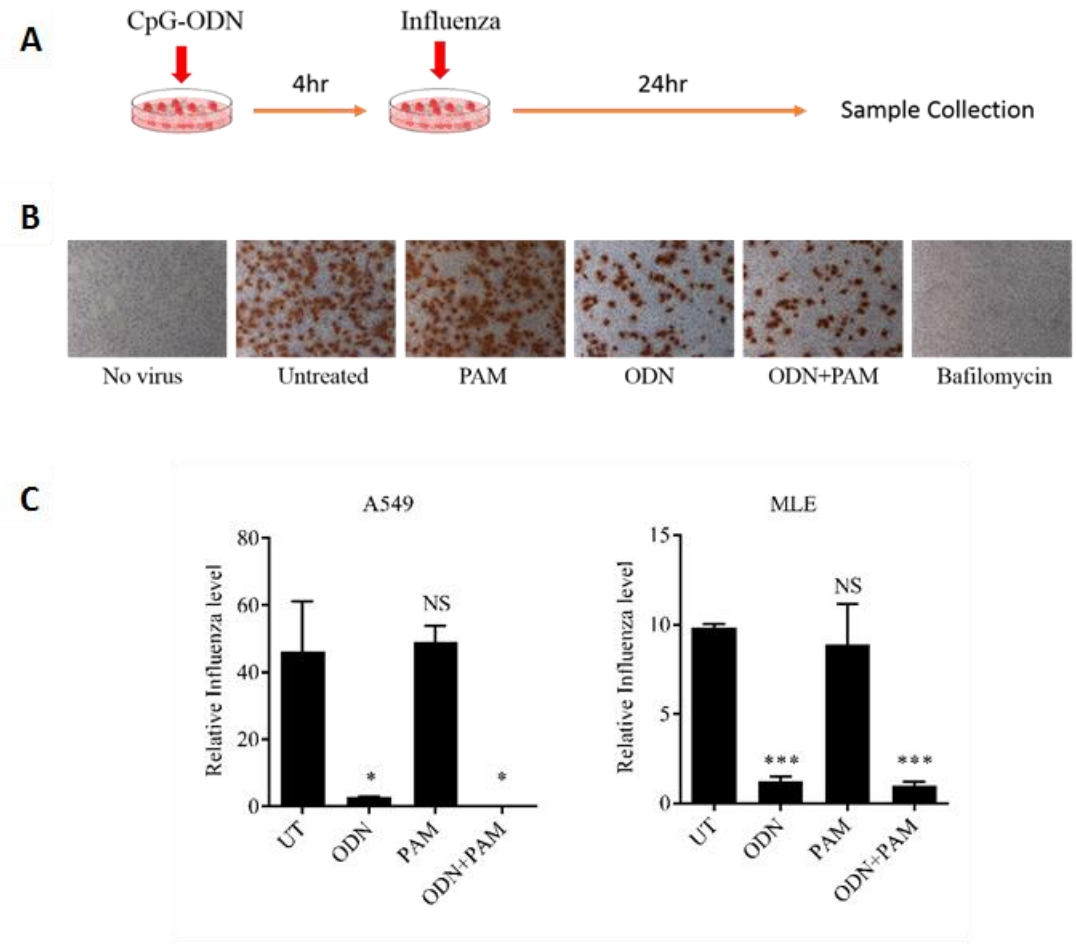

Figure 23. Anti-influenza activity by CpG-ODN and PAM2CSK4 in respiratory epithelial cells

(A) Schematic diagram of *in vitro* antiviral assays. Cells were pretreated with indicated TLR agonists for 4 hours and infected with influenza virus for additional 24 hours. Cells were then fixed for IHC or lysed for RNA isolation. (B) Representative images of IHC of influenza infected cells with indicated pretreatment. Bafilomycin was shown to inhibit lysosome acidification, thus used as positive control to inhibit influenza replication. (C) Real-time PCR was performed to determine influenza RNA level in A549 and MLE cells with indicated pretreatment. All data represent mean ± SEM. *p<0.05, ***p<0.001. Unpaired *t*-test was used.

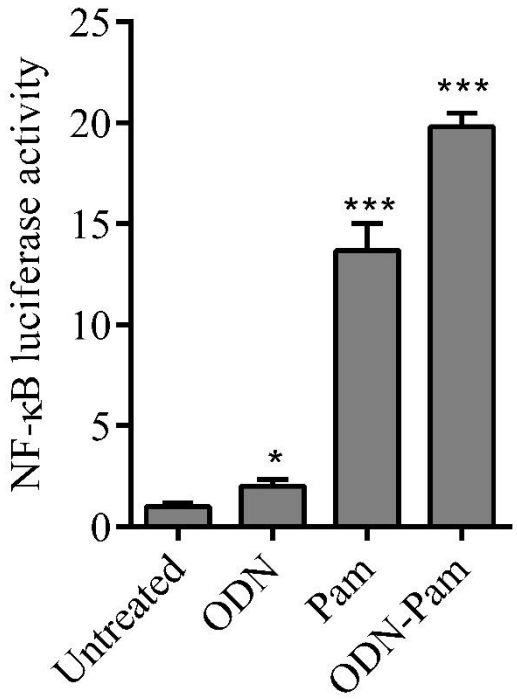

Figure 24. NF-κB activation induced by CpG-ODN and Pam2CSK4 in MLE15 cells

MLE15 cells were transfected with NF-κB reporter plasmid and analyzed for luciferase activity with indicated stimulation. All data represent mean ± SEM. *p<0.05, ***p<0.001. Unpaired *t*-test was used.

CpG-ODN-induced protection against influenza virus infection in lung epithelial cells

We first used class A, B and C CpG-ODN to investigate whether they could induce antiviral activity in A549 cells (Figure 25A). Class B CpG-ODN ODN2006, Class C CpG-ODN ODN2395, and ODN M362 exhibited strong antiviral activity against influenza virus infection, whereas class A CpG-ODN ODN2216 was less effective (Figure 25B). Class A CpG-ODNs strongly activate pDCs and induce high amount of IFN-α production, but are weak stimulators of TLR9 dependent NF-κB signaling (Krug et al., 2001). Class B CpG-ODNs strongly activate B cells but stimulate very small amounts of IFN-α secretion (Krieg et al., 1995). Class C CpG-ODNs combine the features of both Class A and B CpG-ODNs. A previous report showed that a class C CpG-ODN in combination with Pam2CSK, provided the most efficient protection against lethal influenza infection *in vivo* (Tuvim et al., 2012). Our observation in the antiviral assays here is consistent with previous result.

To confirm the antiviral effect by class C CpG-ODN, the influenza virus RNA was evaluated by qRT-PCR. A reduction of 85% to 95% in influenza virus RNA was observed in ODN M362 pretreated cells (Figure 26A and B). Immunohistochemistry and immunofluorescence staining of influenza virus protein showed that fewer cells were infected after pretreatment with ODN M362 (Figure 25B and Figure 26C). Taken together, these data reveal a protective effect against influenza virus infection by CpG-ODN in lung epithelial cell lines.

A

ODN 2216 (class A): 5'- ggGGGACGA:TCGTCgggggg-3' (20 mer)

ODN 2006 (class B): 5'- tcgtcgttttgtcgttttgtcgtt-3' (24 mer)

ODN 2395 (class C): 5'- tcgtcgttttcggcgc:gcgccg-3' (22 mer)

ODN M362 (class C): 5'-tcgtcgtcgttc:gaacgacgttgat-3' (25 mer)

B

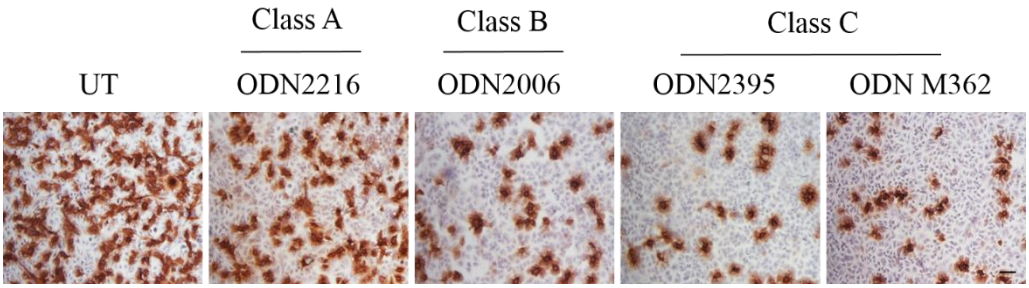

Figure 25. CpG-ODN-induced protection against influenza virus infection in lung epithelial cells

(A) CpG-ODNs were used in antiviral assays. (B) Representative IHC images of influenza virus-infected cells with indicated CpG-ODN pretreatment. Brown color indicates infected cells. Scale bar, 10μm.

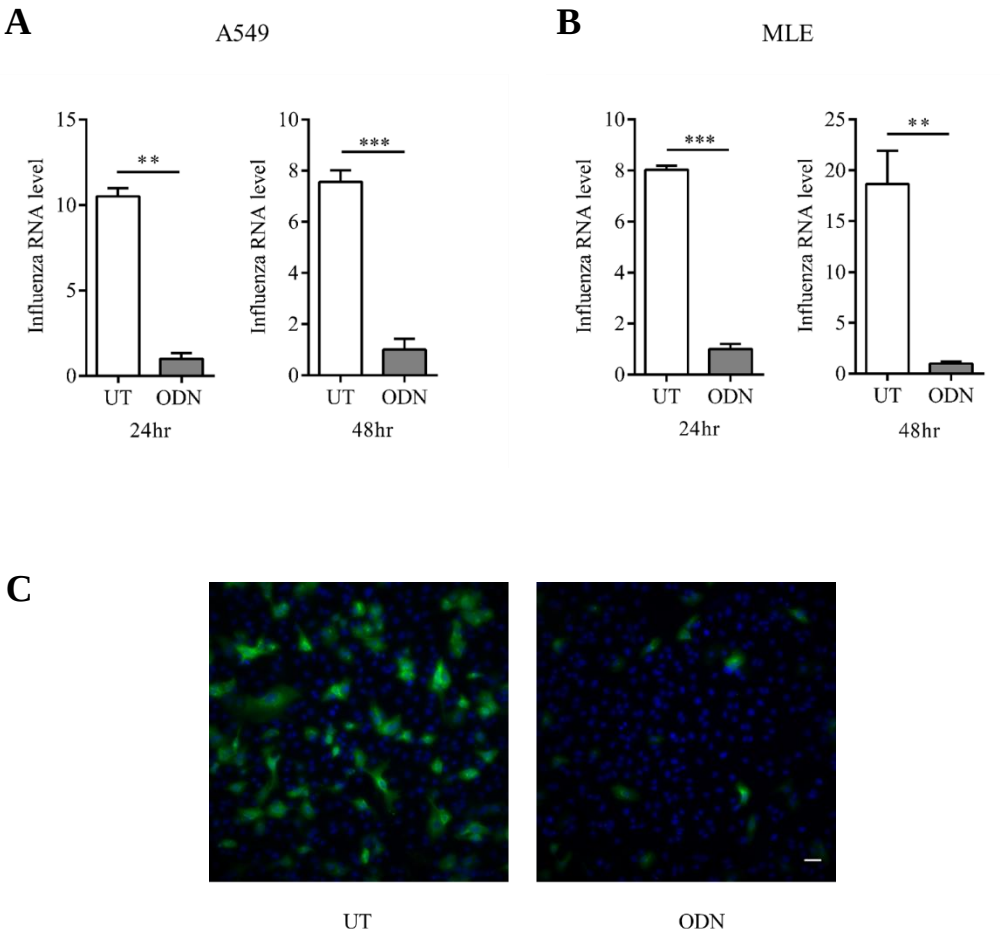

Figure 26. Protection with class C ODN M362 against influenza virus infection in A549 cells

Influenza RNA level in untreated and CpG-ODN pretreated (A) A549 cells and (B) MLE cells after influenza infection for 24 hours and 48 hours. All data represent mean ± SEM. **p<0.01, ***p<0.001. Unpaired *t*-test was used. (C) Representative images of untreated and CpG-ODN pretreated A549 cells after influenza infection for 24 hours. Scale bar, 10 μm.

ODN M362 pretreatment decreased IAV-induced type III interferon secretion in lung epithelial cells

Type I and type III interferons are activated rapidly during influenza virus infections (Killip et al., 2015; Lazear et al., 2015). The interferons upregulate hundreds of interferon-stimulated genes that efficiently inhibit virus replication and spread, thus restrict the early stage of influenza infections before the activation of adaptive immune responses (Iwasaki and Pillai, 2014). We detected limited upregulation of type I interferon after influenza infection in A549 cells (data not shown). However, type III interferon expression was robustly regulated (Figure 27A and B). Type III IFN is the predominant IFN produced by the epithelium and is only produced in infected cells (Crotta et al., 2013; Jewell et al., 2010; Okabayashi et al., 2011; Sommereyns et al., 2008). We found that IFN lambda expression was significantly less in CpG-ODN M362 (hence "CpG-ODN") pretreated cells after influenza virus infection at both protein level and mRNA level (Figure 27A and B). Since CpG-ODN is not sufficient to induce IFN lambda expression (Ioannidis et al., 2013), the results suggested that antiviral effect by CpG-ODN and the related antiviral mechanism might occur before interferon secretion.

Cell viability is not affected by CpG-ODN pretreatment

To investigate if the observed protective effect is due to decreased cell viability possibly induced by CpG-ODN pretreatment, we evaluated A549 cell viability after CpG-ODN stimulation. Although it was reported that CpG-ODN induced apoptosis in other cell types (Arunkumar et al., 2013; Zhang et al., 2014), we found no significant difference

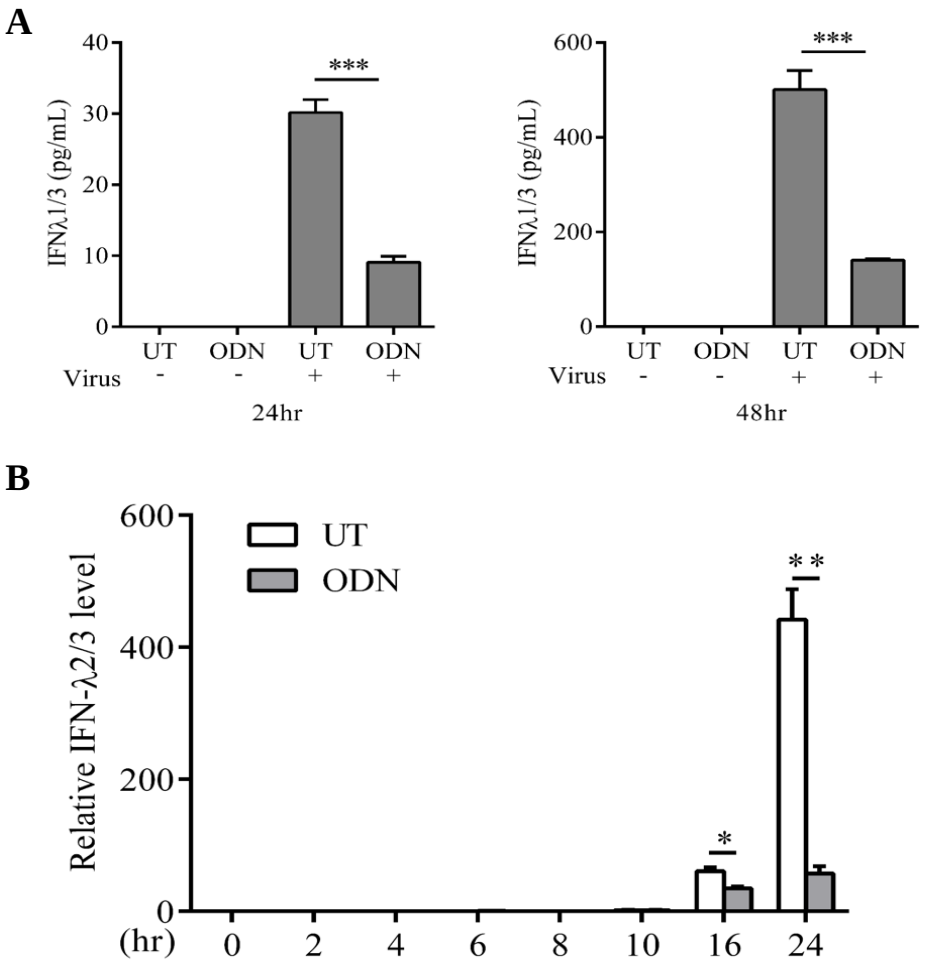

Figure 27. CpG-ODN pretreatment decreased IAV induced IFN-λ expression in A549 cells

(A) IFN lambda level in the supernatant of untreated and CpG-ODN pretreated A549 cells with or without influenza infection, as detected by ELISA. (B) IFN lambda mRNA level in untreated and CpG-ODN pretreated A549 cells after influenza infection, as detected by real-time RT-qPCR. Time point indicates infection time. All data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Unpaired *t*-test was used.

between untreated and CpG-ODN-treated cells (Figure 28). Thus, the CpG-ODN induced protection against influenza virus infection *in vitro* is an early innate immune response irrelevant with cell viability.

CpG-ODN induced antiviral effect is independent of TLR9-MyD88 signaling

Since TLR9 can be functionally activated in A549 cells, we next wondered whether CpG-ODN-activated TLR9 signaling was responsible for the protective effect against influenza virus infection. Activation of TLR9 downstream signaling requires the key adaptor protein MyD88 (Akira and Takeda, 2004; Warner and Nunez, 2013). And MyD88 is indispensable for immune protection during influenza A virus infection *in vivo* (Seo et al., 2010). We first knocked down MyD88 in A549 cells by siRNA to evaluate if MyD88 was essential in CpG-ODN induced protection. Surprisingly, in MyD88 knockdown A549 cells, CpG-ODN still elicited protection against influenza virus infection at a comparable level to control cells (Figure 29A, B and C). Fewer cells were infected of both control and MyD88 knockdown cells after CpG-ODN pretreatment, and also there was no significant difference in influenza RNA level.

To further confirm this result to rule out the possibility that the observation is due to incomplete deletion of the functional MyD88 gene, we then generated MyD88 knockout A549 cells by CRISPR/Cas9 techniques to determine if MyD88 was not required in CpG-ODN induced protection. With this method, MyD88 protein level was completely diminished in knockout cells (Figure 30B). Consistent with MyD88 knockdown results, CpG-ODN induced antiviral effect was not impaired in MyD88 knockout cells

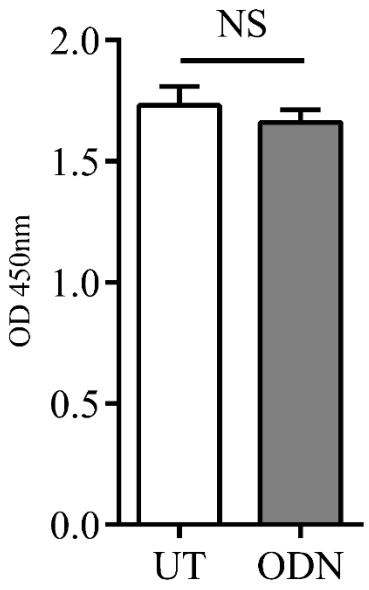

Figure 28. CpG-ODN treatment does not affect A549 cell viability

A549 cell viability was measured 24 hours after CpG-ODN treatment, as detected by CCK-8 kit. NS, non-significant.

(Figure 30A). Taken together, we concluded that CpG-ODN induced protective effect in lung epithelial cell lines is independent of TLR9-MyD88 signaling.

Non-TLR9 stimulatory GpC-ODN also confer antiviral effect

TLR9 recognizes CpG motifs of ODN (Hemmi et al., 2000). To ask whether CpG-ODN induced protection is characterized by TLR9 recognition, we used non-TLR9 stimulatory GpC-ODN instead of CpG-ODN in the antiviral assays. The GpC-ODN contains GpC dinucleotides instead of CpGs and can be used as negative controls with their respective CpG-ODN. Notably, all GpC-ODN showed comparable antiviral effect with their CpG-ODN (Figure 31A, B and Figure 25B). We found that Class B and C GpC-ODN induced stronger protection than Class A GpC-ODN, which was consistent with their respective CpG-ODN. These results showed that CpG-ODN induced protection did not go through TLR9 or its downstream signaling.

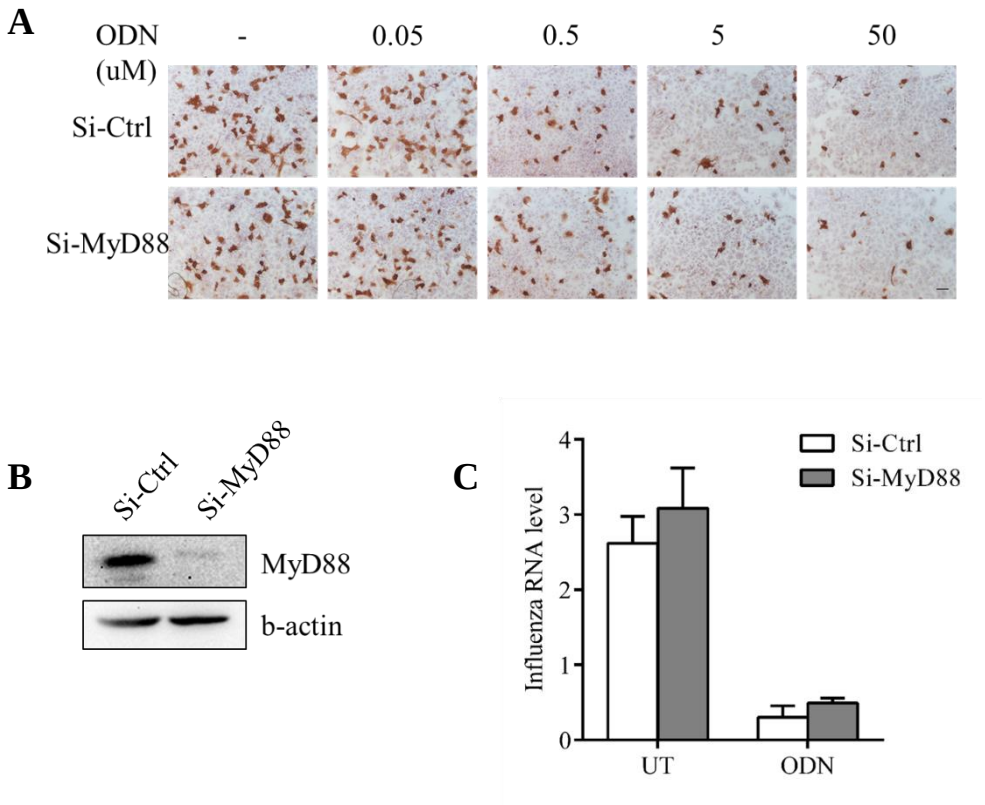

Figure 29. Protection by CpG-ODN from influenza infection in MyD88 knockdown A549 cells

A549 cells were transfected with control siRNA or MyD88 siRNA. (A) Representative images of antiviral activity after CpG-ODN pretreatment in control or MyD88 knockdown cells. Different concentrations of ODN were used. (B) Knockdown efficiency determined by Western blot. (C) Influenza RNA level in control or MyD88 knockdown cells were detected by real-time RT-qPCR.

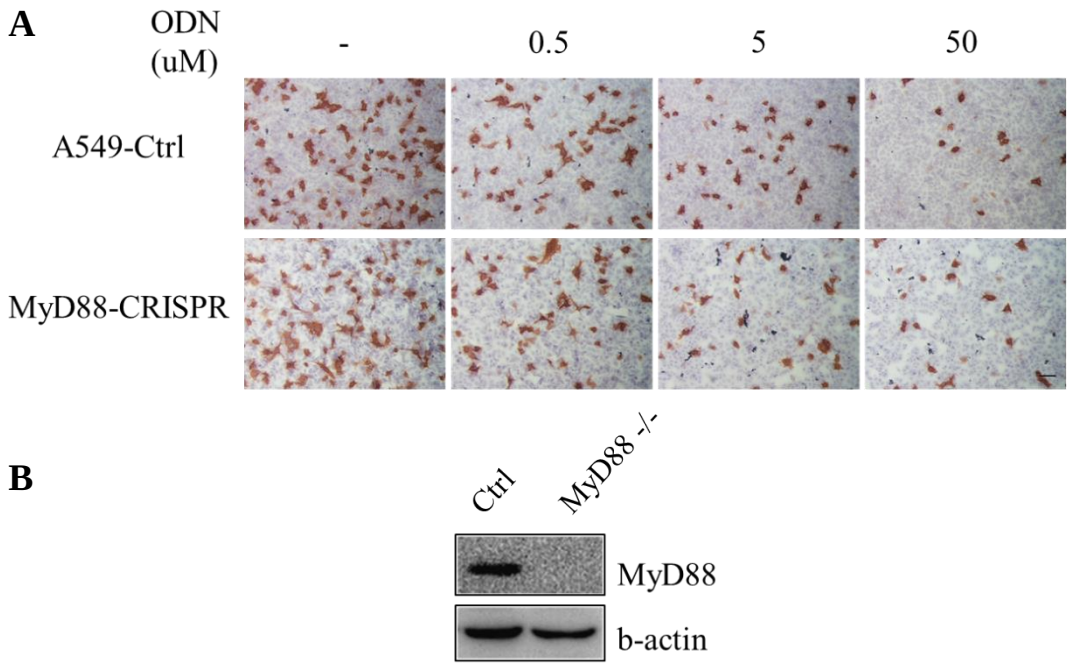

Figure 30. Protection by CpG-ODN in MyD88 knockout A549 cells. MyD88 deficient cells were generated by CRISPR/Cas9 technique

(A) Representative images of antiviral activity after CpG-ODN pretreatment in control and MyD88 knockout cells. (B) Protein level in control and MyD88 knockout cells were detected by Western blot.

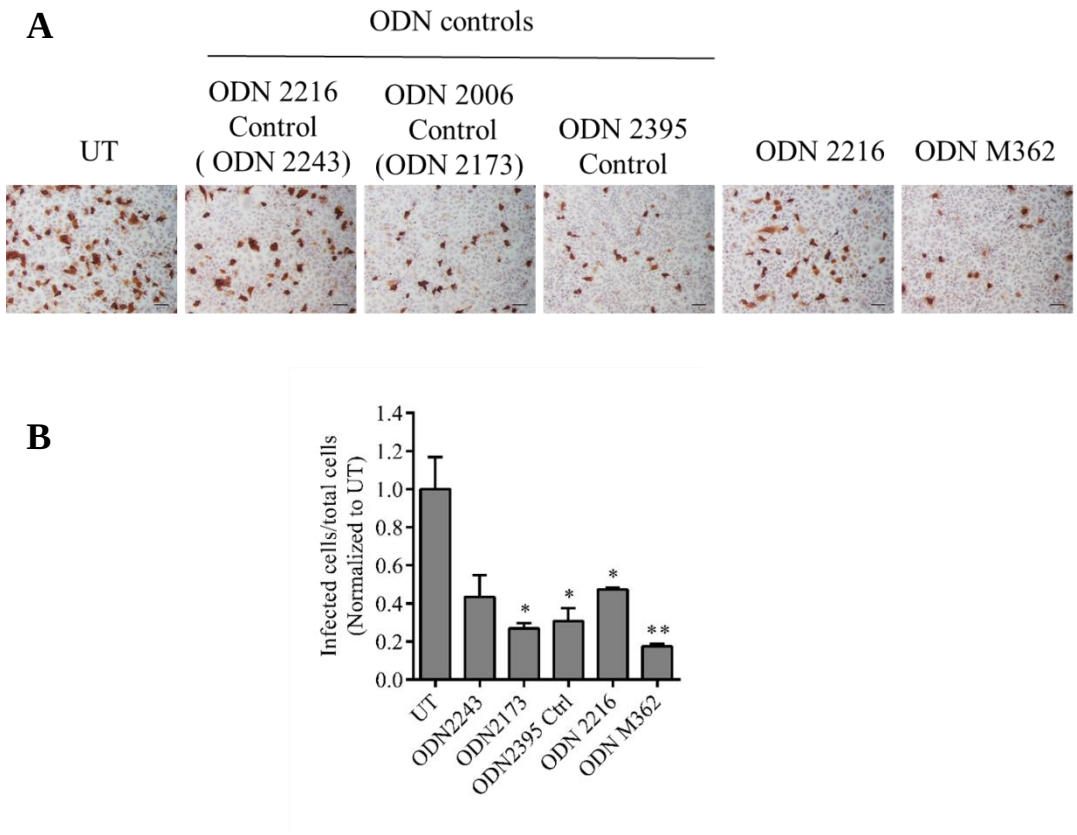

Figure 31. Non-TLR9 stimulatory GpC-ODNs also protect cells from influenza virus infection

(A) Representative IHC images of antiviral activity after ODN or ODN controls pretreatment. (B) Quantification of virus-infected cells/total cells in (A). All data represent mean ± SEM. *p<0.05, **p<0.01. Unpaired *t*-test was used.

TBK1, DDX58 and STING are not responsible for CpG-ODN-induced antiviral effect in lung epithelial cells

Next, we sought to investigate if other intracellular DNA sensors are responsible for CpG-ODN recognition and induced protection. It has been reported that DDX58 (also known as retinoic acid-induced gene I (RIG-I)), DAI (DNA-dependent activator of IFN-regulatory factors) and STING (Stimulator of interferon genes) could also detect intracellular DNA and activate immune responsive signaling (Ablasser et al., 2009; Diner et al., 2013; Sun et al., 2013; Takaoka et al., 2007). DDX58 induces type I interferon production after cytosolic DNA detection by RNA polymerase III. DAI senses cytoplasmic DNA and recruits IRF3 and TBK1, leading to interferon induction. Cyclic GMP-AMP senses cytosolic DNA and activates its adaptor protein STING to trigger antiviral immune responses. We generated DDX58, and TBK1 (the downstream effector of DAI) knockdown cells, and evaluated the antiviral effect of CpG-ODN pretreatment. We found no difference in the antiviral activity of control cells and knockdown cells. CpG-ODN protected both knockdown cells from influenza infection (Figure 32A). In addition, STING-deficient cells showed no impairment in CpG-ODN induced antiviral activity (Figure 32B and C). As a result, CpG-ODN induced antiviral effect might not go through canonical DNA sensing pathways. Notably, these cytosolic DNA sensors mainly triggers type I interferon induction, while CpG-ODN induced antiviral mechanism appears to be interferon-independent.

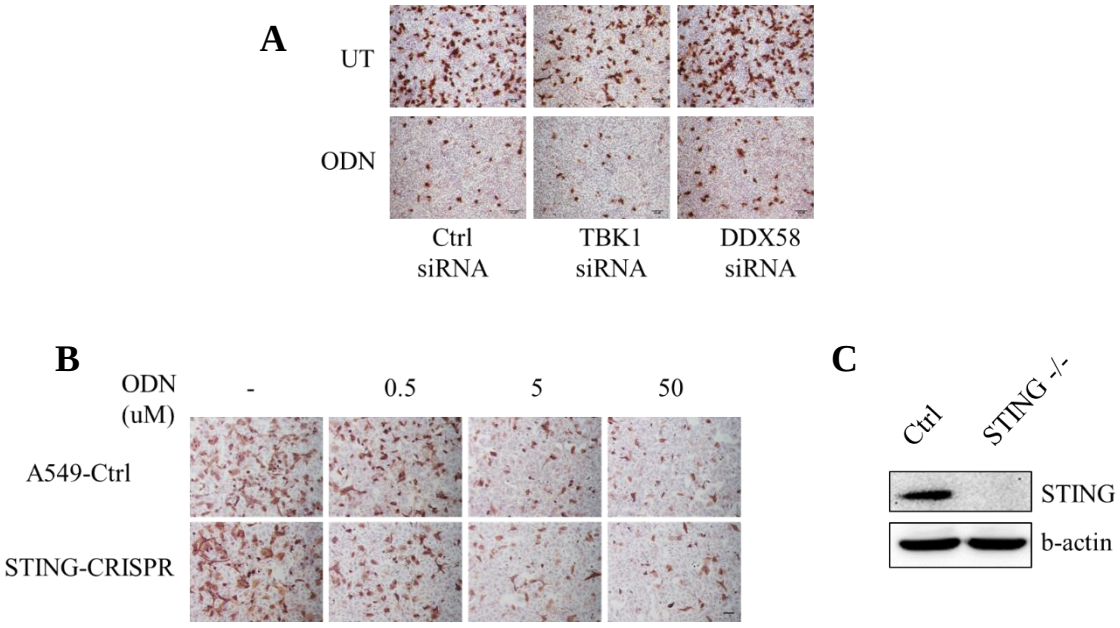

Figure 32. TBK1, DDX58, and STING are not responsible for ODN-induced anti-IAV activity

(A) Representative IHC images of antiviral activity in CpG-ODN pretreated control, TBK1 knockdown or DDX58 knockdown A549 cells. (B) STING-deficient A549 cells were generated by CRISPR/Cas9. Representative IHC images of antiviral activity in CpG-ODN-pretreated control cells or STING deficient cells. (C) Protein level in control and STING-deficient cells were detected by Western blot.

CpG-ODN induced antiviral effect is NF-κB and STAT1 signaling independent

Our results so far indicated that a non-canonical antiviral mechanism elicited by CpG-ODN, resulting in less cell infection and reduced viral particles. We wondered whether two most important antiviral pathways: NF-κB signaling and STAT1 signaling are dispensable for this phenomenon. Interestingly, knockdown of RELA (p65) in NF-κB signaling cascades, a key component for nuclear translocation and activation showed no impairment in CpG-ODN induced protection against influenza virus infection (Figure 33A and B). Knockdown of STAT1 did not affect CpG-ODN-induced protection against IAV either. (Figure 33C and D). These data further suggested that an unpredicted mechanism plays a role in CpG-ODN induced protection of lung epithelial cells from influenza virus infection. The activation of NF-κB activation after CpG-ODN stimulation and induction of type III interferon after influenza virus infection may be one of the explanations for the CpG-ODN-Pam2CSK4 induced protection *in vivo*, but unlikely to be the mechanism for the antiviral effect we observed *in vitro*. Given the data that fewer cells were infected in pretreated cells, CpG-ODN is likely to prevent infection events rather than eliminate the virus. ODN designed to target either influenza polymerase or translation complex has been reported to inhibit influenza virus replication (Kwok et al., 2009; Rodriguez et al., 2016). However, CpG-ODN were designed for immunostimulatory effect rather than targeting influenza or translational component. Its effect on the different stages of viral infection is unknown.

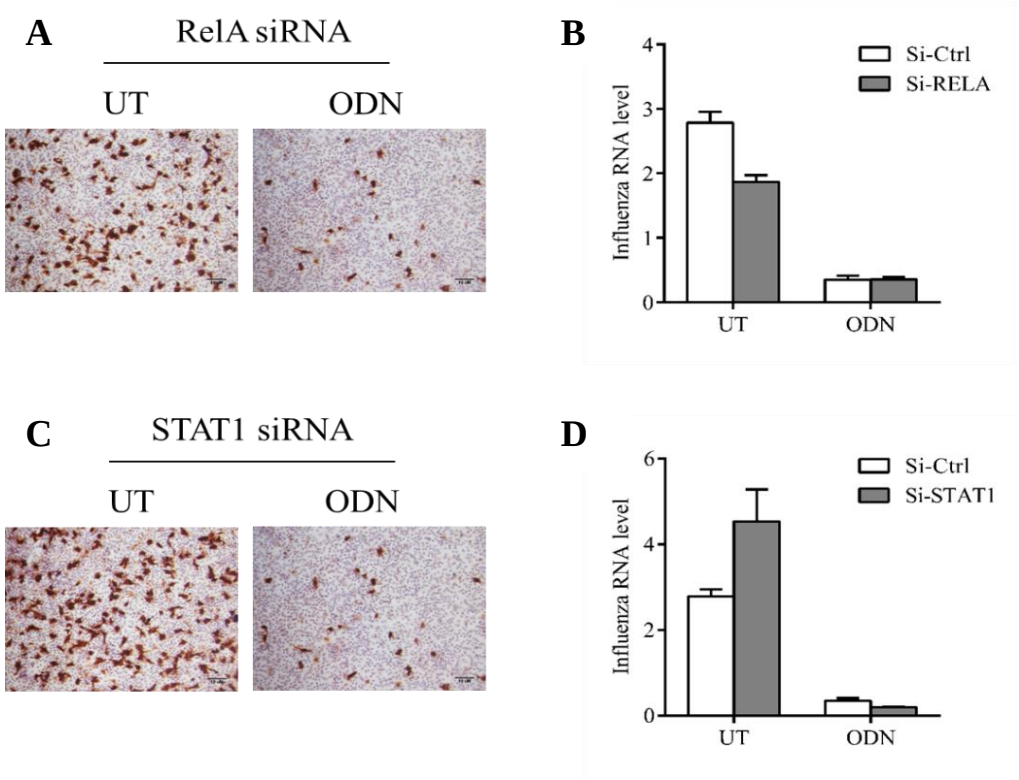

Figure 33. CpG-ODN-induced anti-IAV activity does not go through NF-kB or STAT1 signaling

(A) and (B) A549 cells were transfected with control or RelA siRNA and CpG-ODN induced antiviral activity was detected by (A) IHC or (B) real-time RT-qPCR. (C) and (D) A549 cells were transfected with control or STAT1 siRNA and CpG-ODN induced antiviral activity was detected by (C) IHC or (D) real-time RT-qPCR.

CpG-ODN-induced antiviral activity in lung epithelial cells occurs at early stage

The life cycle of the influenza A virus from entering a cell to exiting a cell is a rapid the process involving multiple steps. To further characterize the infection process, we performed the time-lapse infection experiment. The viral particles started to increase markedly from 6 hours after infection (Figure 34A and B). The data were consistent with the previous report (Kamps et al., 2006). Comparing with untreated group, fewer cells in CpG-ODN pretreated group were infected at all-time points from 6 hours after infection (Figure 34A). The influenza virus RNA level in CpG-ODN pretreated group was also dramatically reduced than untreated group starting from 6 hours after infection (Figure 34B). These data indicated that CpG-ODN induced antiviral effect occurred within the first life cycle of influenza virus replication, perhaps much earlier than the typical antiviral immune responses.

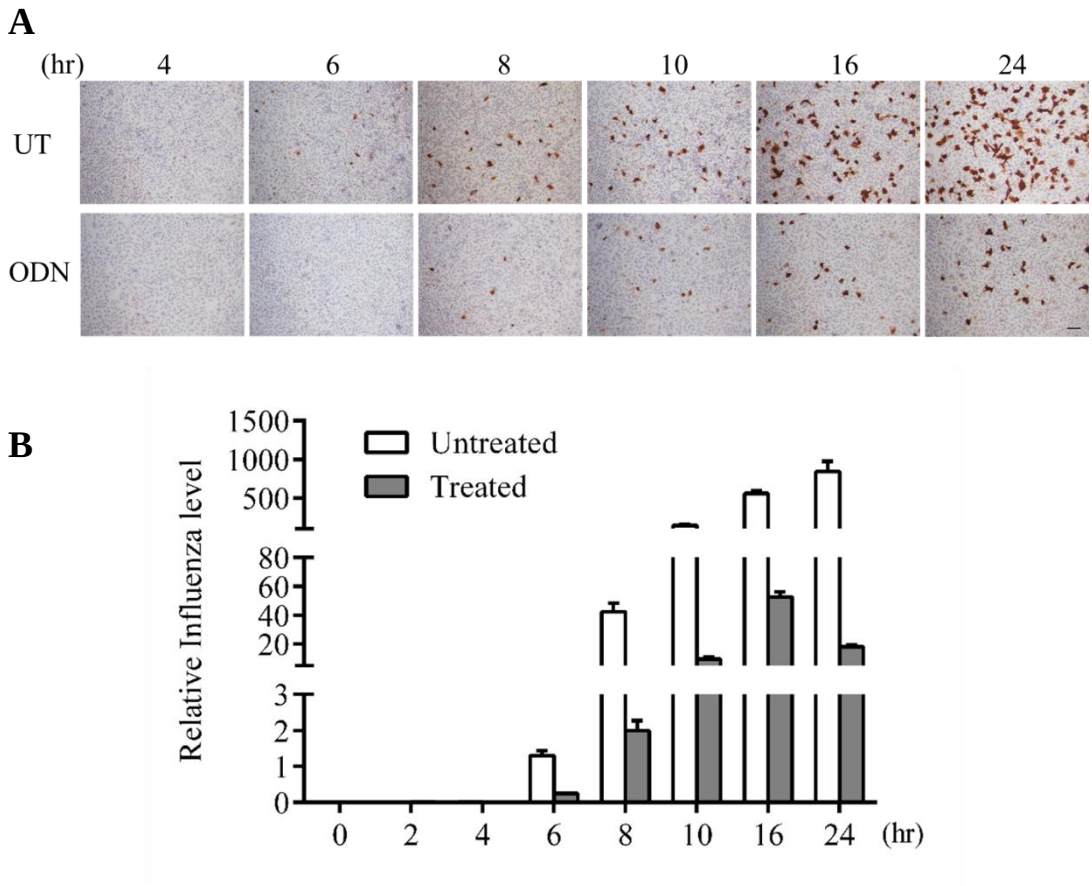

Figure 34. Time-lapse influenza virus infection in A549 cells

A549 cells were pretreated with CpG-ODN for 4hr and then infected with influenza virus for indicated time period. Antiviral activity was evaluated by (A) IHC and (B) real-time RT-qPCR. Time point indicates infection time.

Discussion

During the last few decades, boosting immune responses to induce protection against infection has been a central point of many studies. As one of the potent immunostimulatory agents, bacterial lysates have been introduced to evaluate the capacity of protection against infectious damages. In fact, immunization with bacterial lysates seem to be effective against microbial infections, especially in respiratory system infections (Evans et al., 2010a; Kikuchi et al., 2014; Mak et al., 1983; Spencer et al., 1977; Van Daal et al., 1991; van Daal et al., 1990; van Daal et al., 1992). Bacterial lysates are a mixture of bacterial antigens derived from one or more species. These antigens are recognized by PRRs and stimulate innate immune responses. The protection was associated with activation of innate immune cells, expression of inflammatory cytokines and chemokines, interferons and rapid pathogen killing. The rationale is further expanded by investigating the protective effect of the different combination of TLR agonists. We and multiple groups demonstrate that pretreatment with a combination of Pam2CSK4 (TLR2/6) and CpG-ODN (TLR9) could induce a high level of resistance to lung infection by bacteria, influenza and fungi (Duggan et al., 2011; Tuvim et al., 2012).

Similar innate immune responses are observed with other combinations, however, no dramatic protective effect is comparable with that of Pam2CSK4 and CpG-ODN. The mechanisms are not fully elucidated. It is suggested that Pam2CSK4 and CpG-ODN induced innate resistance to bacteria pneumonia appears to be mainly mediated by activated epithelial cells rather than recruited immune cells (Cleaver et al., 2014; Evans et

al., 2011; Evans et al., 2010b). Our studies show that pretreatment with Pam2CSK4 and CpG-ODN indeed confers an inhibitory effect on influenza virus infection in lung epithelial cells. Surprisingly, the inhibition is mainly induced by CpG-ODN, while Pam2CSK4 almost shows no effect. On the other hand, Pam2CSK4 induces higher activation of NF-κB signaling than CpG-ODN. Since single component could not induce innate resistance *in vivo*, it suggests that Pam2CSK4 induced signaling might work in an unknown mechanism synergistically with CpG-ODN. We further investigated the antiviral effect with different classes of CpG-ODN. Class B and C ODN induced comparable significant antiviral effect, while Class A ODN exhibited limited antiviral activity.

TLR9 is functionally expressed in lung epithelial cells and activated by CpG-ODN (Droemann et al., 2005). Unexpectedly, CpG-ODN induced the antiviral effect in A549 cells appears to be TLR9-MyD88 signaling independent. GpC-ODN controls exhibited similar resistance against influenza virus infection as CpG-ODN. GpC motifs do not stimulate TLR9 signaling and can be used as a negative control. Moreover, MyD88 deficiency did not compromise CpG-ODN induced antiviral effect. Of note, we only found expression of type III IFN, but barely type I IFN. Type III IFN expression was significantly decreased in CpG-ODN pretreated cells. Since type III IFN is produced only in infected cells, it suggests that fewer cells are infected after CpG-ODN pretreatment. The IHC images also showed that CpG-ODN pretreatment significantly decreased the number of infected cells.

We checked other intracellular DNA sensors including STING, DDX58, TBK1 and DNA-PKcs, and none of them was indispensable for CpG-ODN induced antiviral activity in lung epithelial cells. Furthermore, neither of pro-inflammatory NF-κB signaling or ISG related STAT1 signaling is involved in CpG-ODN induced antiviral effect, suggesting that it is not mediated by late produced cytokines or interferons. Indeed, by characterizing the dynamics of antiviral effect in a time-lapse fashion, we found that CpG-ODN induced antiviral effect occurred within several hours after infection. Considering that the life cycle of influenza virus is only several hours, CpG-ODN seems to function very quickly.

Currently, we are investigating the mechanism of how CpG-ODN contribute to the induced resistance to influenza infection in lung epithelial cells. Our observation from *in vitro* antiviral assay suggests that CpG-ODN pretreatment is more likely to prevent influenza virus from infecting cells or inhibit influenza virus replication rather than directly kill the virus. It is reasonable to assume that any step of the influenza viral life cycle could be inhibited. One possibility is that CpG-ODN pretreatment stimulates a rapid intracellular response to inhibit influenza virus replication. This process may involve upregulation of specific antiviral effectors. Another possibility is that CpG-ODN may directly interfere with influenza viral replication. Thus, it is important to investigate which step of influenza viral life cycle (binding, entry, uncoating, genome releasing and replication, transcription, translation, assembly, and egress) is inhibited. This CpG-ODN induced antiviral effect observed in vitro may contribute to Pam2CSK4 and CpG-ODN induced protection against influenza infection *in vivo*.

Our study shows that Pam2CSK4 and CpG-ODN induce a high innate resistance to a lethal dose of influenza virus infection in mice. Our study also shows that CpG-ODN inhibits influenza virus infection in lung epithelial cells, which is independent of TLR9-MyD88 signaling. Our work provides implications for the therapeutic use of TLR agonist to boost innate resistance against infections.

CHAPTER IV

CONCLUSIONS

In the first part, we characterized an autophagy related-protein MAP1S in the regulation of TLR signaling. In macrophages, we found that MAP1S deficiency resulted in impaired cytokine secretion after activation of cell surface TLRs, but not endosomal TLRs. We also found that MAP1S deficiency decreased the efficiency of phagocytosis of bacteria in macrophages. Interestingly, MAP1S was shown to directly interact with a critical adaptor protein, MyD88. More intriguingly, we found that MAP1S appeared to affect p38 MAPK signaling, but not NF-kB signaling. Also, after TLR stimulation in macrophages, MAP1S facilitated MyD88 recruitment to autophagy protein LC3 associated structures. These data suggest a previously unrecognized aspect in the connection between autophagy and TLR signaling

In the second part, we concentrated on elucidating the mechanism underlying CpG-ODN induced resistance against influenza A virus in lung epithelial cells. CpG-ODN in combination with Pam2CSK4 protected mice from lethal influenza A virus infection. The combination also inhibited influenza A virus infection in vitro, and we noted that CpG-ODN was the active component. We found that pretreatment with Class B and C CpG-ODN significantly inhibited influenza A virus infection in lung epithelial cell lines. We found that CpG-ODN induced antiviral effect was TLR9-MyD88 signaling independent. We also showed that the antiviral effect did not go through other DNA sensors including DNA-PKcs, TBK1, DDX58, and STING. Furthermore, we noted that

CpG-ODN induced antiviral activity occurs at early stage during influenza life cycle. Thus, our data suggest a novel mechanism through which CpG-ODN protects lung epithelial cells from influenza infection.

REFERENCES

Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K.A., and Hornung, V. (2009). RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat Immunol *10*, 1065-1072.

Akira, S. (2009). Pathogen recognition by innate immunity and its signaling. Proc Jpn Acad Ser B Phys Biol Sci *85*, 143-156.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat Rev Immunol *4*, 499-511.

Akira, S., Takeda, K., and Kaisho, T. (2001). Toll-like receptors: Critical proteins linking innate and acquired immunity. Nat Immunol *2*, 675-680.

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

Aliprantis, A.O., Yang, R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., Klimpel, G.R., Godowski, P., and Zychlinsky, A. (1999). Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science *285*, 736-739.

Anand, P.K., Tait, S.W., Lamkanfi, M., Amer, A.O., Nunez, G., Pages, G., Pouyssegur, J., McGargill, M.A., Green, D.R., and Kanneganti, T.D. (2011). TLR2 and RIP2 pathways mediate autophagy of Listeria monocytogenes via extracellular signal-regulated kinase (ERK) activation. J Biol Chem *286*, 42981-42991.

Arango Duque, G., and Descoteaux, A. (2014). Macrophage cytokines: Involvement in immunity and infectious diseases. Front Immunol *5*, 491.

Arroyo, D.S., Soria, J.A., Gaviglio, E.A., Garcia-Keller, C., Cancela, L.M., Rodriguez-Galan, M.C., Wang, J.M., and Iribarren, P. (2013). Toll-like receptor 2 ligands promote microglial cell death by inducing autophagy. FASEB J *27*, 299-312.

Arunkumar, N., Liu, C., Hang, H., and Song, W. (2013). Toll-like receptor agonists induce apoptosis in mouse B-cell lymphoma cells by altering NF-kappaB activation. Cell Mol Immunol *10*, 360-372.

Ayabe, T., Satchell, D.P., Wilson, C.L., Parks, W.C., Selsted, M.E., and Ouellette, A.J. (2000). Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol *1*, 113-118.

Ballas, Z.K., Rasmussen, W.L., and Krieg, A.M. (1996). Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol *157*, 1840-1845.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Baum, A., Sachidanandam, R., and Garcia-Sastre, A. (2010). Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. Proc Natl Acad Sci U S A *107*, 16303-16308.

Bell, J.K., Mullen, G.E., Leifer, C.A., Mazzoni, A., Davies, D.R., and Segal, D.M. (2003). Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol *24*, 528-533.

Belvin, M.P., and Anderson, K.V. (1996). A conserved signaling pathway: The Drosophila toll-dorsal pathway. Annu Rev Cell Dev Biol *12*, 393-416.

Birchler, T., Seibl, R., Buchner, K., Loeliger, S., Seger, R., Hossle, J.P., Aguzzi, A., and Lauener, R.P. (2001). Human Toll-like receptor 2 mediates induction of the antimicrobial peptide human beta-defensin 2 in response to bacterial lipoprotein. Eur J Immunol *31*, 3131-3137.

Blander, J.M., and Medzhitov, R. (2004). Regulation of phagosome maturation by signals from toll-like receptors. Science *304*, 1014-1018.

Blander, J.M., and Medzhitov, R. (2006). On regulation of phagosome maturation and antigen presentation. Nat Immunol *7*, 1029-1035.

Bonilla, D.L., Bhattacharya, A., Sha, Y., Xu, Y., Xiang, Q., Kan, A., Jagannath, C., Komatsu, M., and Eissa, N.T. (2013). Autophagy regulates phagocytosis by modulating the expression of scavenger receptors. Immunity *39*, 537-547.

Borregaard, N. (2010). Neutrophils, from marrow to microbes. Immunity *33*, 657-670.

Bouvier, N.M., and Palese, P. (2008). The biology of influenza viruses. Vaccine *26 Suppl 4*, D49-53.

Bridges, C.B., Kuehnert, M.J., and Hall, C.B. (2003). Transmission of influenza: Implications for control in health care settings. Clin Infect Dis *37*, 1094-1101.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. Science *303*, 1532-1535.

Brinkmann, V., and Zychlinsky, A. (2012). Neutrophil extracellular traps: Is immunity the second function of chromatin? J Cell Biol *198*, 773-783.

Brubaker, S.W., Bonham, K.S., Zanoni, I., and Kagan, J.C. (2015). Innate immune pattern recognition: A cell biological perspective. Annu Rev Immunol *33*, 257-290.

Cartwright, G.E., Athens, J.W., and Wintrobe, M.M. (1964). The kinetics of granulopoiesis in normal man. Blood *24*, 780-803.

Castillo, E.F., Dekonenko, A., Arko-Mensah, J., Mandell, M.A., Dupont, N., Jiang, S., Delgado-Vargas, M., Timmins, G.S., Bhattacharya, D., Yang, H., *et al.* (2012). Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. Proc Natl Acad Sci U S A *109*, E3168-3176.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., *et al.* (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol *4*, 702-707.

Chuang, T., and Ulevitch, R.J. (2001). Identification of hTLR10: A novel human Toll-like receptor preferentially expressed in immune cells. Biochim Biophys Acta *1518*, 157-161.

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

Clark, R.A. (1999). Activation of the neutrophil respiratory burst oxidase. J Infect Dis *179 Suppl 2*, S309-317.

Cleaver, J.O., You, D., Michaud, D.R., Pruneda, F.A., Juarez, M.M., Zhang, J., Weill, P.M., Adachi, R., Gong, L., Moghaddam, S.J., *et al.* (2014). Lung epithelial cells are essential effectors of inducible resistance to pneumonia. Mucosal Immunol *7*, 78-88.

Cooney, R., Baker, J., Brain, O., Danis, B., Pichulik, T., Allan, P., Ferguson, D.J., Campbell, B.J., Jewell, D., and Simmons, A. (2010). NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat Med *16*, 90-97.

Crotta, S., Davidson, S., Mahlakoiv, T., Desmet, C.J., Buckwalter, M.R., Albert, M.L., Staeheli, P., and Wack, A. (2013). Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia. PLoS Pathog *9*, e1003773.

Dahlgren, C., and Karlsson, A. (1999). Respiratory burst in human neutrophils. J Immunol Methods *232*, 3-14.

Dallol, A., Cooper, W.N., Al-Mulla, F., Agathanggelou, A., Maher, E.R., and Latif, F. (2007). Depletion of the Ras association domain family 1, isoform A-associated novel microtubule-associated protein, C19ORF5/MAP1S, causes mitotic abnormalities. Cancer Res *67*, 492-500.

Davies, L.C., Jenkins, S.J., Allen, J.E., and Taylor, P.R. (2013). Tissue-resident macrophages. Nat Immunol *14*, 986-995.

Delgado, M.A., and Deretic, V. (2009). Toll-like receptors in control of immunological autophagy. Cell Death Differ *16*, 976-983.

Delgado, M.A., Elmaoued, R.A., Davis, A.S., Kyei, G., and Deretic, V. (2008). Toll-like receptors control autophagy. EMBO J *27*, 1110-1121.

Deretic, V., Saitoh, T., and Akira, S. (2013). Autophagy in infection, inflammation and immunity. Nat Rev Immunol *13*, 722-737.

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

Diner, E.J., Burdette, D.L., Wilson, S.C., Monroe, K.M., Kellenberger, C.A., Hyodo, M., Hayakawa, Y., Hammond, M.C., and Vance, R.E. (2013). The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. Cell Reports *3*, 1355-1361.

Ding, L., Linsley, P.S., Huang, L.Y., Germain, R.N., and Shevach, E.M. (1993). IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. J Immunol *151*, 1224-1234.

Doyle, S.E., O'Connell, R.M., Miranda, G.A., Vaidya, S.A., Chow, E.K., Liu, P.T., Suzuki, S., Suzuki, N., Modlin, R.L., Yeh, W.C., *et al.* (2004). Toll-like receptors induce a phagocytic gene program through p38. J Exp Med *199*, 81-90.

Droemann, D., Albrecht, D., Gerdes, J., Ulmer, A.J., Branscheid, D., Vollmer, E., Dalhoff, K., Zabel, P., and Goldmann, T. (2005). Human lung cancer cells express functionally active Toll-like receptor 9. Respir Res *6*, 1.

Du, X., Poltorak, A., Wei, Y., and Beutler, B. (2000). Three novel mammalian toll-like receptors: Gene structure, expression, and evolution. Eur Cytokine Netw *11*, 362-371.

Duggan, J.M., You, D., Cleaver, J.O., Larson, D.T., Garza, R.J., Guzman Pruneda, F.A., Tuvim, M.J., Zhang, J., Dickey, B.F., and Evans, S.E. (2011). Synergistic interactions of TLR2/6 and TLR9 induce a high level of resistance to lung infection in mice. J Immunol *186,* 5916-5926.

Evans, S.E., Scott, B.L., Clement, C.G., Larson, D.T., Kontoyiannis, D., Lewis, R.E., Lasala, P.R., Pawlik, J., Peterson, J.W., Chopra, A.K., *et al.* (2010a). Stimulated innate resistance of lung epithelium protects mice broadly against bacteria and fungi. Am J Respir Cell Mol Biol *42,* 40-50.

Evans, S.E., Tuvim, M.J., Fox, C.J., Sachdev, N., Gibiansky, L., and Dickey, B.F. (2011). Inhaled innate immune ligands to prevent pneumonia. Br J Pharmacol *163,* 195-206.

Evans, S.E., Xu, Y., Tuvim, M.J., and Dickey, B.F. (2010b). Inducible innate resistance of lung epithelium to infection. Annu Rev Physiol *72*, 413-435.

Falloon, J., and Gallin, J.I. (1986). Neutrophil granules in health and disease. J Allergy Clin Immunol *77*, 653-662.

Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W., and O'Garra, A. (1991). IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol *146*, 3444-3451.

Fitzgerald, K.A., Palsson-McDermott, E.M., Bowie, A.G., Jefferies, C.A., Mansell, A.S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M.T., *et al.* (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature *413,* 78-83.

Flannagan, R.S., Jaumouille, V., and Grinstein, S. (2012). The cell biology of phagocytosis. Annu Rev Pathol *7*, 61-98.

Freeman, S.A., and Grinstein, S. (2014). Phagocytosis: Receptors, signal integration, and the cytoskeleton. Immunol Rev *262*, 193-215.

Fu, J., Liang, J., Kang, H., Lin, J., Yu, Q., and Yang, Q. (2013). Effects of different CpG oligodeoxynucleotides with inactivated avian H5N1 influenza virus on mucosal immunity of chickens. Poult Sci *92*, 2866-2875.

Gateva, V., Sandling, J.K., Hom, G., Taylor, K.E., Chung, S.A., Sun, X., Ortmann, W., Kosoy, R., Ferreira, R.C., Nordmark, G., *et al.* (2009). A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. Nat Genet *41*, 1228-1233.

Gething, M.J., Bye, J., Skehel, J., and Waterfield, M. (1980). Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature *287*, 301-306.

Gilliet, M., Cao, W., and Liu, Y.J. (2008). Plasmacytoid dendritic cells: Sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol *8*, 594-606.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J., and Sansonetti, P.J. (2003). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem *278*, 8869-8872.

Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R.A., Diamond, M.S., and Colonna, M. (2006). Essential role of mda-5 in type I IFN responses to polyriboinosinic:Polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci U S A *103*, 8459-8464.

Glick, D., Barth, S., and Macleod, K.F. (2010). Autophagy: Cellular and molecular mechanisms. J Pathol *221*, 3-12.

Godwin, J.W., Pinto, A.R., and Rosenthal, N.A. (2013). Macrophages are required for adult salamander limb regeneration. Proc Natl Acad Sci U S A *110*, 9415-9420.

Gong, L., Cullinane, M., Treerat, P., Ramm, G., Prescott, M., Adler, B., Boyce, J.D., and Devenish, R.J. (2011). The Burkholderia pseudomallei type III secretion system and BopA are required for evasion of LC3-associated phagocytosis. PLoS One *6*, e17852.

Goodridge, H.S., Wolf, A.J., and Underhill, D.M. (2009). Beta-glucan recognition by the innate immune system. Immunol Rev *230*, 38-50.

Gordon, S. (2016). Phagocytosis: An Immunobiologic Process. Immunity *44*, 463-475.

Greenberg, S., and Grinstein, S. (2002). Phagocytosis and innate immunity. Curr Opin Immunol *14*, 136-145.

Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., and Amigorena, S. (2002). Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol *20*, 621-667.

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., and Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell *119*, 753-766.

Hacker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.C., Wang, G.G., Kamps, M.P., Raz, E., Wagner, H., Hacker, G., *et al.* (2006). Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature *439*, 204-207.

Hager, M., Cowland, J.B., and Borregaard, N. (2010). Neutrophil granules in health and disease. J Intern Med *268*, 25-34.

Haldar, M., and Murphy, K.M. (2014). Origin, development, and homeostasis of tissue-resident macrophages. Immunol Rev *262*, 25-35.

Hampe, J., Franke, A., Rosenstiel, P., Till, A., Teuber, M., Huse, K., Albrecht, M., Mayr, G., De La Vega, F.M., Briggs, J., *et al.* (2007). A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet *39*, 207-211.

Han, J.W., Zheng, H.F., Cui, Y., Sun, L.D., Ye, D.Q., Hu, Z., Xu, J.H., Cai, Z.M., Huang, W., Zhao, G.P., *et al.* (2009). Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. Nat Genet *41*, 1234-1237.

Hartmann, G., Battiany, J., Poeck, H., Wagner, M., Kerkmann, M., Lubenow, N., Rothenfusser, S., and Endres, S. (2003). Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. Eur J Immunol *33*, 1633-1641.

Hartmann, G., and Krieg, A.M. (2000). Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J Immunol *164*, 944-953.

Hartmann, G., Weeratna, R.D., Ballas, Z.K., Payette, P., Blackwell, S., Suparto, I., Rasmussen, W.L., Waldschmidt, M., Sajuthi, D., Purcell, R.H., *et al.* (2000). Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. J Immunol *164*, 1617-1624.

Hashimoto, C., Hudson, K.L., and Anderson, K.V. (1988). The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell *52*, 269-279.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature *410*, 1099-1103.

He, C., and Klionsky, D.J. (2009). Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet *43*, 67-93.

He, L., and Marneros, A.G. (2013). Macrophages are essential for the early wound healing response and the formation of a fibrovascular scar. Am J Pathol *182*, 2407-2417.

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

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., *et al.* (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740-745.

Hertz, C.J., Kiertscher, S.M., Godowski, P.J., Bouis, D.A., Norgard, M.V., Roth, M.D., and Modlin, R.L. (2001). Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. J Immunol *166*, 2444-2450.

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., and Fitzgerald, K.A. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature *458*, 514-518.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M., *et al.* (2006). 5'-Triphosphate RNA is the ligand for RIG-I. Science *314*, 994-997.

Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., *et al.* (2003). Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J Biol Chem *278*, 5509-5512.

Inomata, M., Niida, S., Shibata, K., and Into, T. (2012). Regulation of Toll-like receptor signaling by NDP52-mediated selective autophagy is normally inactivated by A20. Cell Mol Life Sci *69*, 963-979.

International Consortium for Systemic Lupus Erythematosus, G., Harley, J.B., Alarcon-Riquelme, M.E., Criswell, L.A., Jacob, C.O., Kimberly, R.P., Moser, K.L., Tsao, B.P., Vyse, T.J., Langefeld, C.D., *et al.* (2008). Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. Nat Genet *40*, 204-210.

Into, T., Inomata, M., Niida, S., Murakami, Y., and Shibata, K. (2010). Regulation of MyD88 aggregation and the MyD88-dependent signaling pathway by sequestosome 1 and histone deacetylase 6. J Biol Chem *285*, 35759-35769.

Into, T., Inomata, M., Takayama, E., and Takigawa, T. (2012). Autophagy in regulation of Toll-like receptor signaling. Cell Signal *24*, 1150-1162.

Ioannidis, I., Ye, F., McNally, B., Willette, M., and Flano, E. (2013). Toll-like receptor expression and induction of type I and type III interferons in primary airway epithelial cells. J Virol *87*, 3261-3270.

Ishikawa, T., Itoh, F., Yoshida, S., Saijo, S., Matsuzawa, T., Gonoi, T., Saito, T., Okawa, Y., Shibata, N., Miyamoto, T., *et al.* (2013). Identification of distinct ligands for the C-type lectin receptors Mincle and Dectin-2 in the pathogenic fungus Malassezia. Cell Host Microbe *13*, 477-488.

Italiani, P., and Boraschi, D. (2014). From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. Front Immunol *5*, 514.

Ito, T., and Kawaoka, Y. (2000). Host-range barrier of influenza A viruses. Vet Microbiol *74*, 71-75.

Ito, T., Wang, Y.H., and Liu, Y.J. (2005). Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9. Springer Semin Immunopathol *26*, 221-229.

Iwasaki, A., and Pillai, P.S. (2014). Innate immunity to influenza virus infection. Nat Rev Immunol *14*, 315-328.

Jagannath, C., Lindsey, D.R., Dhandayuthapani, S., Xu, Y., Hunter, R.L., Jr., and Eissa, N.T. (2009). Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. Nat Med *15*, 267-276.

Janeway CA Jr, T.P., Walport M, et al. (2001). Principles of innate and adaptive immunity. In immunology: The immune system in health and disease 5th edition (New York: Garland Science).

Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol *54 Pt 1*, 1-13.

Janeway, C.A., Jr., and Medzhitov, R. (2002). Innate immune recognition. Annu Rev Immunol *20*, 197-216.

Jewell, N.A., Cline, T., Mertz, S.E., Smirnov, S.V., Flano, E., Schindler, C., Grieves, J.L., Durbin, R.K., Kotenko, S.V., and Durbin, J.E. (2010). Lambda interferon is the predominant interferon induced by influenza A virus infection in vivo. J Virol *84*, 11515-11522.

Jiang, S., Li, X., Hess, N.J., Guan, Y., and Tapping, R.I. (2016). TLR10 Is a Negative Regulator of Both MyD88-Dependent and -Independent TLR Signaling. J Immunol *196*, 3834-3841.

Jiang, X., Zhong, W., Huang, H., He, H., Jiang, F., Chen, Y., Yue, F., Zou, J., Li, X., He, Y., *et al.* (2015). Autophagy defects suggested by low levels of autophagy activator MAP1S and high levels of autophagy inhibitor LRPPRC predict poor prognosis of prostate cancer patients. Mol Carcinog *54*, 1194-1204.

Jin, M.S., Kim, S.E., Heo, J.Y., Lee, M.E., Kim, H.M., Paik, S.G., Lee, H., and Lee, J.O. (2007). Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell *130*, 1071-1082.

Jin, Y., Sun, C., Feng, L., Li, P., Xiao, L., Ren, Y., Wang, D., Li, C., and Chen, L. (2014). Regulation of SIV antigen-specific CD4+ T cellular immunity via autophagosome-mediated MHC II molecule-targeting antigen presentation in mice. PLoS One *9*, e93143.

Johnson, N.P., and Mueller, J. (2002). Updating the accounts: Global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med *76*, 105-115.

Jutras, I., and Desjardins, M. (2005). Phagocytosis: At the crossroads of innate and adaptive immunity. Annu Rev Cell Dev Biol *21*, 511-527.

Kamps, B.S., Hoffmann, C., and Preiser, W. (2006). Influenza report 2006 (Flying Publisher).

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O., *et al.* (2005). Cell type-specific involvement of RIG-I in antiviral response. Immunity *23*, 19-28.

Kaur, J., and Debnath, J. (2015). Autophagy at the crossroads of catabolism and anabolism. Nat Rev Mol Cell Biol *16*, 461-472.

Kawagoe, T., Sato, S., Matsushita, K., Kato, H., Matsui, K., Kumagai, Y., Saitoh, T., Kawai, T., Takeuchi, O., and Akira, S. (2008). Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. Nat Immunol *9*, 684-691.

Kerkmann, M., Costa, L.T., Richter, C., Rothenfusser, S., Battiany, J., Hornung, V., Johnson, J., Englert, S., Ketterer, T., Heckl, W., *et al.* (2005). Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon-alpha induction by CpG-A in plasmacytoid dendritic cells. J Biol Chem *280*, 8086-8093.

Kikuchi, Y., Kunitoh-Asari, A., Hayakawa, K., Imai, S., Kasuya, K., Abe, K., Adachi, Y., Fukudome, S., Takahashi, Y., and Hachimura, S. (2014). Oral administration of Lactobacillus plantarum strain AYA enhances IgA secretion and provides survival protection against influenza virus infection in mice. PLoS One *9*, e86416.

Killip, M.J., Fodor, E., and Randall, R.E. (2015). Influenza virus activation of the interferon system. Virus Res *209*, 11-22.

Kim, C., Rockenstein, E., Spencer, B., Kim, H.K., Adame, A., Trejo, M., Stafa, K., Lee, H.J., Lee, S.J., and Masliah, E. (2015). Antagonizing neuronal Toll-like receptor 2 prevents synucleinopathy by activating autophagy. Cell Rep *13*, 771-782.

Kobe, B., and Kajava, A.V. (2001). The leucine-rich repeat as a protein recognition motif. Curr Opin Struct Biol *11*, 725-732.

Kofinger, J., Ragusa, M.J., Lee, I.H., Hummer, G., and Hurley, J.H. (2015). Solution structure of the Atg1 complex: implications for the architecture of the phagophore assembly site. Structure *23*, 809-818.

Koh, T.J., and DiPietro, L.A. (2011). Inflammation and wound healing: The role of the macrophage. Expert Rev Mol Med *13*, e23.

Kong, L., and Ge, B.X. (2008). MyD88-independent activation of a novel actin-Cdc42/Rac pathway is required for Toll-like receptor-stimulated phagocytosis. Cell Res *18*, 745-755.

Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A., and Klinman, D.M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature *374*, 546-549.

Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z.K., Endres, S., Krieg, A.M., and Hartmann, G. (2001). Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol *31*, 2154-2163.

Kwok, T., Helfer, H., Alam, M.I., Heinrich, J., Pavlovic, J., and Moelling, K. (2009). Inhibition of influenza A virus replication by short double-stranded oligodeoxynucleotides. Arch Virol *154*, 109-114.

Kwon, S., Gewirtz, A.T., Hurley, D.J., Robertson, T.P., Moore, J.N., and Vandenplas, M.L. (2011). Disparities in TLR5 expression and responsiveness to flagellin in equine neutrophils and mononuclear phagocytes. J Immunol *186*, 6263-6270.

Lacy, P. (2015). Editorial: Secretion of cytokines and chemokines by innate immune cells. Front Immunol *6*, 190.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., *et al.* (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860-921.

Lazear, H.M., Nice, T.J., and Diamond, M.S. (2015). Interferon-lambda: Immune Functions at Barrier Surfaces and Beyond. Immunity *43*, 15-28.

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

Lee, H.K., Lund, J.M., Ramanathan, B., Mizushima, N., and Iwasaki, A. (2007). Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science *315*, 1398-1401.

Lee, H.K., Mattei, L.M., Steinberg, B.E., Alberts, P., Lee, Y.H., Chervonsky, A., Mizushima, N., Grinstein, S., and Iwasaki, A. (2010). In vivo requirement for Atg5 in antigen presentation by dendritic cells. Immunity *32*, 227-239.

Lees, C.W., Barrett, J.C., Parkes, M., and Satsangi, J. (2011). New IBD genetics: Common pathways with other diseases. Gut *60*, 1739-1753.

Lehrer, R.I., and Ganz, T. (2002). Defensins of vertebrate animals. Curr Opin Immunol *14*, 96-102.

Leibovich, S.J., and Ross, R. (1975). The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. Am J Pathol *78*, 71-100.

LeibundGut-Landmann, S., Waldburger, J.M., Reis e Sousa, C., Acha-Orbea, H., and Reith, W. (2004). MHC class II expression is differentially regulated in plasmacytoid and conventional dendritic cells. Nat Immunol *5*, 899-908.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973-983.

Lerena, M.C., and Colombo, M.I. (2011). Mycobacterium marinum induces a marked LC3 recruitment to its containing phagosome that depends on a functional ESX-1 secretion system. Cell Microbiol *13*, 814-835.

Levine, B., and Deretic, V. (2007). Unveiling the roles of autophagy in innate and adaptive immunity. Nat Rev Immunol *7*, 767-777.

Li, X., Ranjith-Kumar, C.T., Brooks, M.T., Dharmaiah, S., Herr, A.B., Kao, C., and Li, P. (2009). The RIG-I-like receptor LGP2 recognizes the termini of double-stranded RNA. J Biol Chem *284*, 13881-13891.

Liao, Y., and Hung, M.C. (2003). Regulation of the activity of p38 mitogen-activated protein kinase by Akt in cancer and adenoviral protein E1A-mediated sensitization to apoptosis. Mol Cell Biol *23*, 6836-6848.

Liu, K., Victora, G.D., Schwickert, T.A., Guermonprez, P., Meredith, M.M., Yao, K., Chu, F.F., Randolph, G.J., Rudensky, A.Y., and Nussenzweig, M. (2009). In vivo analysis of dendritic cell development and homeostasis. Science *324*, 392-397.

Liu, L., Botos, I., Wang, Y., Leonard, J.N., Shiloach, J., Segal, D.M., and Davies, D.R. (2008). Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science *320*, 379-381.

Liu, L., McKeehan, W.L., Wang, F., and Xie, R. (2012). MAP1S enhances autophagy to suppress tumorigenesis. Autophagy *8*, 278-280.

Liu, L., Vo, A., Liu, G., and McKeehan, W.L. (2005). Putative tumor suppressor RASSF1 interactive protein and cell death inducer C19ORF5 is a DNA binding protein. Biochem Biophys Res Commun *332*, 670-676.

Liu, Y.J. (2005). IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu Rev Immunol *23*, 275-306.

Lopez, A.M., Hecker, R., Mutwiri, G., van Drunen Littel-van den Hurk, S., Babiuk, L.A., and Townsend, H.G. (2006). Formulation with CpG ODN enhances antibody responses to an equine influenza virus vaccine. Vet Immunol Immunopathol *114*, 103-110.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Iwasaki, A., and Flavell, R.A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A *101*, 5598-5603.

Mak, N.K., Schiltknecht, E., and Ada, G.L. (1983). Protection of mice against influenza virus infection: enhancement of nonspecific cellular responses by Corynebacterium parvum. Cell Immunol *78*, 314-325.

Mallick, A.I., Parvizi, P., Read, L.R., Nagy, E., Behboudi, S., and Sharif, S. (2011). Enhancement of immunogenicity of a virosome-based avian influenza vaccine in chickens by incorporating CpG-ODN. Vaccine *29*, 1657-1665.

Mari, M., Tooze, S.A., and Reggiori, F. (2011). The puzzling origin of the autophagosomal membrane. F1000 Biol Rep *3*, 25.

Marshall, J.D., Fearon, K., Abbate, C., Subramanian, S., Yee, P., Gregorio, J., Coffman, R.L., and Van Nest, G. (2003). Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. J Leukoc Biol *73*, 781-792.

Martinez-Pomares, L., and Gordon, S. (2007). Antigen presentation the macrophage way. Cell *131*, 641-643.

Martinez, F.O., and Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep *6*, 13.

Martinez, J., Almendinger, J., Oberst, A., Ness, R., Dillon, C.P., Fitzgerald, P., Hengartner, M.O., and Green, D.R. (2011). Microtubule-associated protein 1 light chain

3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proc Natl Acad Sci U S A *108*, 17396-17401.

Martinez, J., Malireddi, R.K., Lu, Q., Cunha, L.D., Pelletier, S., Gingras, S., Orchard, R., Guan, J.L., Tan, H., Peng, J., *et al.* (2015). Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nat Cell Biol *17*, 893-906.

Mathur, R., Oh, H., Zhang, D., Park, S.G., Seo, J., Koblansky, A., Hayden, M.S., and Ghosh, S. (2012). A mouse model of Salmonella typhi infection. Cell *151*, 590-602.

McKenzie, H.A., and White, F.H., Jr. (1991). Lysozyme and alpha-lactalbumin: Structure, function, and interrelationships. Adv Protein Chem *41*, 173-315.

Medina, K.L. (2016). Overview of the immune system. Handb Clin Neurol *133*, 61-76.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.

Michelsen, K.S., Aicher, A., Mohaupt, M., Hartung, T., Dimmeler, S., Kirschning, C.J., and Schumann, R.R. (2001). The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCS). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. J Biol Chem *276*, 25680-25686.

Miles, K., Clarke, D.J., Lu, W., Sibinska, Z., Beaumont, P.E., Davidson, D.J., Barr, T.A., Campopiano, D.J., and Gray, M. (2009). Dying and necrotic neutrophils are anti-inflammatory secondary to the release of alpha-defensins. J Immunol *183*, 2122-2132.

Militello, R.D., and Colombo, M.I. (2011). A membrane is born: Origin of the autophagosomal compartment. Curr Mol Med *11*, 197-203.

Mills, C.D. (2012). M1 and M2 Macrophages: Oracles of Health and Disease. Crit Rev Immunol *32*, 463-488.

Mizushima, N. (2007). Autophagy: Process and function. Genes Dev *21*, 2861-2873.

Moore, M.A., Sheridan, A.P., Allen, T.D., and Dexter, T.M. (1979). Prolonged hematopoiesis in a primate bone marrow culture system: Characteristics of stem cell production and the hematopoietic microenvironment. Blood *54*, 775-793.

Moscat, J., and Diaz-Meco, M.T. (2009). p62 at the crossroads of autophagy, apoptosis, and cancer. Cell *137*, 1001-1004.

Moscat, J., Diaz-Meco, M.T., Albert, A., and Campuzano, S. (2006). Cell signaling and function organized by PB1 domain interactions. Mol Cell *23*, 631-640.

Murthy, A., Li, Y., Peng, I., Reichelt, M., Katakam, A.K., Noubade, R., Roose-Girma, M., DeVoss, J., Diehl, L., Graham, R.R., *et al.* (2014). A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. Nature *506*, 456-462.

Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., *et al.* (2004). Autophagy defends cells against invading group A Streptococcus. Science *306*, 1037-1040.

Nakahira, K., Haspel, J.A., Rathinam, V.A., Lee, S.J., Dolinay, T., Lam, H.C., Englert, J.A., Rabinovitch, M., Cernadas, M., Kim, H.P., *et al.* (2011). Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol *12*, 222-230.

Narita, M., Young, A.R., Arakawa, S., Samarajiwa, S.A., Nakashima, T., Yoshida, S., Hong, S., Berry, L.S., Reichelt, S., Ferreira, M., *et al.* (2011). Spatial coupling of mTOR and autophagy augments secretory phenotypes. Science *332*, 966-970.

Nicas, M., Nazaroff, W.W., and Hubbard, A. (2005). Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. J Occup Environ Hyg *2*, 143-154.

O'Neill, L.A., and Bowie, A.G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol *7*, 353-364.

Okabayashi, T., Kojima, T., Masaki, T., Yokota, S., Imaizumi, T., Tsutsumi, H., Himi, T., Fujii, N., and Sawada, N. (2011). Type-III interferon, not type-I, is the predominant interferon induced by respiratory viruses in nasal epithelial cells. Virus Res *160*, 360-366.

Oldenburg, M., Kruger, A., Ferstl, R., Kaufmann, A., Nees, G., Sigmund, A., Bathke, B., Lauterbach, H., Suter, M., Dreher, S., *et al.* (2012). TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. Science *337*, 1111-1115.

Oosting, M., Cheng, S.C., Bolscher, J.M., Vestering-Stenger, R., Plantinga, T.S., Verschueren, I.C., Arts, P., Garritsen, A., van Eenennaam, H., Sturm, P., *et al.* (2014). Human TLR10 is an anti-inflammatory pattern-recognition receptor. Proc Natl Acad Sci U S A *111*, E4478-4484.

Orban-Nemeth, Z., Simader, H., Badurek, S., Trancikova, A., and Propst, F. (2005). Microtubule-associated protein 1S, a short and ubiquitously expressed member of the microtubule-associated protein 1 family. J Biol Chem *280*, 2257-2265.

Orlikowsky, T.W., Spring, B., Dannecker, G.E., Niethammer, D., Poets, C.F., and Hoffmann, M.K. (2003). Expression and regulation of B7 family molecules on macrophages (MPhi) in preterm and term neonatal cord blood and peripheral blood of adults. Cytometry B Clin Cytom *53*, 40-47.

Park, B.S., Song, D.H., Kim, H.M., Choi, B.S., Lee, H., and Lee, J.O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature *458*, 1191-1195.

Parkin, J., and Cohen, B. (2001). An overview of the immune system. Lancet *357*, 1777-1789.

Paul, S., Kashyap, A.K., Jia, W., He, Y.W., and Schaefer, B.C. (2012). Selective autophagy of the adaptor protein Bcl10 modulates T cell receptor activation of NF-kappaB. Immunity *36*, 947-958.

Paul, W.E. (2011). Bridging innate and adaptive immunity. Cell *147*, 1212-1215.

Perdiguero, E.G., Klapproth, K., Schulz, C., Busch, K., de Bruijn, M., Rodewald, H.R., and Geissmann, F. (2015). The origin of tissue-resident macrophages: When an erythro-myeloid progenitor is an erythro-myeloid progenitor. Immunity *43*, 1023-1024.

Perussia, B., Fanning, V., and Trinchieri, G. (1985). A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses. Nat Immun Cell Growth Regul *4*, 120-137.

Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science *314*, 997-1001.

Pisetsky, D.S., and Reich, C.F., 3rd (1998). The influence of base sequence on the immunological properties of defined oligonucleotides. Immunopharmacology *40*, 199-208.

Plantinga, T.S., Crisan, T.O., Oosting, M., van de Veerdonk, F.L., de Jong, D.J., Philpott, D.J., van der Meer, J.W., Girardin, S.E., Joosten, L.A., and Netea, M.G. (2011). Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. Gut *60*, 1229-1235.

Pollard, J.W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer *4*, 71-78.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., *et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. Science *282*, 2085-2088.

Rane, M.J., Song, Y., Jin, S., Barati, M.T., Wu, R., Kausar, H., Tan, Y., Wang, Y., Zhou, G., Klein, J.B., *et al.* (2010). Interplay between Akt and p38 MAPK pathways in the regulation of renal tubular cell apoptosis associated with diabetic nephropathy. Am J Physiol Renal Physiol *298*, F49-61.

Rankin, R., Pontarollo, R., Ioannou, X., Krieg, A.M., Hecker, R., Babiuk, L.A., and van Drunen Littel-van den Hurk, S. (2001). CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense Nucleic Acid Drug Dev *11*, 333-340.

Redlich, S., Ribes, S., Schutze, S., Eiffert, H., and Nau, R. (2013). Toll-like receptor stimulation increases phagocytosis of Cryptococcus neoformans by microglial cells. J Neuroinflammation *10*, 71.

Revenis, M.E., and Kaliner, M.A. (1992). Lactoferrin and lysozyme deficiency in airway secretions: association with the development of bronchopulmonary dysplasia. J Pediatr *121*, 262-270.

Ribes, S., Ebert, S., Regen, T., Agarwal, A., Tauber, S.C., Czesnik, D., Spreer, A., Bunkowski, S., Eiffert, H., Hanisch, U.K., *et al.* (2010). Toll-like receptor stimulation enhances phagocytosis and intracellular killing of nonencapsulated and encapsulated Streptococcus pneumoniae by murine microglia. Infect Immun *78*, 865-871.

Richards, D.M., Hettinger, J., and Feuerer, M. (2013). Monocytes and macrophages in cancer: Development and functions. Cancer Microenviron *6*, 179-191.

Rioux, J.D., Xavier, R.J., Taylor, K.D., Silverberg, M.S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M.M., Datta, L.W., *et al.* (2007). Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet *39*, 596-604.

Roberts, T.L., Sweet, M.J., Hume, D.A., and Stacey, K.J. (2005). Cutting edge: Species-specific TLR9-mediated recognition of CpG and non-CpG phosphorothioate-modified oligonucleotides. J Immunol *174*, 605-608.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A., and Bazan, J.F. (1998). A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A *95*, 588-593.

Rodriguez, P., Perez-Morgado, M.I., Gonzalez, V.M., Martin, M.E., and Nieto, A. (2016). Inhibition of influenza virus replication by DNA aptamers targeting a cellular component of translation Initiation. Mol Ther Nucleic Acids *5*, e308.

Romao, S., and Munz, C. (2014). LC3-associated phagocytosis. Autophagy *10*, 526-528.

Saitoh, T., Fujita, N., Hayashi, T., Takahara, K., Satoh, T., Lee, H., Matsunaga, K., Kageyama, S., Omori, H., Noda, T., *et al.* (2009). Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. Proc Natl Acad Sci U S A *106*, 20842-20846.

Saitoh, T., Fujita, N., Jang, M.H., Uematsu, S., Yang, B.G., Satoh, T., Omori, H., Noda, T., Yamamoto, N., Komatsu, M., *et al.* (2008). Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature *456*, 264-268.

Sanjana, N.E., Shalem, O., and Zhang, F. (2014). Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods *11*, 783-784.

Sanjuan, M.A., Dillon, C.P., Tait, S.W., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J.L., Withoff, S., *et al.* (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature *450*, 1253-1257.

Sanz, L., Diaz-Meco, M.T., Nakano, H., and Moscat, J. (2000). The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. EMBO J *19*, 1576-1586.

Sarma, J.V., and Ward, P.A. (2011). The complement system. Cell Tissue Res *343*, 227-235.

Schmid, D., and Munz, C. (2007). Innate and adaptive immunity through autophagy. Immunity *27*, 11-21.

Schmid, D., Pypaert, M., and Munz, C. (2007). Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity *26*, 79-92.

Seo, S.U., Kwon, H.J., Song, J.H., Byun, Y.H., Seong, B.L., Kawai, T., Akira, S., and Kweon, M.N. (2010). MyD88 signaling is indispensable for primary influenza A virus infection but dispensable for secondary infection. Journal of Virology *84*, 12713-12722.

Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T.S., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G., *et al.* (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. Science *343*, 84-87.

Shi, C., and Pamer, E.G. (2011). Monocyte recruitment during infection and inflammation. Nat Rev Immunol *11*, 762-774.

Shi, C.S., and Kehrl, J.H. (2008). MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. J Biol Chem *283*, 33175-33182.

Shi, C.S., Shenderov, K., Huang, N.N., Kabat, J., Abu-Asab, M., Fitzgerald, K.A., Sher, A., and Kehrl, J.H. (2012). Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. Nat Immunol *13*, 255-263.

Shi, M., Yao, Y., Han, F., Li, Y., and Li, Y. (2014). MAP1S controls breast cancer cell TLR5 signaling pathway and promotes TLR5 signaling-based tumor suppression. PLoS One *9*, e86839.

Shi, M., Zhang, Y., Liu, L., Zhang, T., Han, F., Cleveland, J., Wang, F., McKeehan, W.L., Li, Y., and Zhang, D. (2016). MAP1S protein regulates the phagocytosis of bacteria and toll-like receptor (TLR) signaling. J Biol Chem *291*, 1243-1250.

Shortman, K., and Naik, S.H. (2007). Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol *7*, 19-30.

Singh, S.M., Alkie, T.N., Abdelaziz, K.T., Hodgins, D.C., Novy, A., Nagy, E., and Sharif, S. (2016a). Characterization of immune responses to an inactivated avian influenza virus vaccine adjuvanted with nanoparticles containing CpG ODN. Viral Immunol *29*, 269-275.

Singh, S.M., Alkie, T.N., Nagy, E., Kulkarni, R.R., Hodgins, D.C., and Sharif, S. (2016b). Delivery of an inactivated avian influenza virus vaccine adjuvanted with poly(D,L-lactic-co-glycolic acid) encapsulated CpG ODN induces protective immune responses in chickens. Vaccine *34*, 4807-4813.

Sommereyns, C., Paul, S., Staeheli, P., and Michiels, T. (2008). IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS Pathog *4*, e1000017.

Song, W., Wang, J., Han, Z., Zhang, Y., Zhang, H., Wang, W., Chang, J., Xia, B., Fan, S., Zhang, D., *et al.* (2015). Structural basis for specific recognition of single-stranded RNA by Toll-like receptor 13. Nat Struct Mol Biol *22*, 782-787.

Spencer, J.C., Ganguly, R., and Waldman, R.H. (1977). Nonspecific protection of mice against influenza virus infection by local or systemic immunization with Bacille Calmette-Guerin. J Infect Dis *136*, 171-175.

Steinman, R.M. (2001). Dendritic cells and the control of immunity: Enhancing the efficiency of antigen presentation. Mt Sinai J Med *68*, 160-166.

Steinman, R.M., and Witmer, M.D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proc Natl Acad Sci U S A *75*, 5132-5136.

Stow, J.L., Low, P.C., Offenhauser, C., and Sangermani, D. (2009). Cytokine secretion in macrophages and other cells: Pathways and mediators. Immunobiology *214*, 601-612.

Sun, L.J., Wu, J.X., and Chen, Z.J. (2013). Cyclic GMP-AMP Synthase (cGAS) is a cytosolic DNA sensor that activates type I interferon pathway by generating a second messenger. Journal of Immunology *190*.

Suzuki, K., and Ohsumi, Y. (2003). The pre-autophagosomal structure (PAS), required for the formation of autophagosome membrane. Seikagaku *75*, 492-499.

Szewczyk, B., Bienkowska-Szewczyk, K., and Krol, E. (2014). Introduction to molecular biology of influenza a viruses. Acta Biochim Pol *61*, 397-401.

Takaoka, A., Wang, Z., Choi, M.K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., *et al.* (2007). DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature *448*, 501-505.

Takeuchi, O., Kawai, T., Sanjo, H., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Takeda, K., and Akira, S. (1999). TLR6: A novel member of an expanding toll-like receptor family. Gene *231*, 59-65.

Tooze, S.A., and Yoshimori, T. (2010). The origin of the autophagosomal membrane. Nat Cell Biol *12*, 831-835.

Treanor, J. (2004). Influenza vaccine--outmaneuvering antigenic shift and drift. N Engl J Med *350*, 218-220.

Tuvim, M.J., Evans, S.E., Clement, C.G., Dickey, B.F., and Gilbert, B.E. (2009). Augmented lung inflammation protects against influenza A pneumonia. PLoS One *4*, e4176.

Tuvim, M.J., Gilbert, B.E., Dickey, B.F., and Evans, S.E. (2012). Synergistic TLR2/6 and TLR9 activation protects mice against lethal influenza pneumonia. PLoS One *7*, e30596.

Unanue, E.R. (1984). Antigen-presenting function of the macrophage. Annu Rev Immunol *2*, 395-428.

Underhill, D.M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M., and Aderem, A. (1999). The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature *401*, 811-815.

Unterholzner, L., Keating, S.E., Baran, M., Horan, K.A., Jensen, S.B., Sharma, S., Sirois, C.M., Jin, T., Latz, E., Xiao, T.S., *et al.* (2010). IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol *11*, 997-1004.

Van Daal, G.J., Beusenberg, F.D., So, K.L., Fievez, R.B., Sprenger, M.J., Mouton, J.W., Van 't Veen, A., and Lachmann, B. (1991). Protection against influenza A virus infection in mice by oral immunization with a polyvalent bacterial lysate. Int J Immunopharmacol *13*, 831-840.

van Daal, G.J., So, K.L., Mouton, J.W., van 't Veen, A., Tenbrinck, R., Bergmann, K.C., and Lachmann, B. (1990). Oral immunization with a polyvalent bacterial lysate can reduce

mortality by infection with S. pneumoniae or influenza A in mice. Pneumologie *44*, 1180-1182.

van Daal, G.J., van 't Veen, A., So, K.L., Mouton, J.W., Smit, F., van Hal, P.T., Bergmann, K.C., and Lachmann, B. (1992). Oral immunization with polyvalent bacterial lysate and infection with Streptococcus pneumoniae: Influence on interferon-gamma and PMN elastase concentrations in murine bronchoalveolar lavage fluid. Int Arch Allergy Immunol *97*, 173-177.

Warner, N., and Nunez, G. (2013). MyD88: A critical adaptor protein in innate immunity signal transduction. J Immunol *190*, 3-4.

Xie, R., Nguyen, S., McKeehan, K., Wang, F., McKeehan, W.L., and Liu, L. (2011a). Microtubule-associated protein 1S (MAP1S) bridges autophagic components with microtubules and mitochondria to affect autophagosomal biogenesis and degradation. J Biol Chem *286*, 10367-10377.

Xie, R., Wang, F., McKeehan, W.L., and Liu, L. (2011b). Autophagy enhanced by microtubule- and mitochondrion-associated MAP1S suppresses genome instability and hepatocarcinogenesis. Cancer Res *71*, 7537-7546.

Xie, Z., and Klionsky, D.J. (2007). Autophagosome formation: Core machinery and adaptations. Nat Cell Biol *9*, 1102-1109.

Xu, Y., Fattah, E.A., Liu, X.D., Jagannath, C., and Eissa, N.T. (2013). Harnessing of TLR-mediated autophagy to combat mycobacteria in macrophages. Tuberculosis (Edinb) *93 Suppl*, S33-37.

Xu, Y., Jagannath, C., Liu, X.D., Sharafkhaneh, A., Kolodziejska, K.E., and Eissa, N.T. (2007). Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity *27*, 135-144.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., *et al.* (2003a). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640-643.

Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003b). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol *4*, 1144-1150.

Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K., and Saito, T. (2008). Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol *9*, 1179-1188.

Yarovinsky, F., Zhang, D., Andersen, J.F., Bannenberg, G.L., Serhan, C.N., Hayden, M.S., Hieny, S., Sutterwala, F.S., Flavell, R.A., Ghosh, S., *et al.* (2005). TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science *308*, 1626-1629.

Young, L.J., Wilson, N.S., Schnorrer, P., Proietto, A., ten Broeke, T., Matsuki, Y., Mount, A.M., Belz, G.T., O'Keeffe, M., Ohmura-Hoshino, M., *et al.* (2008). Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. Nat Immunol *9*, 1244-1252.

Zhang, Y., Lin, A., Zhang, C., Tian, Z., and Zhang, J. (2014). Phosphorothioate-modified CpG oligodeoxynucleotide (CpG ODN) induces apoptosis of human hepatocellular carcinoma cells independent of TLR9. Cancer Immunol Immunother *63*, 357-367.