Title

Mechanisms by which two different Toll-like receptor ligands induce divergent immune responses in the lungs

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Mechanisms by which two different Toll-like receptor ligands induce divergent immune responses in the lungs

by

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DISSERTATION

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This dissertation is dedicated to my parents, Shui and Sui Lee.

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Contributions to the presented work

Chapters 3 and 4 are manuscripts in preparation. I designed, performed, and analyzed the experiments, and wrote this dissertation. Xiaozhu Huang and Xin Ren from UCSF Lung Biology Core performed pilot experimental asthma model experiments and performed experiments examining airway hyperresponsiveness (Fig. 4.1D). Ming Ji and Matthew Dong provided assistance to performing some experiments. Richard Locksley guided experimental design and contributed mouse strains. Anthony DeFranco helped design and supervised the experiments, and edited the dissertation.

Abstract

Mechanisms by which two different Toll-like receptor ligands induce divergent immune responses in the lungs

By Linda May Lee

The mammalian immune system can mount several different types of innate and adaptive responses. The choice of the type of immune response depends on a variety of factors, including which innate immune receptors recognize conserved pathogen molecules on the invading pathogen. How allergens and other type 2 stimuli, such as parasitic worm infections, induce type 2 immunity have been intensely studied, but it is still unclear which pathways are critical for driving this type of immune response. Interestingly, although Toll-like receptor (TLR) stimulation often induces type 1 immune responses during bacterial or viral infections, it can also promote type 2 immune responses in the lung in some circumstances, and this may be important for development of asthma. The work presented in this dissertation investigate the mechanisms by which two different TLR ligands induce divergent immune responses in the lungs.

In Chapter 3, I describe experiments using an intranasal (i.n.) sensitization and challenge model to characterize and understand how a synthetic TLR9 ligand, unmethylated cytosine followed by guanosine (CpG) oligodeoxyribonucleotide (ODN), which has great promise of being a potent adjuvant in vaccines and an immunomodulator clinically, induces an immune response in the lungs. I demonstrated that the sensitization

i.n. with the model antigen ovalbumin (OVA) plus CpG ODN as adjuvant, and subsequent rechallenge with OVA led to a T_H1 response in the lungs and increased serum OVA-specific IgG2c. Early after CpG ODN and OVA i.n. administration, increased levels mRNA encoding IL-12 p40, and increased numbers of dendritic cells (DCs) and monocytes were present in the lungs. CpG ODN treatment led to the elevation of costimulatory molecules on DCs and monocytes in the lungs, and on the migratory DCs in the draining mediastinal lymph node (LN). I.n. CpG ODN was also able to induce early IFN-γ production in both innate-like lymphocytes such as γδ T cells, natural killer (NK) T cells, and NK cells, and also in adaptive CD4 T cells and CD8 T cells. Strikingly, these innate and adaptive immune responses were dependent on myeloid differentiation primary response 88 (MyD88) signaling in DCs, and most production of IFN-γ was dependent on IL-12, which was produced during the innate phase of the response. Based on these results, we suggest that DCs directly sense CpG ODN in the lungs via TLR9 and that this recognition both induces their maturation and their production of IL-12, which promotes innate production of IFN-γ and the T_H1 response.

Chapter 4 describes studies in which CpG ODN and the TLR5 and TLR11 ligand, flagellin, were compared to examine how TLRs can promote different adaptive immune responses in the lungs. In contrast to mice i.n. sensitized with CpG ODN plus OVA, those sensitized with flagellin plus OVA exhibited an innate inflammatory response dominated by neutrophils and monocytes, developed a predominant T_H2 response to OVA, and made substantial amounts of IgE anti-OVA antibodies. Antigenic re-challenge of the mice sensitized with flagellin plus OVA, but not the mice sensitized with CpG

ODN plus OVA, led to a vigorous lung inflammation dominated by eosinophils, as expected for a T_H2 response. Strikingly, the early cytokine responses in the lung to the two different TLR ligands showed a number of important differences. CpG ODN induced much higher levels of mRNA encoding IL-12 p40, whereas, flagellin preferentially induced TSLP mRNA and secretion of the mature form of IL-33, and also induced elevated levels of mRNAs encoding IL-1α and IL-1β. In both cases, migratory DCs in the draining LN after stimulation with ligands for either TLR5/TLR11 or TLR9 had strong induction of the costimulators CD80 and CD86, but only CpG ODN also induced upregulation of CD40. Interestingly, MyD88 signaling in DCs was partially required for the flagellin-induced upregulation of CD80 on migratory DCs, and for the IL-4 production by CD4 T cells in the draining LN on d6. These results indicate that although TLR5, TLR11, and TLR9 all signal via the signaling adaptor MyD88, the innate cytokine response to these TLRs is quite different, and the distinctive innate cytokine production results in distinctive polarization of the adaptive humoral and cell-mediated immune responses.

These studies have provided insight into how TLR stimulation can drive T_H1 and T_H2 responses in the lungs, and how complex immune responses occur in the lungs, which have important implications for understanding infectious diseases and asthma, and for vaccine design.

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Chapter 1 :Introduction

The immune system protects the body from infections, contributes to tissue repair, and eliminates abnormal and malignant cells, but can also cause autoimmune diseases and allergies. This system employs both hard-wired recognition of characteristic molecular features of infectious agents, known as innate immunity, and highly specific antibodies and T cell antigen receptors, known as adaptive immunity (Medzhitov, 2007). The immune system has evolved to express receptors (pattern recognition receptors or PRRs) that can distinguish between different classes of pathogens by recognizing conserved molecules expressed on the pathogens. Recognition of pathogens through PRRs activates the innate immune response, which can lead to direct activation of immune and non-immune cells to produce cytokines and other factors to combat the infection, and/or recruit other immune cells that can provide additional defense. In the cases in which the innate immune response is not enough to clear an infection, cell-mediated adaptive immunity becomes activated, leading to antibody production by B cells and generation of effector T cells. Both the type of antibody produced and the types of effector T cells produced are typically polarized toward one of several options that are especially effective against a particular type of infection.

Innate immune sensing of infections by Toll-like receptors (TLRs)

Pathogens that are able to breach the body's first line of defense, such as the skin or mucosal barrier of the lungs, are initially recognized by PRRs, expressed on host cells such as immune cells or epithelial cells (Medzhitov, 2007). One important family, and the most characterized, of PRRs, is the Toll-like receptor (TLR) family, which recognize

various components of bacterial cell surfaces and nucleic acids characteristic of viruses and/or microbes (Kawai and Akira, 2011). At least ten TLRs in humans and twelve TLRs in mice have been identified. TLRs are expressed on the cell surface or associated in intracellular vesicles. They are type I transmembrane proteins. Each TLR contains an ectodomain made up of leucine-rich repeats (LRR) that recognizes cognate ligand, a transmembrane domain, and a cytosolic Toll-IL-1 receptor (TIR) domain that is involved in signaling.

The locations of TLRs enable the host to recognize both extracellular and intracellular pathogens (Kawai and Akira, 2011). Cell surface TLRs mostly recognize membrane components on microbes. TLRs located in intracellular vesicles, which include the endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes, recognize nucleic acids. By having TLRs that recognize nucleic acid located in the endocytic pathway, host cells can survey foreign nucleic acids from viruses, bacteria, or infected cells, that have been taken up through the endocytic pathway, thus minimizing the probability of sensing self nucleic acid. The nucleic acid sensing TLRs traffic from the ER to endosomes, which is regulated in part by UNC93B1 (Lee and Barton, 2014). To generate functional receptors that can mediate signaling, the N-terminal region of TLR3, 7, and 9 are processed by lysosomal proteases, including cathepsins and asparagine endopeptidase.

The TLRs that are expressed on the cell surface include TLR2, which heterodimerizes with TLR1 or TLR6 to recognize lipoproteins, TLR4, which recognizes lipopolysaccharide (LPS), and TLR5, which recognizes flagellin (Kawai and Akira, 2011). TLR11 is expressed on both the cell surface and intracellular compartments

(Kawai and Akira, 2011) and recognizes profilin-like protein from *Toxoplasma gondii* (Yarovinsky et al., 2005), and more recently has been shown also to recognize flagellin (Mathur et al., 2012). The TLRs that are expressed in intracellular vesicles include TLR3, which recognizes double-stranded RNA, TLR7 and TLR8, which recognize single-stranded RNA, and TLR9, which recognizes DNA with sequence motifs that include unmethylated cytosine followed by guanosine (CpG) (Kawai and Akira, 2011).

After ligand binding, all TLRs, except TLR3, signal through the common adaptor molecule, myeloid differentiation primary response 88 (MyD88), which ultimately leads to the activation of NF-κB, MAP kinases, and interferon regulatory factor (IRF) 5 (Kawai and Akira, 2011). These signaling events lead to the induction of pro-inflammatory genes, such as cytokines, which induce inflammation and/or affect the responses of other immune cells. Cytokines and activated immune cells provide defense against infection. In a MyD88-independent pathway, TLR3 and TLR4 use TIR domain-containing adaptor inducing IFN-β (TRIF) to signal and activate NF-κB, which leads to inflammatory cytokines, and IRF3, which leads to transcription of type I interferon (IFN) genes.

Different TLRs use different signaling molecules, some move to different cellular compartments during signaling, and some can promote type I IFN induction (Kawai and Akira, 2011). TLR4 uses TIR domain-containing adaptor protein (TIRAP) to recruit MyD88, and subsequent signaling leads to NF-κB activation. After being endocytosed, TLR4 signals through TRIF and TRIF-related adaptor molecule (TRAM) and induces late-stage NF-κB activation. TLR2 also uses TIRAP to recruit MyD88. TLR2-TLR1 and TLR2-TLR6 dimers are endocytosed and recruited to the phagosome. It has been shown in inflammatory monocytes that TLR2 is capable of inducing type I IFN through the

activation of IRF3 and IRF7 (Barbalat et al., 2009). TLR7 and TLR9 can also induce type I IFN in a MyD88-IRF7 pathway that is distinct from the pathway that activates NF-κB. Previously, it was thought that plasmacytoid dendritic cells (pDCs) were the only cell type containing this pathway, which explained how, after TLR7 or TLR9 stimulation, pDC produce large amounts of type I IFN (Barbalat et al., 2011). However, one study suggests that other DC subsets and macrophages can use MyD88-IRF7 type I IFN production if the artificial ligand for TLR9, CpG oligodeoxyribonucleotide (ODN), is manipulated to be retained in the endosome (Honda et al., 2005).

Innate immunity instructing adaptive immunity

The cytokines produced during the innate immune response, which reflect the type of pathogen that is recognized, are important in determining the polarization of the CD4 T cell response (Szabo et al., 2003). In this way, the innate immune response integrates what it has sensed to promote the appropriate adaptive immune response. One important cell that bridges the innate immune response to the adaptive immune response is the dendritic cell (DC) (Medzhitov, 2007).

DCs, which express a wide range of PRRs, act as sentinels for invading pathogens in tissues (Iwasaki and Medzhitov, 2004). Many studies have established that DCs are not only essential for sensing conserved molecules on pathogens or pathogen associated molecular patterns (PAMPs), thus driving the innate immune response, but DCs are also critical for activating and directing the appropriate adaptive immune response. After pathogen recognition, DCs can upregulate expression of costimulatory molecules and produce additional signals, such as cytokines, that reflect the type of pathogen the DCs

have sensed. DCs can present cognate phagocytosed and processed pathogen peptide on major histocompatibility complex (MHC), provide activating costimulation, and communicate through cytokines the nature of the infection, to naïve CD4 T cells. In turn, these three signals activate and polarize CD4 T cells to a specific T helper (T_H) subset to mount the appropriate adaptive immune response.

Although, in many situations, the cytokines produced by DCs in direct response to PAMPs are necessary for T cell polarization, particularly for T_H1 responses, in other situations, the cytokines produced by other cell types may be important, particularly in T_H2 responses.

Cytokines that influence T cell polarization

Since the original Mossman and Coffman (1986) observation that there exist distinct T helper cell lineages, namely T_H1 and T_H2, several other T helper subsets have been discovered, such as T_H17, regulatory T cells, follicular helper T cells (T_{FH}) (Zhu et al., 2010) and T_H9 (Stassen et al., 2012). The cytokines that influence T_H1, T_H2, T_H17 development will be reviewed below.

Interleukin (IL)-12 and IFN-γ have been shown to be important for the polarization of naïve T cells into T_H1 cells, which are important for controlling viral and intracellular bacterial infections (Medzhitov, 2007). After infection, through the engagement of PRRs, particularly TLRs, IL-12 is produced by DCs and in many cases, is essential for T_H1 polarization (Hou et al., 2008; Spörri and Reis e Sousa, 2005). IL-12 promotes IFN-γ production by innate lymphocytes, such as natural killer (NK) cells (Trinchieri, 2003). In turn, IFN-γ signaling in T cells leads to the upregulation of master

transcription factor, T-bet (T-box expressed in T cells) (Szabo et al., 2003). T-bet increases the accessibility of the *Ifng* loci, leading to increase IFN-γ production and creating a positive feedback loop supporting T_H1 polarization. Naïve T cells express low levels of the IL-12 receptor chains, IL-12Rβ1 and IL-12β2, but upregulate both chains after T cell receptor (TCR) stimulation. T-bet upregulates further the IL-12Rβ2 chain, thus allowing IL-12 signaling, through STAT4 signaling, to stabilize IFN-γ production.

Many cytokines are important for differentiation of naïve T cells into T_H17 cells, which are important for controlling extracellular bacteria, protozoa, and fungi (Medzhitov, 2007). IL-6 and transforming growth factor (TGF)-β are important for initiating T_H17 differentiation (Gaffen et al., 2014). Signaling downstream of IL-6 induces the transcription of the gene encoding the master transcription factor, RORγt (retinoic acid receptor-related orphan receptor-γt), and of the T_H17 specific genes, IL-17 and IL-23R. IL-6 also upregulates IL-1R, which allows IL-1β to signal and reinforce RORγt expression and also enhances expansion of T_H17 cells. IL-21, which can be produced by NK cell and T cell subsets, is an important autocrine growth factor for developing T_H17 cells. IL-23 is necessary for development of T_H17 inflammatory characteristics.

T_H2 cells are important for providing host defense against multi-cellular parasites, such as helminthes and bloodsucking insects (Pulendran and Artis, 2012). Unlike T_H1 and T_H17 responses, how cytokines polarize naïve T cells into T_H2 is well less defined. The cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) have emerged as important inflammatory cytokines that can drive type 2 immunity (Bartemes and Kita,

2012). Studies suggest that these cytokines are primarily made by epithelial cells present at environmental interfaces, but how they induce T_H2 responses remain poorly understood. These cytokines act on innate lymphoid type 2 cells (ILC2), which respond by making IL-5 and IL-13 (Licona-Limón et al., 2013), but may stimulate other cell types as well, including DCs. How cytokines produced by epithelial cells, ILC2s, or other cell types can condition DCs to induce polarization of naïve T cells into T_H2 effector cells is still poorly defined. Studies using human DCs have shown that TSLP conditions DCs to prime T_H2 responses (Ito et al., 2005).

In vitro studies have shown that exogenously added IL-4 is important for the initiation of T_H2 differentiation (Ansel et al., 2006). Activation of STAT6 by IL-4 signaling, and activation of NFAT and signaling molecules by TCR stimulation, can lead to stabilization and expression of the T_H2 master transcription factor, GATA-3 (GATA-binding protein 3). In addition, TCR stimulation and GATA-3 expression can lead to production of IL-4, inducing a positive feedback loop to reinforce GATA-3 expression and T_H2 differentiation. However, *in vivo*, it is unknown what is the initial source of IL-4 (Ansel et al., 2006; Pulendran and Artis, 2012). Recent studies have suggested that basophils may provide the initial source of this cytokine, but other studies provide evidence against this possibility (Locksley, 2010). Naïve T cells also express low levels of IL-4, which may be sufficient to induce T_H2 differentiation (Ansel et al., 2006).

Other factors that may influence how different T helper cell responses are induced

Although the cytokine milieu of the innate immune response has been shown to play a major role in determining T cell differentiation, other factors may be involved,

such as the amount of antigen used during immunization, the DC subset that activates and primes naïve T cells, and the expression of particular costimulatory molecules by those DCs (Szabo et al., 2003). The latter two will be discussed, with particular emphasis on T_H2 responses and the lungs.

Studies have shown there may be a DC subset that preferentially primes the T_H2 response, depending on the route of immunization. In the spleen, $CD8\alpha^+$ DCs induce a T_H1 response whereas $CD8\alpha^-$ DCs induce T_H2 responses (Pulendran and Artis, 2012). Two studies have examined which DC subset induced T_H2 responses to subcutaneous (s.c.) allergen immunization (papain) or during helminth infection. They found that an IRF4-dependent, $CD301b^+$ DC population was necessary, but not sufficient, to promote T_H2 responses (Gao et al., 2013a; Kumamoto et al., 2013a). In the lungs, one study demonstrated that $CD11b^+$ DCs and monocyte-derived DCs are important for inducing a T_H2 response to house dust mite (HDM) allergen (Plantinga et al., 2013). In addition, they found that $CD11b^+$ DCs exhibited preferential uptake of fluorescent antigen in the lung and draining lymph node (LN) compared to other DC subsets. Conversely, another study suggested that $CD103^+$ DCs were the important DC subset to induce T_H2 responses to house dust extract sensitization (Nakano et al., 2012a). In this study, $CD11b^+$ DCs again had preferential uptake of fluorescent antigen in the lungs, while in the LNs, $CD103^+$ DCs took up more antigen.

Upregulation of cell surface activation molecules on DCs may also be important for inducing Th1 or Th2 responses. CD40 was shown to be necessary for the T_H2 response induced by *Schistosoma mansoni* soluble egg antigen but not for the T_H1 response induced by *Propionbacterium acnes* antigen (MacDonald et al., 2002). Using an

intranasal (i.n.) papain model of asthma, Halim et. al. (2014) showed that LN DCs preferentially upregulated CD40, but this upregulation did not occur in bone marrow chimeras in which mice lacked ILC2. Kuchroo et. al. (1995) showed that blocking CD80 ameliorates experimental autoimmune encephalomyelitis (EAE) symptoms by inducing a T_H2 response, suggesting that CD80 promoted the induction of the pathological T_H1 response. Conversely, blocking CD86 exacerbates EAE symptoms, suggesting that CD86 may be important in inducing T_H2 responses.

ICOSL expression on DCs may also be important for inducing T_H2 responses. After sensitization intraperitoneally (i.p.) with *S. mansoni* eggs followed by i.n. challenge with *S. manosoni* egg antigen, blocking ICOS/ICOSL interaction using ICOS-Ig led to decreased IL-5 and fewer eosinophils, macrophages, and lymphocytes in the lung airspace (Tesciuba et al., 2001). However, this treatment did not have any effect on IgE production nor IL-5 production in restimulated CD4 T cells.

Both *in vitro* studies and murine models have implicated OX40L as being important in promoting T_H2 responses. Ito et al. (2005) showed that TSLP, but not IL-12, led to the expression of OX40L on human myeloid DCs and promoted naïve T cells to produce T_H2 cytokines. Blocking OX40L or adding IL-12 prevented the production of T_H2 cytokines by T cells. Another study showed that stimulation of OX40 on activated human umbilical cord human T cells upregulated IL-4 mRNA (Ohshima et al., 1998). In murine studies, *in vitro* stimulation of T cells with OX40L from a transfected cell line led to IL-4 production (Flynn et al., 1998; Gramaglia et al., 1998). *Ox40*$^{-/-}$ mice sensitized i.p. with OVA/alum and challenged with aerosol OVA had decreased characteristics of experimental asthma (Jember et al., 2001). Moreover, *in vivo* blocking of OX40L reduced

the T_H2 response to *Leishmania major* (Akiba et al., 2000). Similarly, *Ox40l*$^{-/-}$ mice infected with *Heligmosomoides polygyrus* had decreased worm expulsion, and decreased IL-4 production, as well as a partial decrease in IgE (Ekkens et al., 2003).

Effector functions of T cells during infection and immunopathology

T_H1 cells, via IFN-γ and other cytokines, act on phagocytes to lead to the elimination of viruses and intracellular bacteria (Schroder et al., 2004). IFN-γ induces the activation and upregulation of MHCII on DCs and macrophages, leading to increased activation of T cells. Many of the effects on IFN-γ come from activating macrophages to increase phagocytic activity through upregulation of Fc and complement receptors, and microbiocidal activity to kill ingested pathogens through upregulation of NADPH oxidase and inducible nitric oxide synthase (iNOS). IFN-γ-activated macrophages also produce cytokines and chemokines such as IL-6, TNF, IL-1β, IL-12, IL-18, CCL2, CXCL11, CXCL16, which activate and/or recruit other cell types, such as other macrophages, neutrophils, monocytes, and additional T_H1 cells. IFN-γ also acts on other IFN-γ producing lymphocytes to increase survival, such as CD8 T cells and NK cells, which are important for killing virally infected cells. In addition, IFN-γ can promote class-switching of B cells to isotypes that bind complement well and bind with high affinity to Fc receptors on phagocytes to increase phagocytosis, such as IgG2a (Stevens et al., 1988).

T_H2 cells, via the expression of IL-4, IL-5, IL-9, and IL-13, promote host defense against multi-cellular parasites, such as helminthes and bloodsucking insects, but also cause immunopathology as seen in asthma and allergies (Pulendran and Artis, 2012).

These cytokines can act directly on tissue or through other immune cells to induce changes in the tissue that make it difficult for parasites to feed, and also to expel them from tissue (Nair et al., 2006). In asthma, these cytokines lead to remodeling of lung tissue that leads to airway hyperreactivity and obstruction of airways, making it difficult to breath (Locksley, 2010). IL-5 induces the production and survival of eosinophils and IL-9, along with IL-3, induces the production and survival of basophils and mast cells (Paul and Zhu, 2010). IL-4 can induce B cells to class switch to IgE, which binds to the FcεRI receptors on basophils and mast cells. Cross-linking of IgE bound to FcεRI on these cells leads to their degranulation, and release of vasoactive mediators that can cause tissue edema and constriction of blood vessels. T_H2-derived cytokines also directly act on tissue cells (Paul and Zhu, 2010). IL-4 and IL-13 increase mucus production in epithelial cells and smooth muscle contractility. IL-4 and IL-13 can induce differentiation of alternatively activated macrophages, which are important in producing anti-inflammatory cytokines and promote tissue repair and remodeling (Van Dyken and Locksley, 2013).

T_H17 cells, which produce the cytokines IL-17A, IL-17F, IL-22, IL-6, and TNF (Gaffen et al., 2014), are important for providing defense against extracellular bacteria and fungi (Medzhitov, 2007) through attraction and activation of phagocytes (McKenzie et al., 2006). In tissues, IL-17 stimulates stromal, epithelial, and endothelial cells to produce cytokines and chemokines such as IL-6, CXCL8, CXCL1, CXCL6, G-CSF, and GM-CSF, which promote the production of neutrophils and monocytes, and their recruitment to sites of infection. These potent phagocytes can then phagocytose and kill invading pathogens. While IL-17 induces a more pro-inflammatory response, IL-22 functions more to promote tissue repair (Rutz et al., 2013). IL-22 maintains and repairs

epithelial barriers by inducing epithelial cell proliferation. Along with other cytokines, such as IL-17 and TNF, it can promote the production of antimicrobial peptides from epithelial cells. IL-22 can also induce the production of inflammatory mediators, such as IL-6, G-CSF, and IL-1β.

Many of the T helper cytokines can also be produced by innate immune cells and contribute to host defense and subsequent development of the adaptive immune response. Innate lymphocytes such as NK cells, invariant (i) NKT cells, and γδ T cells can produce IFN-γ, IL-4, and IL-17 (Ansel et al., 2006; Cua and Tato, 2010; Trinchieri, 2003). In addition, recent studies have identified and characterized innate lymphoid cells (ILCs), which come in several different types that express many cytokines produced by T helper cells and provide host defense during the early inflammatory response (Spits and Di Santo, 2011).

TLR ligands, flagellin and CpG ODN, promote different immune responses

Bacterial flagellin is recognized by TLR5, TLR11, and NLRC4

Many bacteria, both commensal and pathogenic, express flagella (Ramos et al., 2004). Since flagella enable bacterium motility and adhesion, which can aid in invasion of mucosal epithelia, such as in the gut and the lungs, flagella are considered a virulence factor. The flagellar structure consists of a basal body, torsion hook, and helical hollow filament that is made up of many monomers of the protein flagellin. Since flagellin is key for success of flagellated bacteria, many regions of flagellin are conserved among different bacteria. Because of these conserved regions, the host innate immune system has developed receptors that recognize flagellin, thus enabling the recognition of a wide

range of bacteria with a few germline encoded receptors. When bacteria shed their flagella either actively by bacteria or induced by the host environment, or when flagellin monomers leak out during formation of the flagella, these flagellin monomers can be recognized by host cells through NLRs (nucleotide-binding oligomerization domain (NOD), LRR receptor), TLR5 (Ramos et al., 2004), and TLR11 (Mathur et al., 2012). Flagellin in the cytosol is recognized by NLR apoptosis-inhibitory proteins 5 and 6 (NAIP5 and NAIP6), which hetero-oligomerize with NLR- and CARD- containing receptor 4 (NLRC4), which is also known as IPAF, to form an inflammasome that activates caspase 1 (Kofoed and Vance, 2011). This mechanism is thought to be important for recognizing flagellated bacteria that infect intracellularly. In contrast, TLR5 and TLR11 recognize flagellin on the cell surface, which is the focus of this dissertation.

Cell types that express TLR5 and TLR11

Many immune cells express TLR5, including neutrophils, NK cells, and macrophages (Honko and Mizel, 2005). Some subsets of T cells express TLR5 but whether or not B cells express TLR5 has been controversial (Honko et al., 2006; Pasare and Medzhitov, 2005). Of relevance to the lungs, primary murine epithelial cells (Wilson et al., 2012) and human airway epithelial cell lines express TLR5 (Prince, 2006). Alveolar macrophages also express TLR5 (Hawn et al., 2007). However, whether DCs express TLR5 is unclear. In the lungs, Wilson et al. (2012) found that $CD103^+$ and $CD11b^+$ DCs isolated from lungs had lower expression of TLR5 than alveolar macrophages and lung epithelial cells. Expression of TLR5 on bone-marrow derived DCs (BMDCs) and splenic DCs has been better characterized. BMDCs upregulate CD40,

CD80, CD86 when treated with flagellin or CpG ODN (Datta et al., 2003). In addition, Didierlaurent et al (2004) found that both murine BMDCs and splenic DCs express TLR5 mRNA, but Means et al. (2003) was not able to detect TLR5 mRNA in either population. Means et al. (2003) did find human DCs to be responsive to flagellin and to express TLR5 mRNA. Both Didierlaurent et al. (2004) and Means et al. (2003) found that *ex vivo* isolated splenic DCs were not responsive to flagellin. In the lamina propria of the gut, DCs express TLR5 and TLR11, but macrophages only express TLR11 (Mathur et al., 2012; Uematsu et al., 2006).

Flagellin provides host protection in the gut

Though flagellated bacteria contain multiple TLR ligands, studies using *Tlr5*$^{-/-}$ and *Tlr11*$^{-/-}$ mice demonstrate that TLR5 and TLR11 provide unique contributions to host immunity. *Tlr5*$^{-/-}$ mice are more susceptible to uropathogenic *E. coli* infection (Andersen-Nissen et al., 2007). In addition, TLR5 has emerged as a key player in maintaining gut homeostasis and immunity. *Tlr5*$^{-/-}$ mice can develop spontaneous colitis (Vijay-Kumar et al., 2007) and metabolic syndrome (Vijay-Kumar et al., 2010), which suggests that TLR5 may have an important role in maintaining gut homeostasis, possibly by eliminating commensal bacteria that breach the epithelial layer, but these phenotypes may be influenced by the nature of the microbiota of mice in a particular housing facility (Letran et al., 2011). TLR5 and TLR11 signaling is also important for protection against *Salmonella typhimurium* infection (Mathur et al., 2012). *Tlr11*$^{-/-}$ but not wild-type mice are particularly susceptible to *S. typhi* infection, the cause of typhoid fever in humans,

suggesting that one reason why humans, not mice, become ill after *S. typhi* infection is because humans do not have a functional copy of *Tlr11*.

Stimulation with flagellin or an agonist to TLR5 also can promote resilience in mice stressed in various ways, such as due to lethal irradiation (Burdelya et al., 2008; Vijay-Kumar et al., 2008) or infection (Jarchum et al., 2011; Kinnebrew et al., 2010; Vijay-Kumar et al., 2008; Zhang et al., 2014). Flagellin-induced general protection may be due in part to flagellin's ability to induce production of IL-22, which promotes epithelial cell repair and defense, as described above (Kinnebrew et al., 2012; Van Maele et al., 2010; Zhang et al., 2014).

Innate immune response to flagellin in the lungs

Although TLR5 may not be entirely important for providing host protection against flagellated bacteria infection in the lungs, such as with *Legionella pneumphila* (Hawn et al., 2007), several studies have shown that flagellin administered i.n. promotes a robust inflammatory response in the lungs. Neutrophils quickly infiltrate into the lungs of mice treated with flagellin via the nasal route (Feuillet et al., 2006; Honko and Mizel, 2004; Janot et al., 2009). In addition, flagellin stimulates production of a variety of cytokines, chemokines, and other proteins such as TNFα, IL-6, GM-CSF, KC, IL-1α, and CCL20. Studies from Feuillet et al. (2006) and Janot et al. (2009) suggest both hematopoietic and non-hematopoietic cells sense flagellin and contribute to the early innate immune response. In contrast, Van Maele et al. (2014) showed that many of the genes induced by flagellin were dependent on TLR5 expression in non-hematopoietic cells, and laser-dissected bronchial epithelial cells expressed many of these genes,

arguing that non-hematopoietic cells may contribute primarily to the innate immune response to flagellin. These differing results may be due to the fact that TLR11 is expressed in the lungs, and the authors generated bone marrow chimeras using only *Tlr5*$^{-/-}$ mice.

Emerging role of flagellin shaping adaptive immunity

Stimulation of most TLRs leads to the development of T_H1 responses (Iwasaki and Medzhitov, 2004). However, several studies demonstrate that stimulation of TLR5 with flagellin has distinct properties for the promotion of adaptive immune responses compared to stimulation of other TLRs. Two independent studies have shown that s.c. immunization of flagellin can lead to a T_H2 response. Didierlaurent et al. (2004) demonstrated that the cells of the draining LNs of mice immunized with flagellin and OVA produced T_H2 cytokines, IL-4, IL-5, and IL-13, which were presumably produced by T cells in the LNs. In contrast, cells of the draining LNs of mice immunized with CpG ODN produced high levels of IFN-γ and no T_H2 cytokines. Cunningham et al. (2004) also showed that flagellin can skew towards a T_H2 response. Draining LNs of mice immunized with flagellin expressed mRNA transcripts characteristic of T_H2 cells, but not T_H1 cells. Additionally, the predominant serum isotype in these mice were IgG1. Also, sensitization of mice i.n. with flagellin and OVA leads to a T_H2 response in the lungs (Van Maele et al., 2014; Wilson et al., 2012).

Interestingly, in contrast to s.c. immunization, mice intravenously (i.v.) immunized with flagellin and OVA peptide develop a typical T_H1 response characteristic of other TLRs (McSorley et al., 2002). Furthermore, lamina propria DCs treated with

flagellin and OVA *in vitro* promoted the differentiation of naïve CD4 T cells into T_H1 and T_H17 cells (Uematsu et al., 2008). Hence, the immune response induced by flagellin may be due in part to the tissue environment, and which cell types in that tissue express TLR5 and/or TLR11.

CpG ODN is a synthetic ligand of TLR9

Bacterial and viral DNAs typically contain unmethylated CpG motifs that are recognized by TLR9. The sequences of CpG ODNs that were immunostimulatory were originally found in bacterial DNA (Klinman, 2006). There are three types of synthetic CpG ODN. CpG "B" or "K" type ODNs, which contain a phosphorothioate backbone, induce TNFα production by pDCs and induce B cells to proliferate and secrete antibodies. CpG "A" or "D" type ODNs, which contain either phosphodiester and phosphorothioate backbones, induce IFN-α production by pDC, but are not stimulatory for B cells. CpG "C" type ODNs, which contain a phosphorothioate backbone represent an intermediate type as they can induce IFN-α production by pDCs and induce B cells to produce IL-6 (Klinman, 2006). The differences between the A and B type CpG ODN have been attributed to their physical state of aggregation and the rate at which they move through the endocytic pathway, which may affect which signaling pathways TLR9 can access (Honda et al., 2005). In addition to the unmethylated CpG sequence, the surrounding sequence affects the potency of CpG ODNs, and this sequence preference differs from species to species due to differences in the fine specificity of TLR9 (Klinman, 2006). In my studies, I used a B-type CpG ODN (CpG 1826) that optimally stimulates murine cells (Ballas et al., 2001).

Cell types that express TLR9

Murine splenic DCs have been shown to express TLR9 mRNA and are responsive to CpG ODN or CpG-containing plasmid DNA stimulation (Chen et al., 2006; Edwards et al., 2003). Like murine pDCs, human pDCs express TLR9, but whether other human DC subsets express TLR9 is unclear (discussed further in Chapter 5). *In vitro* stimulated murine lung DCs have been shown to be responsive to CpG ODN (Pesce et al., 2010; Sullivan et al., 2011). B cells in human and mice express TLR9 (Bekeredjian-Ding and Jego, 2009). Mouse monocyte and macrophages express TLR9, but not human monocytes (Krieg, 2002). Whether alveolar macrophages or lung epithelial cells express TLR9 is unclear (Cho et al., 2006; Pesce et al., 2010; Suzuki et al., 2005).

CpG ODNs promote type 1 immunity and T_H1 responses

Immunization of mice with an antigen plus stimulatory CpG ODN leads to the rapid production of IL-12 and IFN-γ and induces robust T_H1 responses when administered systemically or s.c. (Krieg, 2002). Previous studies have shown that MyD88 signaling in DCs is necessary for early cytokine production, T_H1 response, and serum antibodies induced by CpG administered systemically (Hou et al., 2008). Immunization with CpG ODN as adjuvant also has the propensity to drive T_H1 responses in the lungs after i.n. administration (Chen et al., 2006; Pesce et al., 2010). This treatment was associated with production of IL-12 p40 and IFN-γ mRNA in the lungs within hours of administration (Pesce et al., 2010).

CpG ODNs use for vaccines, general host protection, and immunomodulation

CpG ODNs potent ability to induce a robust type 1 and T_H1 response has motivated many studies to examine whether immunization with CpG ODN plus antigen can protect the host from subsequent infection (Klinman, 2006). For example, i.n. vaccination with *Aspergillus fumigatus* antigen, *Asp f 16*, provided protection against lethal infection of this fungi (Bozza et al., 2002). Many studies have also examined the ability of CpG ODNs to provide general host protection and modulate existing immune responses, particularly asthma and allergy (Klinman, 2006; Krieg, 2002). Interestingly, these studies have revealed multiple aspects of CpG ODN-induced immune response that can be functionally important. For example, i.n. administration of CpG ODN three days prior to *Cryptococcus neoformans* infection reduced fungal burden in a manner that was independent of IL-12, NK cells, and T cells, although the ability of CpG ODN to reduce lung eosinophilia was dependent on IL-12 and CD8 T cells (Edwards et al., 2005). Many studies have also examined whether CpG ODN is able to block T_H2 responses in the lungs (Fonseca and Kline, 2009). In a chronic ragweed pollen asthma model, i.n. CpG ODN administration was able to induce long-lasting decrease in asthma-related symptoms (Campbell et al., 2014). Interestingly, neutralizing IFN-γ during the challenge phase did not prevent CpG ODN from inhibiting the asthma response. In contrast, another study found that mice that are deficient in both IFN-γ and IL-12 still had inhibition of *S. mansoni* egg antigen-induced asthma when CpG ODN was administered i.n. However, greater quantities of CpG ODN were needed to inhibit the Th2 response in these mice, and the inhibition of IL-4 and IL-5 production was not as great (Kline et al.,

1998). Thus, CpG ODNs consistently promote a type 1 immune response and typically inhibit a type 2 immune response in the lungs.

Chapter 2 : Materials and Methods

Mice

Mice were used between the ages of 8 and 20 weeks. B6 (000664; C57BL/6J) and B6 (C57BL/6NCr) were purchased from Jackson Laboratory and National Cancer Institute, respectively. B6 mice from Jackson were bred and maintained in the laboratory's colony. *Myd88*$^{fl/fl}$ mice (008888; B6.129P2(SJL)-*Myd88*^{tm1Defr}/J) were crossed to *CD11c-Cre* (008068; B6.Cg-Tg(Itgax-cre)1-1Reiz/J) as previously described (Hou et al., 2008). *Myd88*$^{fl/fl}$ and *Myd88*$^{fl/fl}$ *CD11c-Cre* mice were crossed to the following reporter mice provided by R. Locksley: GREAT – *Ifng*$^{GREAT/GREAT}$ (017581; B6.129S4-*Ifng*$^{tm3.1Lky}$/J) (Reinhardt et al., 2009) to generate *Myd88*$^{fl/fl}$ *Ifng*$^{GREAT/GREAT}$ and *Myd88*$^{fl/fl}$ *Ifng*$^{GREAT/GREAT}$ *CD11c-Cre;* 4get – *Il4*$^{4get/4get}$ (Mohrs et al., 2001) and KN2 – *Il4*$^{KN2/KN2}$ (Mohrs et al., 2005) to generate *Myd88*$^{fl/fl}$ *Il4*$^{4get/KN2}$ and *Myd88*$^{fl/fl}$ *Il4*$^{4get/KN2}$ *CD11c-Cre;* SMART-17A – *Il17a*$^{Smart/Smart}$ (Price et al., 2012) to generate *Myd88*$^{fl/fl}$ *Il17a*$^{Smart/Smart}$. For Figure 4.2A-C, two of three experiments used mice that were *Myd88*$^{fl/+}$ *Il4*$^{4get/KN2}$ *Mcpt8*$^{Basopho8/+}$ (017578; B6.129S4-*Mcpt8*$^{tm1(cre)Lky}$/J) (Sullivan et al., 2011). These mice gave similar results to one experiment using *Myd88*$^{fl/fl}$ *Il4*$^{4get/KN2}$ *mice.* All mice had been backcrossed for at least 8 generations to B6. Unless specified in the figure legend, control mice used included B6 mice and may have contained 1 or 2 alleles of the *Myd88*^{fl} allele or reporter alleles described above. Previous experiments have indicated that the *Myd88*^{fl} allele provides normal MyD88 function (Hou et al., 2008, 2011). All mice were maintained in specific-pathogen free conditions at UCSF, following UCSF and NIH animal use guidelines. Mice were used under UCSF Institutional Animal Care and Use Committee approval (IACUC).

Reagents

Chicken ovalbumin (OVA) Grade VI (Sigma) was used both for i.n. administration and to coat ELISA plates. According to a protocol from Aida and Pabst (1990), Triton X114 (EMD Millipore) was used to remove endotoxin from OVA prior to i.n. administration. Endotoxin-depleted OVA was labeled with Alexa Fluor 647 using Alexa Fluor 647 carboxylic acid succinimidyl ester (Invitrogen). CpG ODN 1826 (TCCATGACGTTCCTGACGTT), with a phosphorothioate-backbone, was purchased from IDT.

Flagellin preparation

Flagellin was purified from *Salmonella typhimurium* TH4778, provided by K. Hughes (University of Utah), using a modified protocol from Smith et al. (2003). Briefly, bacteria were stabbed into the middle of a 0.3% agar plate made of tryptic soy broth (TSB) for 8-12 hours. Highly motile bacteria were isolated from the front edge of growth and inoculated again into another plate. Highly motile bacteria were then inoculated into liquid TSB in 2L flasks, grown overnight at 37^{o} C, with shaking at 80-100rpm. Bacteria from this large culture were then used to inoculate fresh liquid TSB in 2L flasks. When cultures reached optical densities representing exponential growth and prior to stationary growth (approximately 2-3 hours), the bacteria were collected and pelleted by centrifugation. The pellets were washed once with 10mM Tris buffer (pH8.0), pelleted by centrifugation a second time as before, resuspended in Tris buffer, and blended in a Waring blender for 2 minutes. The blended suspension was then centrifuged for 15 minutes at 8,000xg. The supernatant fraction was collected and ultracentrifuged for

1.25hr at 105,000xg. The supernatant fraction was then discarded and the pellet containing flagella and flagellin filaments was resuspended in Dulbecco's (D)-PBS without $Ca^{2+}Mg^{2+}$ overnight at 4° C. This suspension was then heat depolymerized for 25 minutes at 70° C to dissociate flagellin polymers into monomeric flagellin subunits. The solution was then passed through a 100kDa MW cut-off filter (Amicon). The filtrate contained the flagellin monomers. To remove endotoxin, the flagellin preparation was passed through an endotoxin removal column (EndoTrap; Hyglos) according to the manufacturer's instructions. The flagellin concentration was quantified using the BCA Protein Assay Kit (Pierce). The endotoxin level was assayed using the *Limulus* amebocyte lysate (Lonza) and the preparation was found to contain <0.013EU/μg of protein.

In vivo treatment

For intranasal (i.n.) administration, mice were briefly anesthetized with isofluorane and sensitized i.n. with OVA (100μg), OVA plus flagellin, or OVA plus CpG ODN in a total volume of 50μl in D-PBS according to schedule and doses described in the figure legends. For mice challenged i.n. with OVA, 25μg of OVA was used. The timepoints in which the mice were assayed were chosen based on the most optimal timepoint for detecting reporter positive CD4 T cells (data not shown). For IL-12 blocking experiments, mice were given anti-IL-12 p40 (C17.8) or control antibody rat IgG2a (2A3), one day before initial sensitization (d-1; 700μg i.p.). For d6 and d16 time points, mice were again treated on d2 (300μg i.p.). These purified antibodies were purchased from the UCSF Monoclonal Antibody Core.

Airway hyperresponsiveness

Airway hyperresponsiveness was performed on d21 as previously described (Nakagami et al., 2008).

Bronchoalveolar lavage fluid and tissue preparation of lungs and lymph nodes for flow cytometry

For collection of bronchioalveolar lavage fluid (BALF), the lungs were lavaged serially 3 times each with 1mL D-PBS. The lungs of mice were perfused by cardiac puncture using 10 mL D-PBS, then each individual lobe was dissected into a digestion solution containing Liberase TM (0.15-0.3U/mL) from Roche, DNase (40-50U/mL) from Sigma or Worthington in HBSS with Ca^{2+}, Mg^{2+}/Pen-Strep/HEPES in a total volume of 5 mL. Lungs were either minced with scissors or dissociated with a tissue dissociator (Miltenyi Biotec), using program "lung_01", then incubated for 30 minutes in a 37^{o} C water bath, rotating tubes every 5 minutes. For lungs minced by scissors, after 15 minutes, lung suspensions were pipetted up and down using a serological pipette. To stop digestion, EDTA and FBS were added, and lungs were incubated at 37^{o} C for another 5 minutes. If using the tissue dissociator, samples were further dissociated using "lung_02". The samples were then further dispersed by mashing any remaining lung tissue chunks that were not already in suspension using the end of a serological pipette over a 70μm filter (BD Falcon), then washing with HBSS/2%FBS/Pen-Strep/HEPES. After centrifugation, the cells were resuspended in staining buffer (D-PBS/2%FBS/2mM EDTA/0.1% sodium azide) and filtered again through a 70μm filter. Whole cells counts were obtained using the NucleoCounter (Chemometec). For assessing migratory DC

phenotypes, mediastinal lymph nodes (LN) were dissected and teased open and placed in tubes with 1mL solution containing Liberase TM and DNase as described above. The LNs were incubated in a 37^{o} C water bath for 30 minutes, rotating tubes every 5 minutes. To stop digestion, EDTA and FBS were added, and LNs were incubated at 37^{o} C for another 5 minutes. Digested LNs and LNs directed taken out of mice without digestion (Fig. 4.3 and 4.5) were mashed with the plunger of a 3 mL syringe over a 70μM filter and washed with buffer. After centrifugation, the cells were counted as described above for the cells from lungs and BALF. In cases in which mediastinal LN could not be detected or when the percentage of DCs were <0.25%, these mice were excluded from analysis.

Lung and LN cells resuspended in staining buffer were first incubated with anti-CD16/32 (2.4G2) (UCSF Monoclonal Antibody Core) for at least 20 minutes at 4^{o} C to block non-specific antibody binding. Then, the cell suspensions were stained with a combination of antibodies for 30 minutes at 4^{o} C. The following antibodies were from BD Biosciences: B220-APC-Cy7 (RA3-6B2); CD11c-biotin and CD11c-BUV395 (HL3); CD3-biotin (145-2C11); CD4-PE-Cy7 (RMA4-4); CD40-PE (3/23); CD80-PE (16-10A1); CD86-PE (GL1); CXCR5-biotin (2G8); Ly6G-APC (1A8); NK1.1-biotin, -PE, -PE-Cy7 (PK136); and SiglecF-PE (E50-2440). The following antibodies were from BioLegend: CD11b-PE-Cy7 (M1/70); CD11c-APC, -PerCp-Cy5.5 (N418); CD4-APC-Cy7, -PerCp-Cy5.5 (GK1.5); CD44-APC (IM7); CD49b-PerCp-Cy5.5 (DX5); CD62L-APC-Cy7 (MEL-14); CD8-PerCp-Cy5.5 (53-6.7); CD86-biotin (GL1); γ/δ TCR-APC, -FITC (GL3); human NGFR-PE (ME20.4); I-A^{b}-PerCp-Cy5.5 (AF6-120.1); Ly6C-APC-Cy7 (HK1.4); and PD-1-PE-Cy7 (RMP1-14). The following antibodies were from eBiosciences: CD103-biotin (2E7); CD49b-biotin (DX5), Ly6C-APC-e780, -PerCp-Cy5.5 (HK1.4). The following

antibodies were from Invitrogen: Mouse anti-human CD2-PE, and mouse IgG2a-PE. The following antibodies were either from BD or BioLegend: B220-APC-Cy7 (RA3-6B2); Ly6G-PE (1A8); and CD117-biotin (2B8). The following antibody was from either BioLegend or Tonbo Biosciences: CD3-PE-Cy7 (145-2C11). The tetramer for mCD1d-PE and -APC (PBSH7) was obtained from the NIH Tetramer Core Facility. Biotinylated antibodies were labeled by incubation with streptavidin-Qdot605 (Invitrogen). For samples stained for CXCR5-biotin, after incubation with CD16/32, cells were incubated with CXCR5-biotin at room temperature for 45-60 minutes. After washing, the cells were then were incubated with fluorophore-conjugated antibodies and streptavidin-Qdot605 for 30 minutes at 4^{o} C. Prior to flow cytometry analysis, the cells were resuspended in DAPI (Invitrogen) diluted in staining buffer to facilitate exclusion of dead cells. Flow cytometry was performed using a LSRII (BD).

Flow cytometry data analysis

Data was analyzed using FlowJo software (TreeStar). Representative gating strategies are shown in the figures or in the Materials and Methods (Fig. 2.1 and 2.2). For analysis of lung and BALF cell suspensions, after initial gating to exclude debris, $DAPI^{-}$ and $DAPI^{int}$ cells were gated as "live", because alveolar macrophages and monocytes are autofluorescent, and this $DAPI^{-/int}$ gate was used to extrapolate total live cells. In subsequent gating, other cell types were then identified as "live" based on lack of staining with DAPI (See Fig. 2.1 for representative plots). For analysis of lymph node cell suspensions, after using an initial gate to exclude debris, $DAPI^{-}$ cells were gated as "live", and this gate was used to extrapolate total live cells. Doublets were excluded from

analysis by using FSC-HxFSC-A and SSC-HxSSC-A. Gates for reporter positive cells were based on cells from treated non-reporter control mice. In experiments using the 4get/KN2 reporter (*Il4*$^{4get/KN2}$) mice, percent hCD2^{+} cells was calculated by percentage hCD2^{+} minus percentage hCD2^{+} in non-KN2 reporter mouse (*Il4*$^{4get/4get}$) or percentage hCD2^{+} minus percentage isotype control positive.

For Figure 4.2A-C, two of three experiments used mice that were *Myd88*$^{fl/+}$ *Il4*$^{4get/KN2}$ *Mcpt8*$^{Basopho8/+}$, which is shown in Fig. 2.2A. *Mcpt8*^{Basopho8} mice express both YFP and Cre in basophils (Sullivan et al., 2011). Both GFP from 4get reporter and YFP from Basopho8 reporter were read using the same filter/channel on the flow cytometer, and additional markers were used to distinguish these cell populations.

For gating of cell populations in the BALF (Fig. 4.1B), eosinophils, monocytes and neutrophils were similarly identified as in Fig. 2.4. For gating lymphocytes in the BALF, SiglecF^{+} cells were first excluded, and lymphocytes were identified as follows: B cells (B220^{+}TCRβ^{-}), NK cells (CD49b^{+}B220^{-}TCRβ^{-}) and GFP^{-} to exclude basophils), CD4 T cells (TCRβ^{+}CD4^{+}B220^{-}CD8^{-}), and CD8 T cells (TCRβ^{+}CD8^{-}B220^{-}CD4^{-}).

Quantification of serum OVA-specific antibodies

High-binding polystyrene 96 half-well plates (Corning 3690, Sigma-Aldrich), were coated with OVA in D-PBS at 4^{o} C overnight. 1% BSA in D-PBS was used to block plates, dilute serum samples, and dilute detection antibodies. Plates were washed 3 times in wash buffer (D-PBS/0.05% Tween-20), then blocked for at least 1 hour at room temperature (RT). Plates were washed 3 times in wash buffer and incubated with dilutions of serum samples at 4^{o} C overnight. After overnight incubation, the plates were

washed 5 times with wash buffer, then incubated with anti-total IgG and anti-IgG2c conjugated with horseradish peroxidase (HRP) (Southern Biotech), or anti-IgE-biotin (BD Biosciences) for 1 hour at RT. For detection of anti-IgE-biotin, the plates were washed five times and incubated with streptavidin-HRP (Southern Biotech) for 1 hour at RT. After incubation with HRP-conjugated antibodies or streptavidin, the plates were washed 7 times. A substrate for HRP, 3,3',5,5'-Tetramethylbenzidine (TMB) (Vector Labs or KPL), was added to plates, and the reaction was stopped by adding 2N sulfuric acid. Plates were read on a VERSAmax Microplate Reader (Molecular Devices) at OD 450nm and 570nm. Adjusted absorbance was calculated by subtracting A570 from A450. Relative titers were calculated by first plotting absorbance versus antibody dilutions, and then calculating the antibody dilution (titer) at an absorbance that fell in the linear range of all samples. Relative titers were normalized by running simultaneously pooled sera (standard) that were known to contain OVA-specific antibody isotypes. In the cases where there was no or very low levels of a particular isotype detected in the least diluted serum samples, then titers were calculated using the slope from the standard and absorbance at the lowest dilution.

Quantification of IL-33 protein in whole lungs

At the indicated timepoints after i.n. treatment, whole lungs were harvested into tubes and snap frozen in liquid nitrogen, then stored at -80^{o} C until processing. Whole lungs were homogenized using a disperser/homogenizer (IKA) in 500ul TNT (0.1M Tris-Cl, 150mM NaCl, 0.1% Tween 20) lysis buffer and cOmplete mini protease inhibitors (Roche). The samples were centrifuged at maximum speed in a microcentrifuge to pellet

insoluble contents. Supernatants were transferred to a new tube and protein concentration was assayed using the BCA Protein Assay Kit (Pierce). Amounts of mature IL-33 protein were assayed using Mouse IL-33 DuoSet ELISA Kit (R&D Systems) according to the manufacturer's instructions.

RNA isolation and quantitative PCR of whole lung samples

At the indicated timepoints after i.n. treatment, whole lungs were harvested, lobes divided into two tubes, and snap frozen in liquid nitrogen, then stored at -80^{o} C until processing. Using the RNasy Mini Kit (Qiagen), the contents of each tube were homogenized using a disperser/homogenizer (IKA) in 600µl Buffer RLT (provided in the kit) containing 2-mercaptoethanol. Samples from the same mouse were combined to a total volume of 1200µl, and RNA was isolated from only 700µl. RNA was then isolated according to manufacturer's instructions, with on-column DNase digestion. RNA was reverse transcribed to generate cDNA using iScript cDNA synthesis kit (Bio-Rad). Quantification was performed using iTaq Univeral SYBR Green Supermix (Bio-Rad) on a Step-One Plus real-time PCR machine (Applied Biosystems) using the following primers:

Ccl20 forward 5'-GCCTCTCGTACATACAGACGC-3'and reverse 5'-CCAGTTCTGCTTTGGATCAGC-3'; *Csf2* forward 5'-AGCAGGGTCTACGGGGC-3' and reverse 5'-TGAAATCCGCATAGGTGG-3'; *Hprt* forward 5'-AGGTTGCAAGCTTGCTGGT-3' and reverse 5'-TGAAGTACTCATTATAGTCAAGGGCA-3'; *Il1a* forward 5' CCCATGATCTGGAAGAGACCA-3' and reverse 5'-CAAACTTCTGCCTGACGAGC-

3’; *Il1b* forward 5’-GCCACCTTTTGACAGTGATGAG-3’ and reverse 5’-GACAGCCCAGGTCAAAGGTT-3’; *Il12a* forward 5’-GAATCACAACCATCAGCAGA-3’ and reverse 5’-TGCTTCTCCCACAGGAGG-3’; *Il12b* forward 5’-TGCTGGTGTCTCCACTCAT-3’ and reverse 5’-CTTCAGGCGTGTCACAGG-3’; *Il6* forward 5’-GTTCTCTGGGAAATCGTGGA-3’ and reverse 5’-TGTACTCCAGGTAGCTATGG-3’; *Il33* forward 5’-GCTGCGTCTGTTGACACATTGAG-3’ and reverse 5’-GGTCTTGCTCTTGGTCTTTTCCAG-3’; *Tnfa* forward 5’-TCTTCTGTCTACTGAACTTCGGGGT-3’ and reverse 5’ GGCCATAGAACTGATGAGAGGG-3’; *Tslp* forward 5’-TCGAGGACTGTGAGAGCAAGCCAG-3’ and reverse 5’-CTGGAGATTGCATGAAGGAATACCA-3’

Quantification cycles (Cq) of samples were normalized to *Hprt* (DeltaCq = Cq_{sample}-Cq_{Hprt}), and relative expression ($2^{-deltaCq}$) was calculated and plotted.

Statistical tests

Statistics were performed using Prism 5. One-way anova with Bonferroni post-test was used for all experiments except Fig 3.2E, in which unpaired Student’s *t*-test was used. Outliers identified using Grubb’s test after pooling data from all experiments of one type were removed from analysis and noted in figure legend.

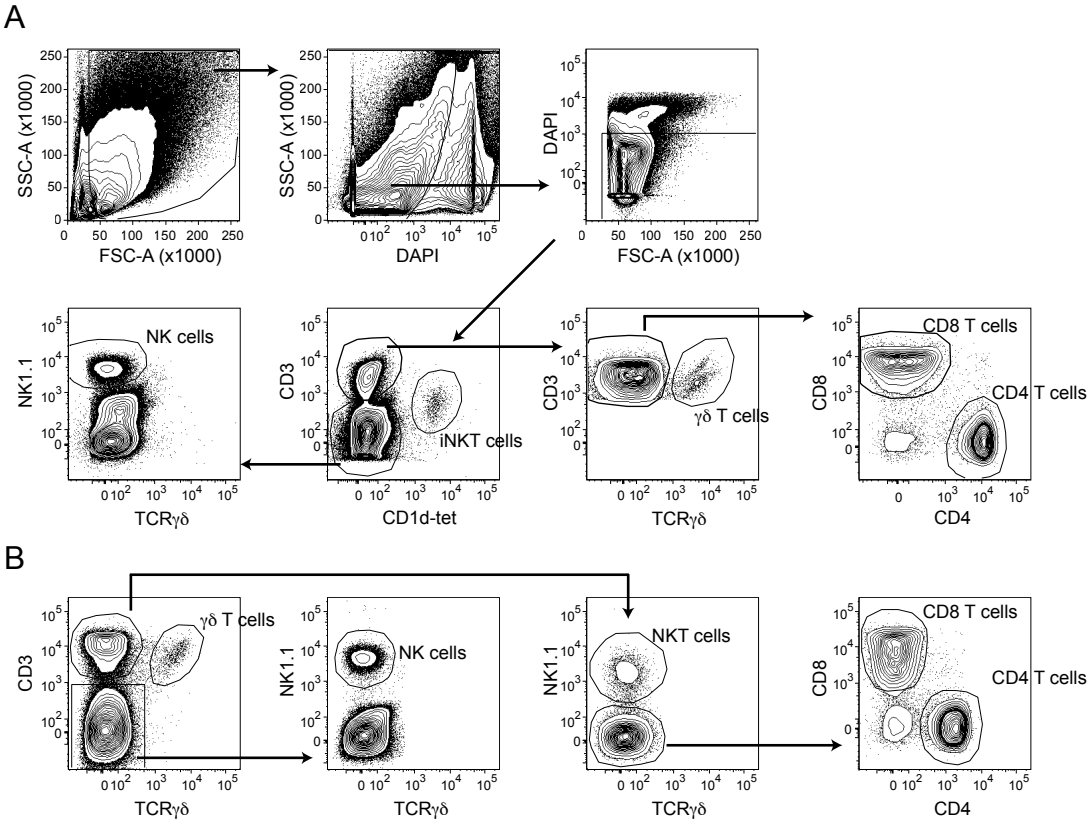

Figure 2.1 Gating strategy for defining lymphocyte populations from the lungs of GREAT and SMART reporter mice.

"Live" cells were gated as described in Materials and Methods. After gating for specific cell populations, the expression of reporter alleles by those cell types was determined, as shown in representative flow cytometry plots in Fig. 3.1 and Fig. 4.2. **(A)** Gating strategy using CD1d-tetramer (CD1d-tet) to identify invariant (i) NKT cells in the experiments shown in Fig. 3.1, 3.3, 4.2G-I, and 4.4B. Cells were identified by the following cell surface markers: iNKT cells (CD1d-tet$^+$CD3$^+$), NK cells (NK1.1$^+$CD3$^-$CD1d-tet$^-$TCRγδ$^-$), γδ T cells (TCRγδ$^+$CD3$^+$CD1d-tet$^-$), CD4 T cells (CD4$^+$CD3$^+$CD1d-tet$^-$TCRγδ$^-$CD8$^-$), and CD8 T cells (CD8$^+$CD3$^+$CD1d-tet$^-$TCRγδ$^-$CD4$^-$). **(B)** Gating strategy using NK1.1 and CD3 to identify NKT cells in the experiments shown in Fig. 4.2D-F and 4.4C. For

these experiments, cells were identified by the following cell surface markers: γδ T cells ($TCR\gamma\delta^+CD3^+$), NK cells ($NK1.1^+TCR\gamma\delta^-CD3^-$), NKT cells ($NK1.1^+CD3^+TCR\gamma\delta^-$), CD4 T cells ($CD4^+CD3^+TCR\gamma\delta^-NK1.1^-CD8^-$), and CD8 T cells ($CD8^+CD3^+CD1d\text{-}tet^-TCR\gamma\delta^-NK1.1^-CD4^-$).

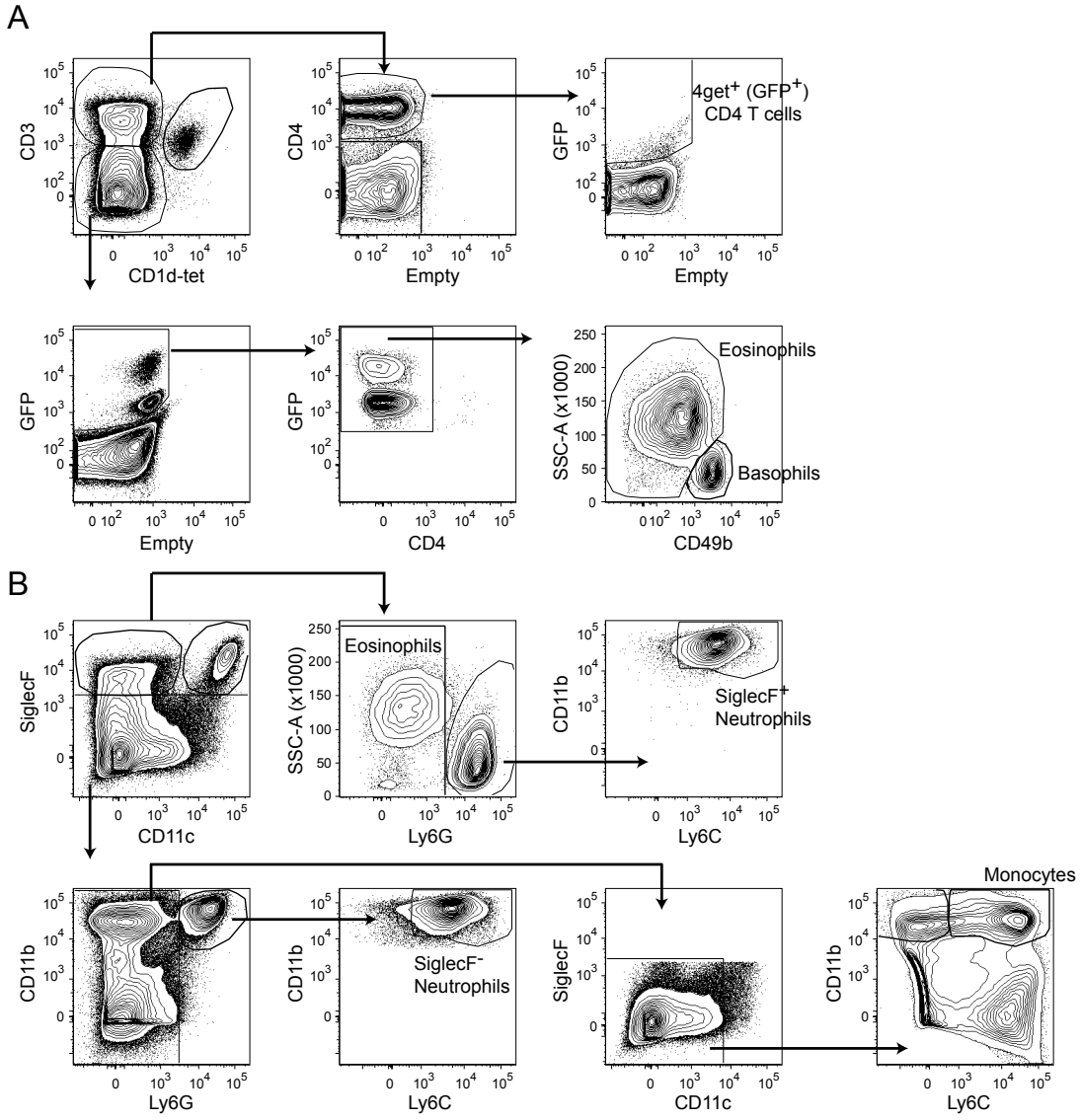

Figure 2.2 Gating strategy for 4get reporter$^+$ cells and non-lymphocyte populations in the lungs and BALF

"Live" cells were gated as described in Materials and Methods. **(A)** Gating strategy for 4get reporter$^+$ CD4 T cells, basophils, and eosinophils in the lungs of 4get/KN2 reporter mice as shown in Fig. 4.2A-C. Cells were identified by using the following markers: 4get$^+$(GFP$^+$) CD4 T cells (GFP$^+$CD4$^+$CD3$^+$CD1d-tet$^-$) and basophils (CD49b$^+$SSC^{lo}GFP$^+$CD3$^-$CD1d-tet$^-$CD4$^-$), and eosinophils (GFP$^+$CD3$^-$CD1d-tet$^-$CD4$^-$

CD49b$^-$). Basophils and eosinophils are constitutively 4get$^+$ (Voehringer et al., 2004). After gating for specific cell populations, they were gated for expression of IL-4 protein reporter, human CD2, as shown in representative flow cytometry plots in Fig. 4.2B. The gating strategy shown is from *Myd88*$^{fl/+}$ *Il4*$^{4get/KN2}$ *Mcpt8*$^{Basopho8/+}$. *Mcpt8*^{Basopho8} mice express both YFP and Cre in basophils (Sullivan et al., 2011). Both GFP from 4get reporter and YFP from Basopho8 reporter were read using the same filter/channel on the flow cytometer, and additional markers were used to distinguish these cell populations as described above. **(B)** Gating strategy for identify non-lymphocyte populations in the lungs and BALF in experiments shown in Fig. 4.1B and Fig. 4.4A. Cells were identified by using the following cell surface markers: eosinophils (SiglecF$^+$CD11b$^+$CD11c$^-$Ly6G$^-$), neutrophils (SiglecF$^+$Ly6G$^+$Ly6C$^+$CD11b$^+$CD11c$^-$ and Ly6G$^+$Ly6C$^+$CD11b$^+$SiglecF$^-$), monocytes (Ly6C$^+$CD11b$^+$CD11c$^{-/int}$SiglecF$^-$Ly6G$^-$).

Chapter 3 : MyD88 signaling in DCs is required for CpG oligodeoxyribonucleotide-induced T_H1 response in the lungs

Introduction

In order for vaccines to provide effective immunity against pathogens, adjuvants are often added to increase the magnitude of the immune response and to direct the most appropriate type of immune response (Coffman et al., 2010). Recent progress in understanding innate immunity have made it clear that the immune system has evolved to express receptors that recognize conserved microbial molecules. These receptors can induce a rapid inflammatory response that aims to control the invading pathogen. They can also promote the priming of the adaptive immune response, which is often specialized to fight a particular type of infection (Medzhitov, 2007). These conserved molecules of pathogens are called pathogen-associated molecular patterns (PAMPs). Many PAMPs have strong adjuvant activity and are currently being tested for use in vaccines (Coffman et al., 2010).

One of the first discovered and best-studied families of receptors recognizing PAMPs is the Toll-like receptor (TLR) family (Kawai and Akira, 2011). At least ten TLRs in humans and twelve TLRs in mice have been identified. TLR1, 2, 4, 5, and 6 are expressed on the surface of various types of cells and recognize conserved PAMPs from the cell walls of bacteria. In contrast, TLR 3, 7, 8, and 9 are expressed in endocytic compartments of various immune cells and recognize bacterial and viral nucleic acids. TLR9 recognizes unmethylated CpG motifs within DNA, which are present in bacterial and viral DNA. In contrast, in vertebrate DNA, these motifs are underrepresented and

frequently methylated as a mechanism of gene repression, which make them non-stimulatory. Moreover, localization of TLR9 within the endocytic pathway limits its exposure to self DNA and its auto-inflammatory potential (Ewald et al., 2008; Kawai and Akira, 2011). TLR9 is stimulated strongly by various synthetic CpG motif containing oligodeoxyribonucleotides (CpG ODN), including those with phosphorothioate linkages to improve their in vivo stability, and these molecules have been shown to have potent adjuvant properties (Krieg, 2002). Immunization of mice with an antigen plus stimulatory CpG ODN induces robust T_H1 responses when administered systemically or subcutaneously (Krieg, 2002), and can provide protection against a wide variety of pathogens (Klinman, 2006).

Immunization with CpG ODN as adjuvant also has the propensity to drive T_H1 responses in the lungs after intranasal (i.n.) administration (Chen et al., 2006; Pesce et al., 2010). This treatment was associated with production of IL-12 p40 and IFN-γ mRNA in the lungs within hours, although which cells expressed these cytokine mRNAs *in vivo* remains uncertain (Pesce et al., 2010). A number of studies have examined the ability of i.n. administration of CpG ODN to modulate existing immune responses in animal models of asthma (Fonseca and Kline, 2009) or to induce protection against subsequent respiratory infections (Bozza et al., 2002; Deng et al., 2004; Edwards et al., 2005). Although it is clear that use of CpG ODN as an adjuvant for immunization via the airways can drive type 1 immunity, the mechanisms underlying this polarization remain poorly defined.

In the studies described here, I have characterized the innate and adaptive response in the lungs resulting from i.n. sensitization of mice with the model antigen

ovalbumin (OVA) plus CpG ODN and the mechanisms involved in the resultant immune response. By day 1 after immunization, both lung dendritic cells (DCs) and migratory DCs in the draining lymph node (LN) had strongly upregulated the maturation markers CD40, CD80 and CD86. By day 3, multiple T cell and innate lymphoid cell types had upregulated IFN-γ production. Pretreatment with anti-IL-12 largely blocked this innate type 1 immune response. Subsequent rechallenge with OVA alone two weeks after sensitization led to a robust inflammatory response in the lung that included T_H1 cells and IFN-γ-producing CD8 T cells. These innate and adaptive type 1 responses were largely dependent upon myeloid differentiation primary response 88 (MyD88) signaling in DCs, suggesting direct recognition of the CpG ODN adjuvant by TLR9 on DCs, resulting in IL-12 production, which in turn promoted innate and adaptive production of IFN-γ.

Results

CpG ODN promotes a T_H1 response in the lungs

CpG ODN that are capable of stimulating TLR9 have been found to be excellent adjuvants for adaptive immune responses (Coffman et al., 2010). To study the adjuvant effects of a CpG ODN on the adaptive immune response in the lungs, I modified a previously used model of i.n. immunization (Eisenbarth et al., 2002; Herrick et al., 2000). Mice were sensitized by i.n. exposure to the antigen OVA or to OVA plus CpG ODN on three successive days and after 2 weeks, were re-challenged once with OVA alone. Mice sensitized in this way produced higher titers of OVA-specific total IgG than mice sensitized with OVA alone, and in particular, produced the IFN-γ-inducible isotype, IgG2c (Fig. 3.1A). To examine which cells produced IFN-γ in this model, I used the GREAT IFN-γ reporter mice, which express YFP from the IFN-γ locus if cells have recently produced IFN-γ (Reinhardt et al., 2009). Although the numbers of CD4 and CD8 T cells present in lung tissue 1 day after antigen rechallenge (d16) were not greatly altered by inclusion of CpG ODN during sensitization (Fig. 3.1B), the mice that had been sensitized with CpG ODN and rechallenged with OVA had greater percentages and numbers of IFN-γ-producing CD4 and CD8 T cells in the lung (Fig. 3.1C, D). These results indicate that the presence of CpG ODN as adjuvant promoted a strong T_H1 polarization of the humoral and cell-mediated response to the co-administered antigen OVA.

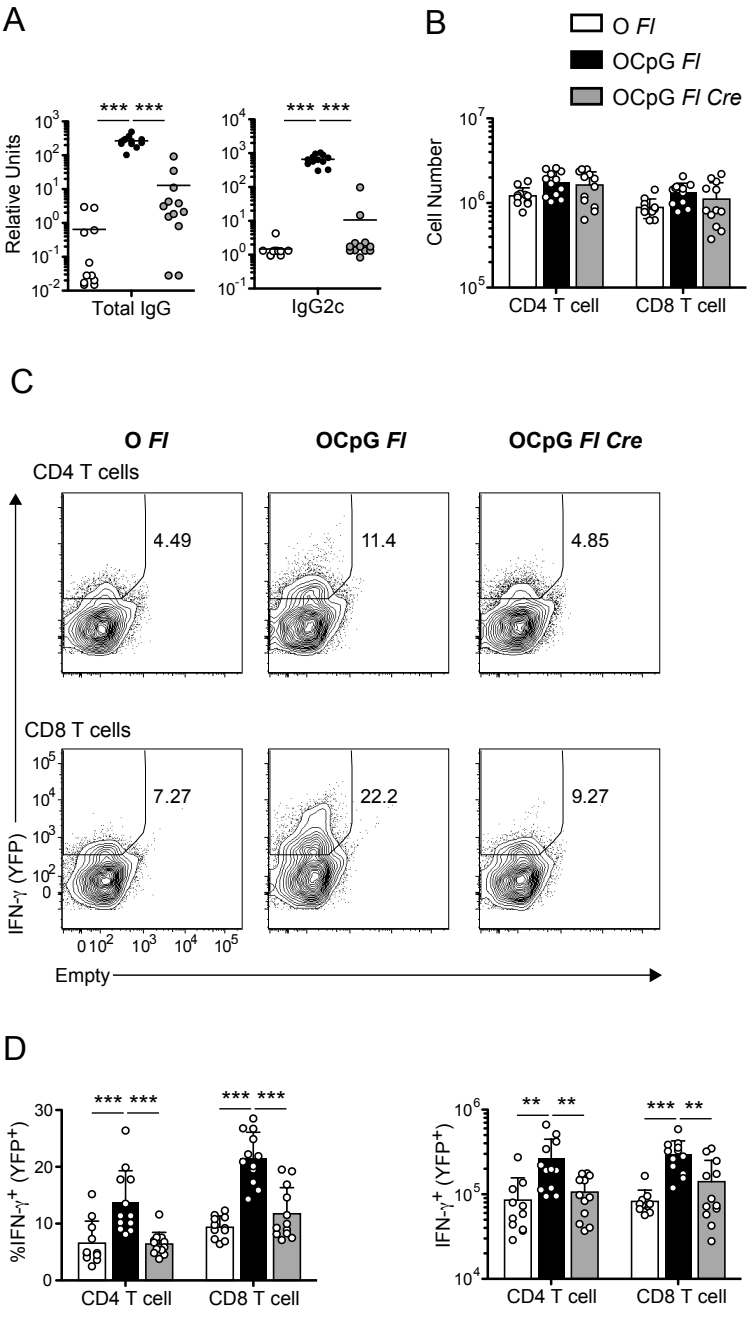

Figure 3.1 CpG ODN leads to a T_H1 response in the lungs that is dependent on MyD88 signaling in DCs.

GREAT reporter mice expressing MyD88 (*Myd88*$^{fl/fl}$ *Ifng*$^{GREAT/GREAT}$ (*Fl*) mice – white and black bars) and reporter mice that are deficient for MyD88 selectively in DCs and

alveolar macrophages (*Myd88*$^{fl/fl}$ *Ifng*$^{GREAT/GREAT}$ *CD11c-Cre* (*Fl/Cre*) mice – gray bars) were sensitized i.n on d 0, 1 and 2 with OVA (O) (white bars) or OVA plus 0.75μg CpG (OCpG) (black and gray bars). On d15, the mice were challenged i.n. with OVA and immune responses were analyzed on d16. **(A)** Serum levels of OVA-specific IgG (total) and IgG2c were measured by ELISA. **(B)** Numbers of CD4 and CD8 T cells obtained from lung tissue on d16. **(C)** Representative flow cytometry plots of IFN-γ reporter (YFP) expression by CD4 (top row) and CD8 (bottom row) T cells from lung. **(D)** Percentages (left panel) and numbers (right panel) of IFN-γ reporter^{+} CD4 and CD8 T cells. Data are pooled from three independent experiments with totals of 11-12 mice per group. 1 outlier mouse treated with OVA was removed from the analysis, as described in the Methods section. Each circle represents one individual mouse. Error bars indicate mean + SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

MyD88 signaling in DCs is required for CpG ODN-induced DC activation and for priming the T_H1 response

DCs may promote T_H1 responses after TLR9 stimulation, as this innate receptor strongly promotes maturation of DCs to a highly stimulatory phenotype and additionally can induce synthesis by DCs of cytokines such as IL-12 that favor T_H1 responses (Hou et al., 2008; Spörri and Reis e Sousa, 2005). One day after the first i.n. sensitization with CpG ODN plus OVA, there were increased numbers of DCs and monocytes in the lung (Fig. 3.2A, B), and these cells had elevated cell surface expression of the costimulatory molecule CD86 (Fig. 3.2C, D). In addition, increased levels of mRNA encoding IL-12 p40 were detected in the lungs of mice that received CpG ODN during initial sensitization (Fig. 3.2E)

OVA fluorescently-labeled with Alexa Fluor 647 was used to track the migratory DC that had taken up the antigen and migrated to the mediastinal LN 1 day later. I.n. administration of a single dose of CpG ODN plus OVA led to the upregulation of CD40, CD80 and CD86 on the surface of migratory DCs and these molecules were upregulated to a greater degree on the migratory DCs that had taken up fluorescently labeled OVA (Fig. 3.2G, H). Taken together, these data indicate that i.n. CpG ODN administration induced maturation of the DCs of the lung and likely stimulated them to produce IL-12.

To gain insight into the mechanisms by which TLR9 promoted the activated phenotype of migratory DCs and T_H1 response, I used *Myd88$^{fl/fl}$ CD11c-Cre* mice, in which MyD88, a signaling adaptor molecule required for TLR9 signaling, was selectively deleted from DCs and from alveolar macrophages (Abram et al., 2014; Hou et al., 2008). In *Myd88$^{fl/fl}$ CD11c-cre* mice, >96% of conventional DCs and ~80% of plasmacytoid

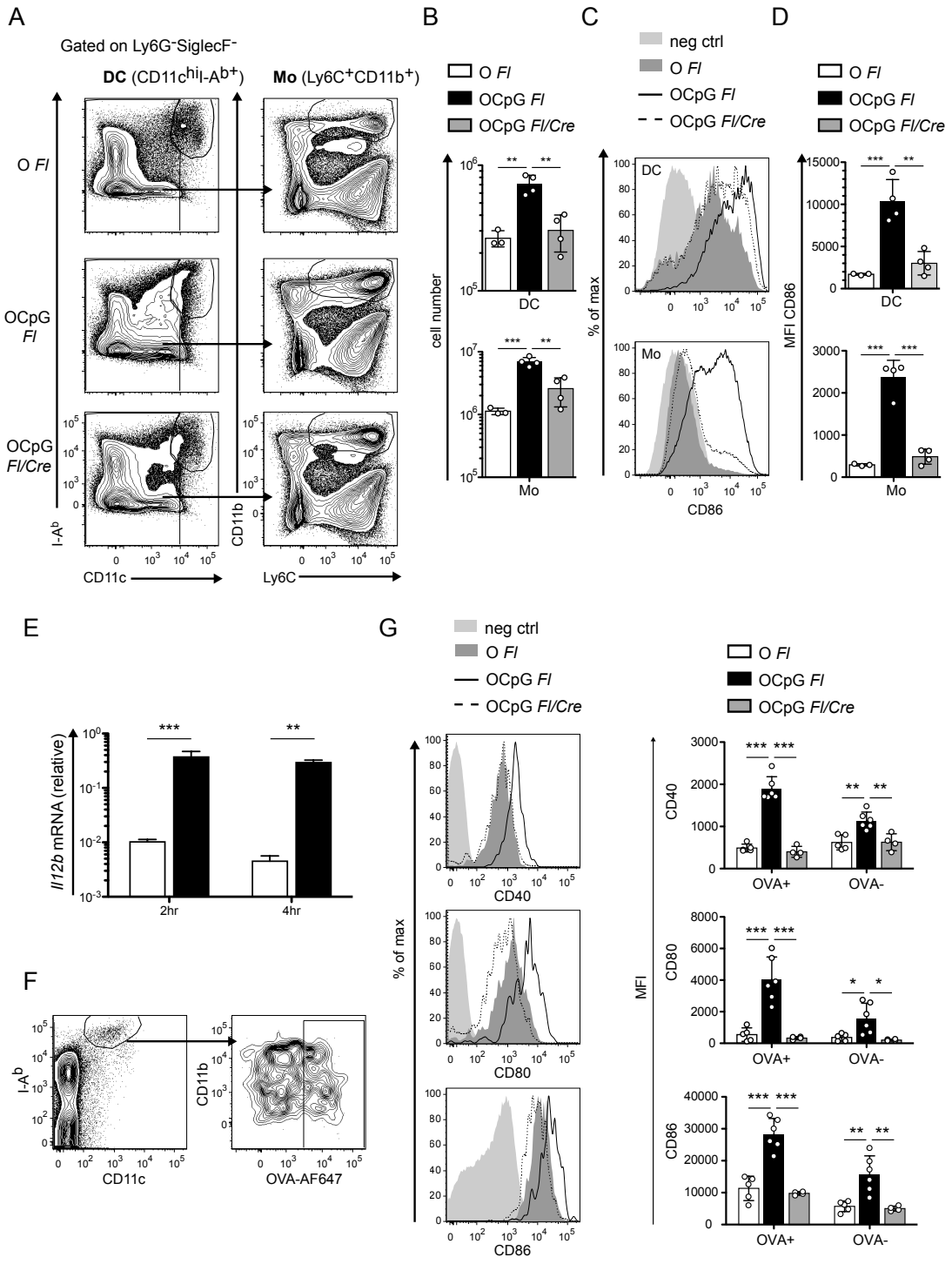

Figure 3.2 MyD88 signaling in DCs is required for DC activation in response to CpG ODN

Figure 3.2 MyD88 signaling in DCs is required for DC activation in response to CpG ODN

(A-D) Mice expressing MyD88 (*Myd88*$^{fl/fl}$ (*Fl*) – white and black bars) or deficient for MyD88 selectively in DCs and alveolar macrophages (*Myd88*$^{fl/fl}$ *CD11c-Cre* (*Fl/Cre*) – gray bars) were sensitized a single time i.n. with OVA (white bars) or OVA plus 3μg CpG ODN (black and gray bars) and analyzed one day later. **(A)** Representative flow cytometry plots and gating strategy for DCs (CD11c^{+}I-A^{b+}) (left column) and monocytes (Mo) (Ly6C^{+}CD11b^{+}) (right column) in the lungs. **(B)** Numbers of lung DCs (top row) and monocytes (bottom row). **(C)** Representative histograms of CD86 expression on lung DCs (top row) and monocytes (bottom row). CD86 expression on Ly6G^{+}SiglecF^{+}CD11c^{-} cells (neutrophils and eosinophils) was used as the negative control histogram. **(D)** Level of expression (MFI) of CD86 on lung DCs (top row) and Mo (bottom row). **(E)** *Il12b* gene induction measured by quantitative PCR of RNA from whole lung tissue at indicated time points after i.n. administration of OVA or OVA plus CpG (3μg). Samples were normalized to *Hprt* mRNA. **(F-H)** *Myd88*$^{fl/fl}$ (*Fl*) and *Myd88*$^{fl/fl}$ *CD11c-Cre* (*Fl/Cre*) mice were exposed i.n. to AlexaFluor647-labeled OVA (OVA-AF647) or OVA-AF647 plus 0.75μg CpG ODN and one day later, migratory DCs from the mediastinal LNs were analyzed for maturation markers. **(F)** Gating strategy for migratory DCs (CD11c^{+}I-A$^{b\ hi}$) from the mediastinal LN that had taken up OVA-AF647. **(G)** Representative histograms of CD40 (top row), CD80 (middle row), and CD86 (bottom row) expression on migratory DCs isolated from the mediastinal LN on d1. Activation marker expressions on CD11c^{-}I-A^{b-} cells were used as negative control histograms. **(H)** Level of expression (MFI) of activation markers on migratory DCs that did or did not take up fluorescent OVA. Data

(A-D) are representative of one of three independent experiments with 3-4 mice per group, data in **(E)** are representative of one of two independent experiments with 4-5 mice per group, and data in **(F-H)** are representative of one of two independent experiments with 4-6 mice per group. Each circle in **(B, D, H)** represents an individual mouse. Error bars indicate mean +SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test or unpaired t-test in **(E)**.

DCs (pDC) lack expression of MyD88 (Hou et al., 2008). Remarkably, in these mice, the T_H1 polarization induced in response to i.n. administration with OVA plus CpG ODN were substantially decreased, both the adaptive cellular immune response, as assessed by IFN-γ production with the GREAT reporter allele, and the class switch to IgG2c (Fig. 3.1). In addition, *Myd88$^{fl/fl}$ CD11c-Cre* mice had attenuated infiltration of the lungs with DCs and monocytes after CpG ODN treatment (Fig. 3.2A-D). Moreover, lung DCs and migratory DCs in the mediastinal LN of these mice had greatly attenuated acquisition of maturation markers (Fig. 3.2F-H).

Early IFN-γ production induced by CpG ODN is dependent on MyD88 signaling in DCs

In addition to the T_H1 response induced by i.n. sensitization with OVA plus CpG ODN, mice sensitized in this way also made IFN-γ during the innate phase of the response. For example, one day after the third i.n. administration of OVA plus CpG ODN, the GREAT reporter was expressed in a variety of T cell and innate lymphoid cell types, and the fraction of reporter positive CD8 T cells, CD4 T cells, γδ T cells, invariant natural killer T (iNKT) cells, and natural killer (NK) cells were all increased in response to OVA plus CpG ODN compared to OVA alone (Fig. 3.3). This innate IFN-γ response was highly dependent on MyD88 signaling in DCs and/or alveolar macrophages as revealed by immunization of the *Myd88$^{fl/fl}$ CD11c-Cre* mice i.n. with OVA plus CpG ODN (Fig. 3.3). Thus, MyD88 signaling in DCs and/or alveolar macrophages was required not only for adaptive IFN-γ production, but also for innate IFN-γ production. Interestingly, when the *Myd88$^{fl/fl}$ CD11c-Cre* mice were immunized with OVA plus a higher dose of CpG ODN (3μg instead of 0.75μg), then innate production of IFN-γ as

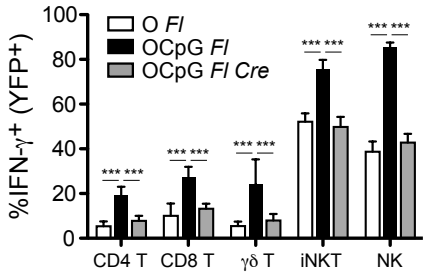

Figure 3.3 Early IFN-γ production induced by i.n. CpG ODN is dependent on MyD88 signaling in DCs.

GREAT reporter mice expressing MyD88 (*Myd88*$^{fl/fl}$ *Ifng*$^{GREAT/GREAT}$ (*Fl*) – white and black bars) or reporter mice that are deficient for MyD88 selectively in DCs and alveolar macrophages (*Myd88*$^{fl/fl}$ *Ifng*$^{GREAT/GREAT}$ *CD11c-Cre* (*Fl/Cre*) – gray bars) were administered i.n. with OVA (white bars) or OVA plus 0.75μg CpG ODN (black and gray bars) on d0, 1, and 2. One day later, expression of the *GREAT* IFN-γ reporter (YFP) was determined in different cell types from digested lung tissue. Data are pooled from two independent experiments with 5 or 8 mice per group. Error bars indicate mean +SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

assessed with the GREAT reporter was affected to a much lesser degree (data not shown). These results suggest that higher CpG ODN levels induced more cytokines from cells other than classical DCs and that these cytokines could promote the innate IFN-γ response to some degree by a secondary pathway.

IFN-γ production induced by CpG ODN is partially dependent on IL-12

IL-12, which can be produced by DCs after TLR stimulation, is a potent inducer of IFN-γ (Trinchieri, 2003). To test whether IL-12 was important for the innate and/or adaptive IFN-γ production induced by i.n. immunization with OVA plus CpG ODN, mice were pre-treated with neutralizing antibody against IL-12 p40 and then sensitized by i.n. administration of OVA plus CpG ODN on three successive days. Anti-IL-12 pretreatment resulted in a marked reduction of IFN-γ production in the various lymphocyte populations one day after the last i.n. exposure compared to mice treated with control antibody (Fig. 3.4A). Therefore, IL-12 was an important contributor to the innate phase IFN-γ production seen on day 3.

I next examined the importance of IL-12 for polarization of CD4 T cells into T_H1 cells during the adaptive immune response. Mice were pretreated with anti-IL-12 sensitized with OVA plus CpG ODN, and then rechallenged with OVA, as before. The antibody response was not greatly affected by anti-IL-12 treatment with regard to titer of anti-OVA IgG or degree of isotype switching to IgG2c (Fig. 3.4B). In contrast, the percentage of IFN-γ-producing CD4 T cells in the lung after antigen rechallenge were decreased in mice pretreated with anti-IL-12 antibody, compared to mice treated with

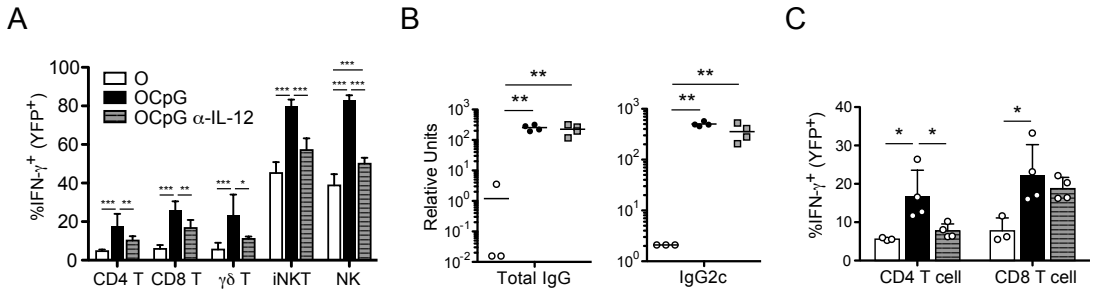

Figure 3.4 CpG ODN-induced IL-12 is largely responsible for the innate IFN-γ response and adaptive T_H1 response.

GREAT reporter mice (*Ifng*$^{GREAT/GREAT}$) were treated with anti-IL-12 p40 (gray striped bars, gray squares) to block IL-12 bioactivity or with control antibody (rat IgG2a) (white and black bars, white and black circles), one day before initial sensitization (d-1; 700μg i.p.) **(A-C)** and again on d2 (300μg i.p.) **(B, C).** These mice were then sensitized i.n. with OVA (white bars and circles) or OVA plus CpG ODN (0.75ug) (black bars and circles, gray striped bars and squares) on day 0, 1 and 2 (**A-C**) and re-challenged on d15 with OVA alone (**B**, **C**). **(A)** Percentages of IFN-γ reporter$^+$ lymphocytes in the lungs on d3. **(B)** Serum levels of OVA-specific IgG (total) (left graph) and IgG2c (right graph) were measured by ELISA on d16. **(C)** Percentages of IFN-γ reporter$^+$ CD4 and CD8 T cells from lung on d16. Each circle or square represents one individual mouse. Data in (**A**) are pooled from two independent experiments with 6-7 mice per group, and similar results were obtained in a third independent experiment, and data in (**B, C**) are representative of one of three independent experiments with 3-4 mice per group. Error bars indicate mean +SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

control antibody (Fig. 3.4C). Anti-IL-12 pretreatment did not have an effect on the percentage IFN-γ -producing CD8 T cells after antigen rechallenge. Thus, some elements of the adaptive immune response (T_H1 response) were inhibited by blockade of IL-12, but other elements (IgG2c production and IFN-γ production by CD8 T cells) were much less affected suggesting that either these responses required lower levels of IL-12 or they were sufficiently promoted by other cytokines, such as type 1 IFNs.

Discussion

The experiments presented here demonstrate that sensitization via the nasal passages with the model antigen OVA plus CpG ODN as adjuvant, and subsequent rechallenge with OVA led to a T_H1 response in the lungs and increased serum OVA-specific IgG2c. Early after CpG ODN and OVA i.n. administration, increased numbers of DCs and monocytes were present in the lungs. CpG ODN treatment led to the elevation of costimulatory molecules on DCs and monocytes in the lungs, and on the migratory DCs in the draining mediastinal LN. I.n. CpG ODN was also able to induce early IFN-γ production in both innate-like lymphocytes such as γδ T cells, iNKT cells, and NK cells, and also in adaptive CD4 T cells and CD8 T cells. Strikingly, these innate and adaptive immune responses were dependent on MyD88 signaling in DCs, and most production of IFN-γ was dependent on IL-12 produced during the innate phase of the response. Based on these results, I suggest that DCs directly sense CpG ODN in the lungs via TLR9 and that this recognition both induces their maturation and their production of IL-12, which promotes innate production of IFN-γ and the T_H1 response.

The simplest model to explain our data is that TLR9-expressing conventional DCs in the lung directly sense CpG ODN and represent the key cell directly responding to this adjuvant to promote the T_H1 response in the lungs. In agreement with this hypothesis, several studies have shown that lung DCs were responsive to CpG ODN *in vitro*. For example, lung DCs stimulated with CpG ODN *in vitro* upregulated activation markers and promoted proliferation of allogeneic T cells (Chen et al., 2006). Another study showed that purified $CD11c^+$ lung cells stimulated with CpG ODN *in vitro* produced similar levels of cytokines compared to total lung cells stimulated with CpG ODN,

suggesting that $CD11c^+$ cells are the primary producers of cytokines in the lungs (Pesce et al., 2010). Moreover, TLR-stimulated DCs are rapid and potent producers of IL-12 (Hou et al., 2008, 2011), which was required for much of the innate IFN-γ production as well as the presence of activated T_H1 cells following re-challenge with OVA.

This hypothesis can explain why deletion of *Myd88* with CD11c-Cre greatly inhibited the innate and adaptive immune responses seen to i.n. CpG ODN plus OVA, since CD11c-Cre deletes floxed alleles primarily in conventional DCs and pDCs (Hou et al., 2008). It should be noted, however, that CD11c-Cre also deletes floxed alleles efficiently in alveolar macrophages (Abram et al., 2014), which express high levels of CD11c, so the defect seen in the *Myd88$^{fl/fl}$ CD11c-Cre* mice could reflect a necessary role for alveolar macrophages. There are conflicting reports as to whether alveolar macrophages express TLR9 and are capable of responding to CpG ODN *in vitro* (Pesce et al., 2010; Suzuki et al., 2005), so it is unclear whether or not alveolar macrophages contributed to the responses seen.

Both TLR and IL-1R family signaling use the MyD88 signaling adaptor, so the defect in the CpG ODN-induced type 1 immune response in *Myd88$^{fl/fl}$ CD11c-Cre* mice could be due to a defect in IL-1R family signaling in DCs or alveolar macrophages, rather than a defect in TLR9 signaling in these cells. IL-1α and IL-1β protein have been detected in lung homogenates after i.n. CpG ODN and in purified lung DCs stimulated with CpG ODN *in vitro* for 24 hours (Pesce et al., 2010). Similarly, I was also able to detect IL-1α and IL-1β transcripts 2 hours after CpG ODN stimulation in the lungs that showed a trend toward increased expression that was not statistically significant compared to OVA stimulation alone (Chapter 4). Thus, it is possible that these cytokines

act on DCs to amplify the response. I do not favor this explanation based on previous studies from Sporri and Reis e Sousa (2005), who used a mixed bone marrow chimera approach to demonstrate that direct recognition of CpG ODN by TLR9 on DCs was required to get a T_H1 response to systemic immunization with antigen plus CpG ODN. In their experimental system, cytokines were able to induce maturation of $TLR9^{-/-}$ DC, leading to clonal explanation of CD4 T cells, but these DCs did not produce IL-12 nor induce CD4 T cells to produce IFN-γ.

When I used a neutralizing anti-IL-12 p40 antibody to block IL-12 action in mice sensitized with OVA plus CpG ODN, I saw a reduction in early IFN-γ production and T_H1 polarization, but this inhibition was less than the major defect seen in the *Myd88*$^{fl/fl}$ *CD11c-Cre* mice. Moreover, the IgG2c response remained intact in the anti-IL-12 p40 treated mice. These data suggest that other cytokines, besides IL-12, may be involved in promoting type 1 immunity in response to CpG administered i.n. The type I IFNs are attractive candidate for cytokines that contributed to the type 1 immune responses that persisted in the presence of blocking anti-IL-12 p40 antibody. Type I IFN may also be produced in the lungs after i.n. administration of CpG ODN. Besides promoting an antiviral state in cells, type I IFNs has been shown to influence subsequent innate and adaptive immunity (Iwasaki and Medzhitov, 2004). Immunization with the antigen chicken gamma globulin and type I IFNs can lead to antibody responses of all subclasses of IgG, including IgG2a (Le Bon et al., 2001). Interesting in this regard, *Ifnα/βr*$^{-/-}$ mice immunized intradermally with CpG ODN and antigen had defective cytotoxic lymphocyte specific lysis and IFN-γ production in restimulated splenocytes compared to control mice (Van Uden et al., 2001). pDCs express both TLR7 and TLR9 and respond

robustly to CpG ODN by producing type I IFN and other cytokines such as TNF (Hou et al., 2008; Kawai and Akira, 2011). CD11c-Cre deletes floxed genes at 80% efficiency in pDCs, so the response of these cells would be attenuated but not totally absent in *Myd88$^{f/f}$ CD11c-Cre* mice (Hou et al., 2008). Previously, it has been shown that systemic administration of CpG ODN can lead to rapid induction of genes encoding type I IFN, IFN-α and IFN-β, in the spleen (Hou et al., 2008).

Interestingly, there was a strong defect in innate IFN-γ production by CpG ODN-stimulated *Myd88$^{fl/fl}$ CD11c-Cre* mice on d1 after a higher dose of CpG ODN (3μg) (data not shown) or on d3 after three daily treatments with a lower dose of CpG ODN (0.75μg each day), but when these mice were treated with the higher dose for three days, they retained a substantial response (data not shown). One explanation for these observations is that at higher doses, the non-deleted 20% of pDCs produced enough type 1 IFNs to take the place of IL-12 from conventional DCs to induce IFN-γ production.

Interestingly, although TLR4, TLR5, and TLR9 all signal through MyD88, they can promote distinctive immune responses in the lungs. In previous studies, i.n. immunization of mice with either antigen and a low dose of the TLR4 ligand lipopolysaccharide (LPS) (Eisenbarth et al., 2002) or with antigen and various doses of the TLR5 ligand flagellin (Van Maele et al., 2014; Wilson et al., 2012) induced robust T_H2 responses in the lungs after rechallenge with antigen, whereas i.n. immunization with antigen and a high dose of LPS induced a T_H1 response (Eisenbarth et al., 2002), similarly to CpG ODN in this study. The reasons for this difference remain to be fully elucidated, but one possibility relates to whether or not the lung epithelial cells and/or conventional DCs directly respond to the adjuvant. Lung epithelial cells are known to

release or produce cytokines that promote T_H2 responses, such as TSLP and IL-33. These cells express TLR4 (Monick et al., 2003; Sha et al., 2004; Zhang et al., 2005) and TLR5 (Prince, 2006; Wilson et al., 2012) but it is unclear whether or not they express TLR9 (Cho et al., 2006; Pesce et al., 2010). T_H2-polarizing cytokines, IL-25, IL-33, and TSLP (Bartemes and Kita, 2012), were found in the lung airspace (Wilson et al., 2012) or lung tissue (Chapter 4) after i.n. administration of flagellin. I.n. administration of low and high doses of LPS Both induced thymic stromal lymphopoietin (TSLP) mRNA in the lungs (Tan et al., 2010). In contrast, CpG ODN did not induce nearly as much TSLP mRNA or mature IL-33 release as did flagellin (Chapter 4). Thus, the low level of T_H2-polarizing cytokines from the lung epithelium after CpG ODN administration may have contributed to the lack of a T_H2 response. In addition, the strong IL-12 production that was induced by CpG ODN, probably through direct recognition by DCs, combined with the potent ability of IL-12 to induce innate IFN-γ production may have greatly favored a T_H1 response in the draining LN. Though mice sensitized to CpG ODN had a trend towards increased mRNA encoding TSLP that was not statistically significant, this presumably was not able to overcome the dominant effect of high IL-12 and/or IFN-γ production resulting from TLR9 stimulation of DCs.

In conclusion, I have shown that CpG ODN acts as an adjuvant in the lungs and induces innate and adaptive immune responses with strong polarization to type 1 immunity. Excellent candidates for vaccines provide both humoral immunity and generate protective CD8 T cell responses (Coffman et al., 2010). I have shown that CpG ODN leads to both increased levels of IgG and IFN-γ producing CD8 T cells. Therefore immunization via the nasal passages may provide strong protection against many

infectious agents not only locally and at other mucosal sites, but also systemically. This type of vaccination may be advantages compared to more conventional immunization routes, such as intramuscular (Neutra and Kozlowski, 2006). Future studies should address whether CpG ODN, when used as adjuvant in the lungs, can provide better protection compared to immunization at other sites.

Chapter 4 : Flagellin-induced type 2 immune response is partially dependent on MyD88 signaling in dendritic cells and is distinct from CpG ODN-induced type 1 immune response in the lungs

Introduction

The mammalian immune system can mount several different types of innate and adaptive responses, each of which are specialized to combat different types of infections. The choice of the type of immune response depends on a variety of factors, including which innate immune receptors recognize conserved pathogen molecules, or pathogen-associated molecular patterns (PAMPs), on the invading pathogen. Often, Toll-like receptor (TLR) recognition is associated with type 1 innate and adaptive immune responses (Medzhitov, 2007), and this is in part due to IL-12 production by TLR-stimulated dendritic cells (DCs) (Chapter 3; Hou et al., 2008; Iwasaki and Medzhitov, 2004; Spörri and Reis e Sousa, 2005). IL-12, in turn, promotes IFN-γ production by innate lymphocytes, such as natural killer (NK) cells (Trinchieri, 2003), and also helps stabilize T_H1 polarization during the initial activation of naïve CD4 T cells (Szabo et al., 2003).

How allergens and other type 2 stimuli, such as parasitic worm infections, induce type 2 immunity have been intensely studied, but it is still unclear which pathways are critical for driving type 2 immunity (Allen and Sutherland, 2014; Locksley, 2010; Pulendran and Artis, 2012). This issue has frequently been studied in the context of inhaled antigens, due to the relevance of such studies to understanding the development of human asthma (Hammad and Lambrecht, 2008). A subset of allergens, such as papain,

Aspergillus extracts, ragweed pollen, and birch pollen, seem to derive their ability to induce type 2 immunity in the lungs from inherent protease activity which in some cases may act through members of the protease-activated receptor family (Hammad and Lambrecht, 2008; Pulendran and Artis, 2012). Other classes of allergens do not contain protease activity and alert the immune system in alternative ways, for example, chitin, a widespread environmental polysaccharide has been associated with occupational asthma in humans (Cartier et al., 2004), and induces a robust type 2 innate immune response in the lungs of mice (Van Dyken et al., 2014; Reese et al., 2007).

The cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) have emerged as important inflammatory cytokines that can drive type 2 immunity (Lambrecht and Hammad, 2014; Locksley, 2010; Saenz et al., 2008). Studies suggest these cytokines are primarily made by epithelial cells present at environmental interfaces, but how allergens induce secretion of these cytokines and how they induce T_H2 responses remain poorly understood. These cytokines have been shown to act on innate lymphoid type 2 cells (ILC2), which respond by making IL-5 and IL-13 (Walker et al., 2013). For example, chitin has been shown to induce IL-25, IL-33, and TSLP, which are necessary to activate ILC2s to produce IL-5 and IL-13, and promote an innate eosinophil-rich inflammation and polarization of macrophages to the alternatively activated state (Van Dyken et al., 2014). In addition, these cytokines may stimulate other cell types as well, including DCs (Besnard et al., 2011; Rank et al., 2009; Willart et al., 2012). How cytokines produced by lung epithelial cells, ILC2s, or other cell types can condition DCs to induce polarization of naïve T cells into T_H2 effector cells is still poorly defined. Studies using human DCs have shown that TSLP conditions DCs to prime a T_H2

response (Ito et al., 2005). Alternatively or additionally, IL-13 produced by ILC2s may induce DCs to migrate to the draining lymph node (LN) and activate antigen-specific T cells (Halim et al., 2014).

Strikingly, although TLR stimulation often induces T_H1 responses, it can also promote type 2 immune responses in the lung in some circumstances and this may be important for development of asthma. House dust extracts can contain house dust mites (HDM), which contain ligands for TLRs, including lipopolysaccharide (LPS), a ligand for TLR4 (Hammad et al., 2009). More recently, flagellin was found in house dust extracts (Wilson et al., 2012), which is recognized by TLR5 (Kawai and Akira, 2011) and TLR11 (Mathur et al., 2012) . Moreover, TLRs can be a sufficient adjuvant for priming a robust T_H2 response via intranasal (i.n.) immunization, as shown initially by Bottomly and colleagues (Eisenbarth et al., 2002; Herrick et al., 2000). They showed that i.n. sensitization of mice with a low dose of LPS and the model antigen ovalbumin (OVA) induced a robust TLR4-dependent T_H2 response in the lungs following antigen challenge. In contrast, the use of a high dose of LPS as adjuvant led to a T_H1 response. More recently, it has been shown that i.n. sensitization with flagellin and OVA induces a TLR5-dependent T_H2 response in the lungs after antigen challenge (Van Maele et al., 2014; Wilson et al., 2012). Thus, TLR stimulation can lead to type 2 immune responses or to type 1 immune responses and the how this occurs is not understood.

Here, I describe studies in which I have compared different TLR ligands using an i.n. sensitization and challenge model to examine how TLRs can promote different adaptive immune responses. Mice sensitized i.n. with a TLR9 ligand CpG oligodeoxyribonucleotide (ODN) plus OVA elicited an innate inflammatory response in

the lung characterized by the presence of monocytes and innate IFN-γ-secreting lymphocytes, developed a predominant T_H1 response to OVA, and predominately made IgG2c anti-OVA antibodies. In contrast, mice sensitized i.n. with flagellin plus OVA elicited an innate inflammatory response dominated by neutrophils and monocytes, developed a predominant T_H2 response to OVA, and made substantial amounts of IgE anti-OVA antibodies. Antigenic re-challenge of the mice sensitized with flagellin plus OVA, but not the mice sensitized with CpG ODN plus OVA, led to a vigorous lung inflammation dominated by eosinophils, as expected for a T_H2 response. Strikingly, the early cytokine responses in the lung showed a number of important differences. CpG ODN induced much higher levels of mRNA encoding IL-12 p40, whereas, flagellin preferentially induced TSLP mRNA and secretion of the mature form of IL-33 and also induced elevated levels of mRNAs encoding IL-1α and IL-1β. In both cases, migratory DCs in the draining LN after stimulation with ligands for either TLR5 or TLR9 had strong induction of the costimulators CD80 and CD86, but only CpG ODN also induced upregulation of CD40. Interestingly, myeloid differentiation primary response 88 (MyD88) signaling in DCs was partially required for the flagellin-induced upregulation of CD80 on migratory DCs that had taken up antigen, and for the IL-4 production in CD4 T cells in the draining LN on d6. These results indicate that although TLR5, TLR11, and TLR9 all signal via the signaling adaptor MyD88, the innate cytokine response to these TLRs is quite different, and the distinctive innate cytokine production results in distinctive polarization of the adaptive humoral and cell-mediated immune responses.

Results

Flagellin promotes a type 2 immune response in the lung after i.n. immunization

To study the effects of TLR ligands on the adaptive immune response in the lungs, I slightly modified a model of i.n. immunization, in which mice were sensitized by i.n. exposure to an antigen, OVA, or to OVA plus TLR ligand three times on successive days and after 2 weeks, were re-challenged with OVA alone for up to four times over a six day time period (Fig. 4.1A) (Eisenbarth et al., 2002; Herrick et al., 2000). When I examined the mice two days after the last i.n. OVA challenge, use of either the TLR5 and TLR11 ligand flagellin or the TLR9 ligand CpG ODN as adjuvant led to 2-10-fold increased numbers of inflammatory cells in the lung airspace (as assessed in the bronchoalveolar lavage (BAL) fluid) compared to mice that were sensitized with only OVA and rechallenged (Fig. 4.1B). For mice sensitized with OVA plus flagellin and rechallenged with OVA, the predominant cell type in the lung airspace was eosinophils. In contrast, few eosinophils were seen in the mice sensitized with OVA plus CpG ODN and subsequently rechallenged with OVA, and instead these mice had robust accumulation of CD8 T cells, monocytes, and NK cells. These cell types were attracted in somewhat lower numbers in the mice sensitized with OVA plus flagellin. Use of either TLR ligand as adjuvant in the sensitization phase led to similarly increased numbers of B cells, CD4 T cells, and neutrophils compared to mice sensitized with OVA alone, although some of these increases were not statistically significant.

The predominant infiltration of eosinophils in the lungs of flagellin-treated mice suggests that flagellin may have induced a T_H2 response, whereas the increase of CD8 T cells, monocytes, and NK cells and the absence of eosinophils in the lungs of CpG ODN-

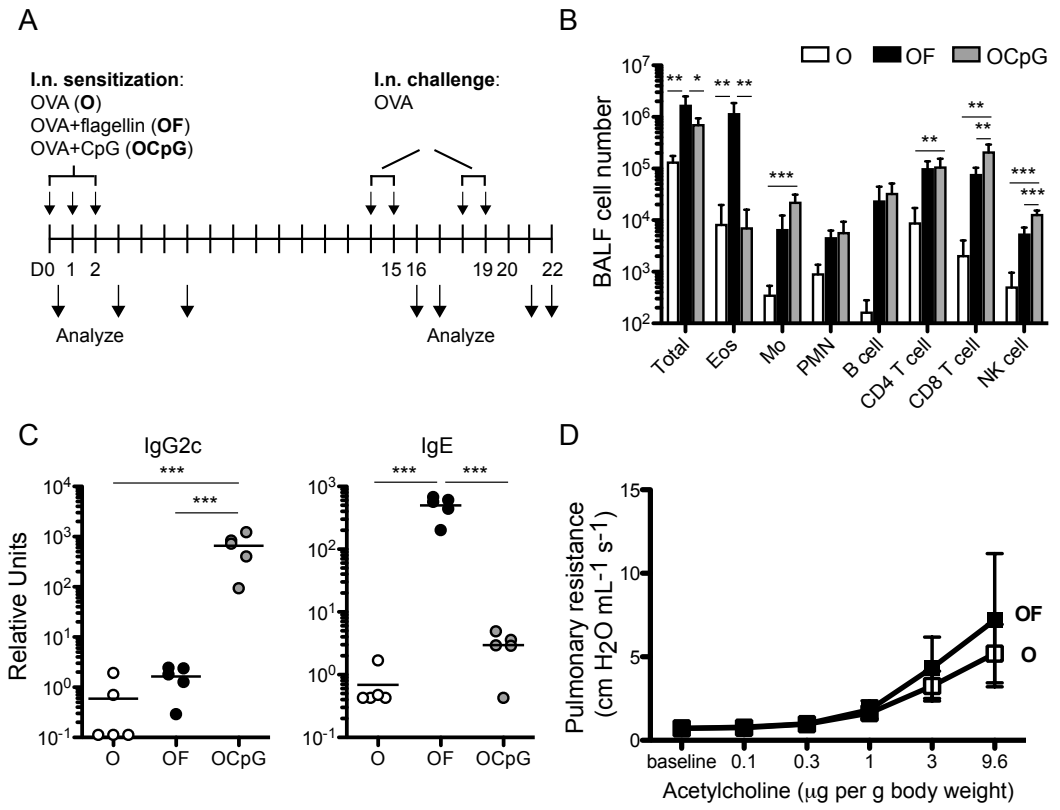

Figure 4.1 I.n. sensitization with OVA plus flagellin induces an IgE response and eosinophilic inflammation in the lungs after antigen challenge

(A) Outline of i.n. sensitization and rechallenge regimen used in this study. Mice received i.n. exposure to OVA (O) (white bars), OVA plus flagellin (OF) (black bars), or OVA plus CpG ODN (OCpG) (gray bars) on days 0, 1, and 2. In experiments examining the adaptive immune response, mice were challenged i.n. with OVA two weeks later on days 15, 16, 19, and/or 20. Immune responses were assessed at one or more of the time points indicated by the arrows below the timeline. **(B)** Airway inflammation after OVA re-challenge on d22 as indicated in **(A)** was assessed by flow cytometry of the cells in the bronchoalveolar lavage fluid (BALF) (eosinophils (Eos), monocytes (Mo), neutrophils (PMN), B cell, CD4 T cells, CD8 T cell, and NK cell). **(C)** Levels of serum OVA-

specific antibodies IgG2c (left panel) and IgE (right panel) were measured by ELISA on d22 after sensitization and rechallenge as shown in **(A)**. Each circle represents one individual mouse. **(D)** The effect of sensitization and rechallenge on pulmonary resistance was measured on d22 of the treatment regime shown in **(A)**. Data shown in (**B**, **C**) are representative of two experiments with at least 5 mice per group, and data in (**D**) are pooled from four experiments with n=20 for OVA and n=27 for OF (except for acetylcholine dose 0.1: O n=14, OF n=21). For these experiments, the mice received 0.2, 1 or 2 μg flagellin for each of the sensitization treatments, and the pulmonary resistance was similar with each dose of flagellin (not shown). Error bars indicate mean +SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

treated mice, suggest that CpG ODN may have induced a T_H1 response. Also, the trend of increased neutrophils seen with either TLR ligand as adjuvant suggests that some T_H17 effectors may have been induced.

The antibody isotypes of anti-OVA antibodies induced in these different i.n. immunizations were also consistent with the possibility that flagellin and CpG ODN promoted adaptive immune responses with different polarization. For example, the i.n. immunization with flagellin as adjuvant led to substantial serum titers of OVA-specific IgE, which were not seen when CpG ODN was the adjuvant or when OVA was administered by itself (Fig. 4.1C). Since class switching to IgE requires high levels of IL-4 (Paul and Zhu, 2010), this suggests that flagellin induced follicular helper T cells (T_{FH}) to produce the T_H2 cytokine IL-4 (Crotty, 2011).

Similar i.n. immunization protocols have been used previously as murine models of asthma, so I also examined airway hyperresponsiveness to acetylcholine as a hallmark feature of asthma-like airway alterations. Although sensitization with OVA plus flagellin induced anti-OVA IgE and led to eosinophilic inflammation in the lungs after repeated rechallenge with OVA, these mice exhibited only a non-significant trend toward greater airway hyperresponsiveness compared to mice sensitized with OVA alone and rechallenged with OVA according to the same schedule (Fig. 4.1D).

To directly address which types of T helper cells were being induced by these different stimulation regimes, I examined cytokine production in CD4 T cells present in lung tissue after rechallenge with OVA by using cytokine reporter mice. In the 4get/KN2 IL-4 reporter mice, cells that have opened up the IL-4 gene locus express GFP (Mohrs et al., 2001), and cells that have recently produced IL-4 additionally express cell surface

human CD2 (Mohrs et al., 2005). In GREAT IFN-γ reporter mice, cells that have recently produced IFN-γ express YFP (Reinhardt et al., 2009), and in the SMART-17A IL-17A reporter mice, cells that have recently produced IL-17A express cell surface human nerve growth factor receptor (hNGFR) (Price et al., 2012). Whereas mice sensitized with OVA plus a TLR ligand as adjuvant had a trend towards increased numbers of CD4 T cells in the BAL fluid that was not statistically significant, the numbers of CD4 T cells recovered from lung tissue were similar after sensitization and rechallenge, regardless of whether or not a TLR ligand was used during the sensitization phase (Fig. 4.2A, D, G). Although the numbers of CD4 T cells present were similar, the production of effector T cell cytokines, as indicated by cytokine reporter expression, was very different, as suggested by the different cellular compositions of the inflammatory infiltrates observed, described above. Use of flagellin as adjuvant induced a higher percentage of IL-4-producing (GFP^+hCD2^+) CD4 T cells in the lungs (Fig 4.2B, C), whereas use of CpG ODN as adjuvant induced an increased percentage of IFN-γ-producing (YFP^+) CD4 T cells and CD8 T cells in the lungs (Fig 4.2E, F). Interestingly, i.n. flagellin also led to increased numbers of basophils in the lungs and increased IL-4 production by basophils. In contrast, immunizations with either flagellin or CpG ODN as adjuvant followed by rechallenge with OVA led to small increases in the percentages of IL-17-producing ($hNGFR^+$) CD4 T cells (Fig 2H, I) and γδ T cells in the lungs. These data demonstrate that i.n. immunization with flagellin as adjuvant and subsequent rechallenge with antigen led to a predominant T_H2 response in the lungs, whereas immunization with CpG ODN as adjuvant and rechallenge led to a predominant T_H1 response in the lungs.

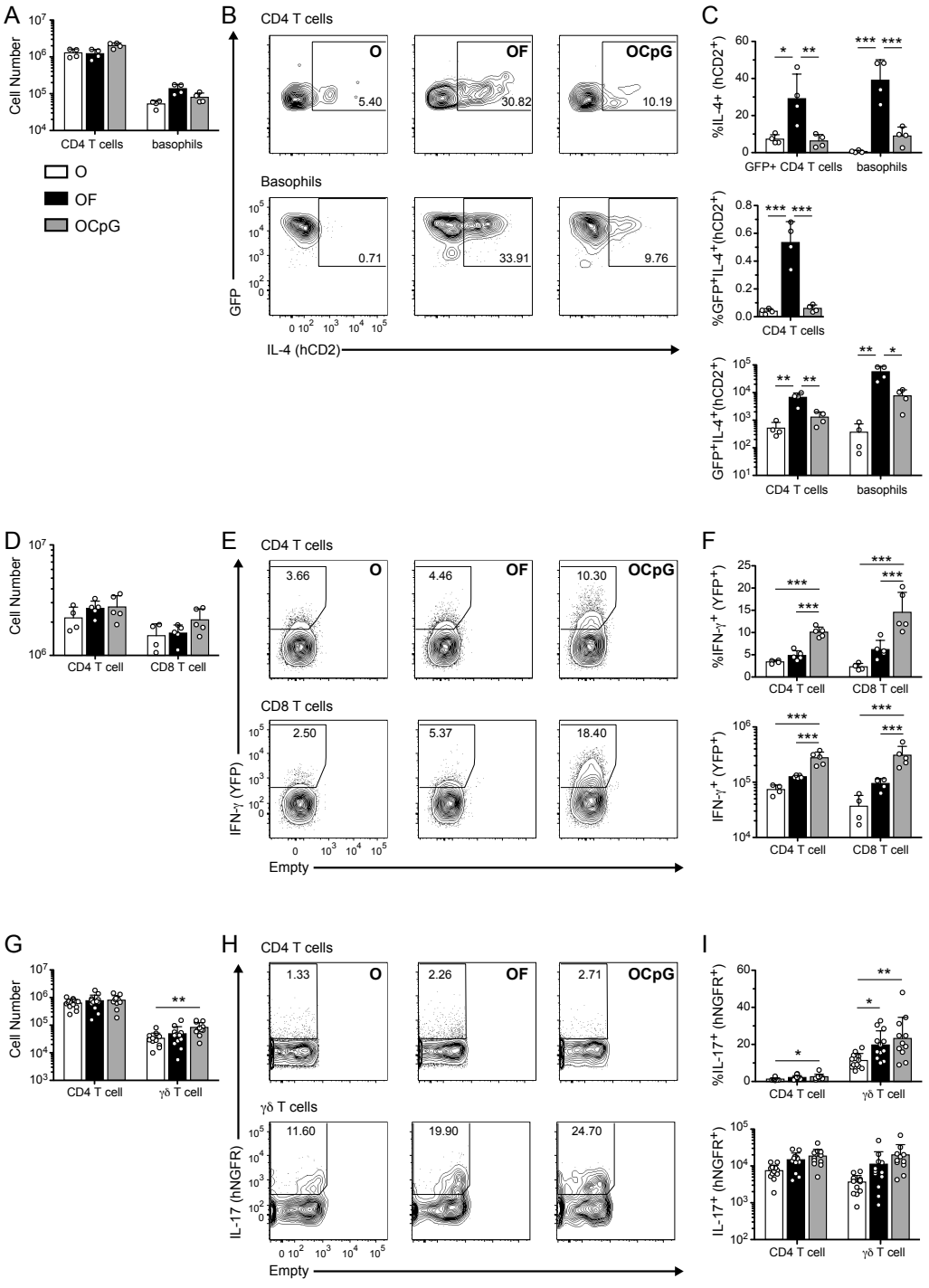

Figure 4.2 Different polarization of CD4 T cells after i.n. sensitization with antigen and either flagellin or CpG ODN.

Figure 4.2. Different polarization of CD4 T cells after i.n. sensitization with antigen and either flagellin or CpG ODN.

(A, B, and C) Presence of activated T_H2 cells and IL-4-producing basophils in the lungs of OVA rechallenged mice. 4get/KN2 reporter mice (*Il4*$^{4get/KN2}$) were administered with OVA (white bars), OVA plus 1μg flagellin (black bars), or OVA plus 0.75μg (1 experiment) or 3μg (2 experiments) CpG ODN (gray bars) i.n., and challenged with i.n. OVA as in Fig. 4.1A. One day after the fourth i.n. OVA challenge (d21), expression of IL-4 reporter (GFP, hCD2) by lung CD4 T cells and basophils was determined. **(A)** Numbers of CD4 T cells and basophils in the lungs on d21. **(B)** Representative flow cytometry plots of IL-4 production (hCD2) by IL-4 competent (GFP^+) CD4 T cells ($CD1d^-CD3^+CD4^+GFP^+$) (top row) and basophils ($CD1d^-CD3^-GFP^+CD49b^+SSC^{lo}$) (bottom row). **(C)** Percentage IL-4^+ ($hCD2^+$) of IL-4 competent (GFP^+) CD4 T cells and basophils (top row), percentage GFP^+IL-4^+ ($hCD2^+$) of total CD4 T cells (middle row), and numbers of GFP^+IL-4^+ ($hCD2^+$) CD4 T cells and basophils (bottom row). **(D, E,** and **F)** Presence of activated T_H1 cells and IFN-γ-expressing CD8 T cells in the lungs of rechallenged mice. GREAT reporter mice (*Ifng*$^{GREAT/GREAT}$) were administered with OVA (white bars), OVA plus 5μg flagellin (black bars), or OVA plus 3μg CpG ODN (gray bars) i.n., and challenged with i.n. OVA as in Fig. 4.1A. One day after the first i.n. OVA challenge (d16), expression of IFN-γ reporter (YFP) by lung CD4 and CD8 T cells was determined. **(D)** Numbers of CD4 and CD8 T cells in the lungs. **(E)** Representative flow cytometry plots of IFN-γ (YFP) by CD4 (top row) and CD8 (bottom row) T cells. **(F)** Percentages (upper row) and numbers (lower row) of IFN-γ^+ CD4 and CD8 T cells. **(G, H,** and **I)** Presence of activated T_H17 cells and IL-17A-expressing γδ T cells in the lungs

of rechallenged mice. SMART-17A (*Il17*$^{SMART/SMART}$) reporter mice were administered with OVA (white bars), OVA plus 1μg flagellin (black bars), or OVA plus 3μg CpG ODN (gray bars) i.n., and challenged with i.n. OVA as in Fig. 4.1A. One day after the second i.n. OVA challenge (d17), expression of the IL-17 reporter (hNGFR) by lung CD4 and γδ T cells was determined. **(G)** Total numbers of CD4 and γδ T cells in the lung. **(H)** Representative flow cytometry plots of IL-17 (hNGFR) expression by CD4 (top row) and γδ (bottom row) T cells. **(I)** Percentages (upper row) and numbers (lower row) of IL-17^{+} CD4 and γδ T cells. Data in **(A, B, C)** are representative of one of three independent experiments with four mice per group, data in **(D, E, F)** are representative one of two experiments with at least 4 mice per group, data in **(G, H, I)** are pooled from three independent experiments with combined totals of 11-13 mice per group. Each circle represents one individual mouse. Mice in **(A, B, C)** were also *Mcpt8*^{Basopho8}, but this additional allele did not affect the results, as described in the Materials and Methods (Chapter 2). 1 outlier mouse in **(D, E, F)** treated with OVA was removed from the analysis, as described in the Materials and Methods (Chapter 2). Error bars indicate mean + SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

Flagellin promotes the appearance of IL-4-producing CD4 T cells and T_{FH} cells in the draining LN

I next examined IL-4-producing cells in the lung-draining LN after i.n. immunization with OVA plus flagellin. Although the numbers of CD4 T cells and activated CD4 T cells in the draining LN on day 6 were similar in the different treatment groups, mice that were sensitized with OVA plus flagellin as shown in Fig. 4.1A had a greater number of LN CD4 T cells that had secreted IL-4 based on hCD2 reporter expression compared to mice sensitized with OVA alone or with OVA plus CpG ODN (Fig. 4.3). T_{FH} are known to be important in formation and maintenance of germinal centers, where B cells differentiate into long-lived plasma cells and affinity matured memory B cells (Crotty, 2011). The fraction of activated CD4 T cells that became T_{FH}, as defined by the expression of CXCR5, the chemokine that attracts these cells to the boundary between the T cell-zone and the follicle and also into the germinal center, was increased by addition of flagellin or CpG ODN as adjuvant (Fig. 4.3A, C). Use of flagellin as adjuvant also strongly increased the fraction of T_{FH} that had secreted IL-4 as assessed with the KN2 reporter. This increase in IL-4 secretion by T_{FH} cells was not seen when CpG ODN was used as adjuvant. Therefore, IL-4 secretion by T_{FH} in the draining LN was selectively enhanced by flagellin and correlated well with IgE production (Fig. 4.1C).

Flagellin induces rapid production of inflammatory and type 2-promoting cytokines in the lungs

To gain insight into the mechanisms by which flagellin and CpG ODN induced opposing adaptive immune responses in the lung, I examined the innate immune

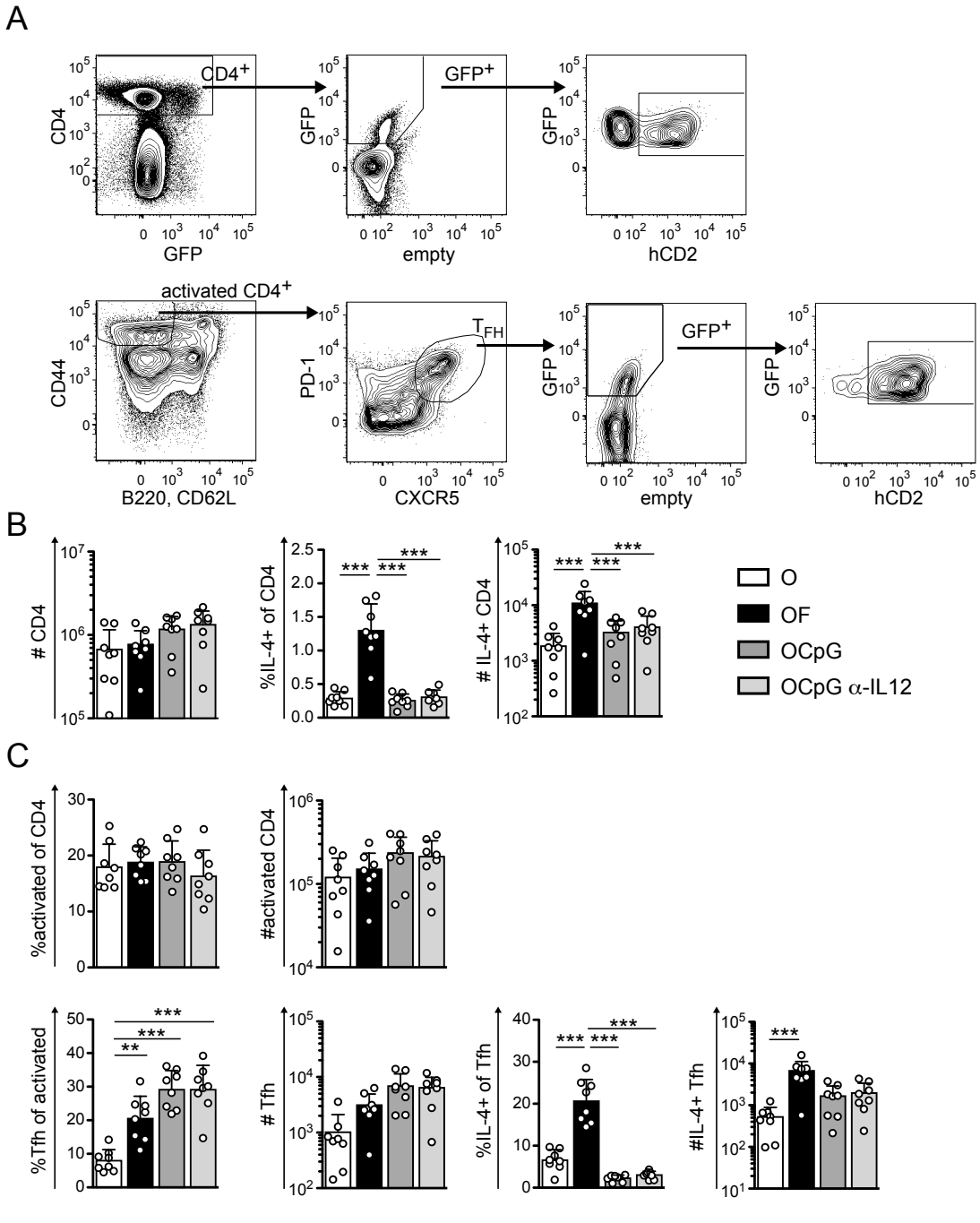

Figure 4.3 Flagellin promotes development of IL-4-producing CD4 T cells in the draining LN.

4get/KN2 reporter mice (*Il4*$^{4get/KN2}$) were administered OVA (white bars), OVA plus

flagellin (1μg) (black bars), or OVA plus CpG (0.75μg) (gray bars) i.n. on day 0, 1 and 2. In addition, to block IL-12 action in some mice, mice were given anti-IL-12 p40 (light gray bars) or control antibody (rat IgG2a) (white, black, and dark gray bars) twice, one day before initial sensitization (d-1; 700μg i.p.) and again on d2 (300μg i.p.). On d6, expression of IL-4 reporters (GFP^+hCD2^+) by CD4 T cells was examined in the mediastinal LN. (**A)** Gating strategy of CD4 T cells ($CD4^+$), activated CD4 T cells ($CD4^+CD44^{hi}B220^-CD62L^-$), and T_{FH} cells ($CD4^+CD44^{hi}B220^-CD62L^-PD\text{-}1^+CXCR5^+$). (**B)** Numbers of CD4 T cells, percentages and numbers of IL-4-producing (GFP^+hCD2^+) CD4 T cells. (**C)** Percentages and numbers of activated CD4 T cells, percentages and numbers of T_{FH} cells, and percentages and numbers of IL-4-producing T_{FH}. Data are pooled from two independent experiments with combined totals of 8 mice per group. Each circle represents one individual mouse. Error bars indicate mean +SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

responses they induced after the first or third i.n. administration. Interestingly, both flagellin and CpG ODN led to robust inflammatory responses one day after the third and last i.n. administration of OVA plus TLR ligand. CpG ODN led to increased numbers of lung-infiltrating monocytes, as did flagellin to a lesser extent (Fig. 4.4A). In contrast, flagellin induced a larger number of neutrophils in the lungs than did CpG ODN. Although i.n. sensitization of mice with flagellin plus OVA and repeated rechallenge with OVA led to robust accumulation of eosinophils in the lung airspace on d22, the early inflammatory infiltrate induced by OVA plus flagellin did not include a substantial number of eosinophils in the lung, and the numbers were not consistently increased above what was seen in mice treated with OVA alone.

Because i.n. administration of OVA plus flagellin induced a robust infiltration of neutrophils in the lungs, I next examined IL-17A production during the innate phase of the response using the *SMART-17A* reporter mice. Flagellin treatment led to an increased percentage of IL-17A-producing γδ T cells on day 3 (Fig. 4.4B). CpG ODN also promoted an increase, but typically to a lesser degree. Also, both flagellin and CpG ODN treatment increased the fraction of CD4 T cells that secreted IL-17A at this early time. Two cytokines that are known to promote expression of IL-17 are IL-1β and IL-23 (Gaffen et al., 2014), and i.n. flagellin treatment induced the mRNAs encoding IL-1β and the p19 subunit of IL-23 (*Il23a)* in the lung within two hours (Fig. 4.4D), which may have contributed to the subsequent IL-17 production by γδ T cells.

I also examined IFN-γ production during the innate immune response. Compared to i.n. administration of OVA alone, administration of OVA plus CpG ODN led to increased production of IFN-γ by several different lymphocyte populations (Fig. 4.4C).

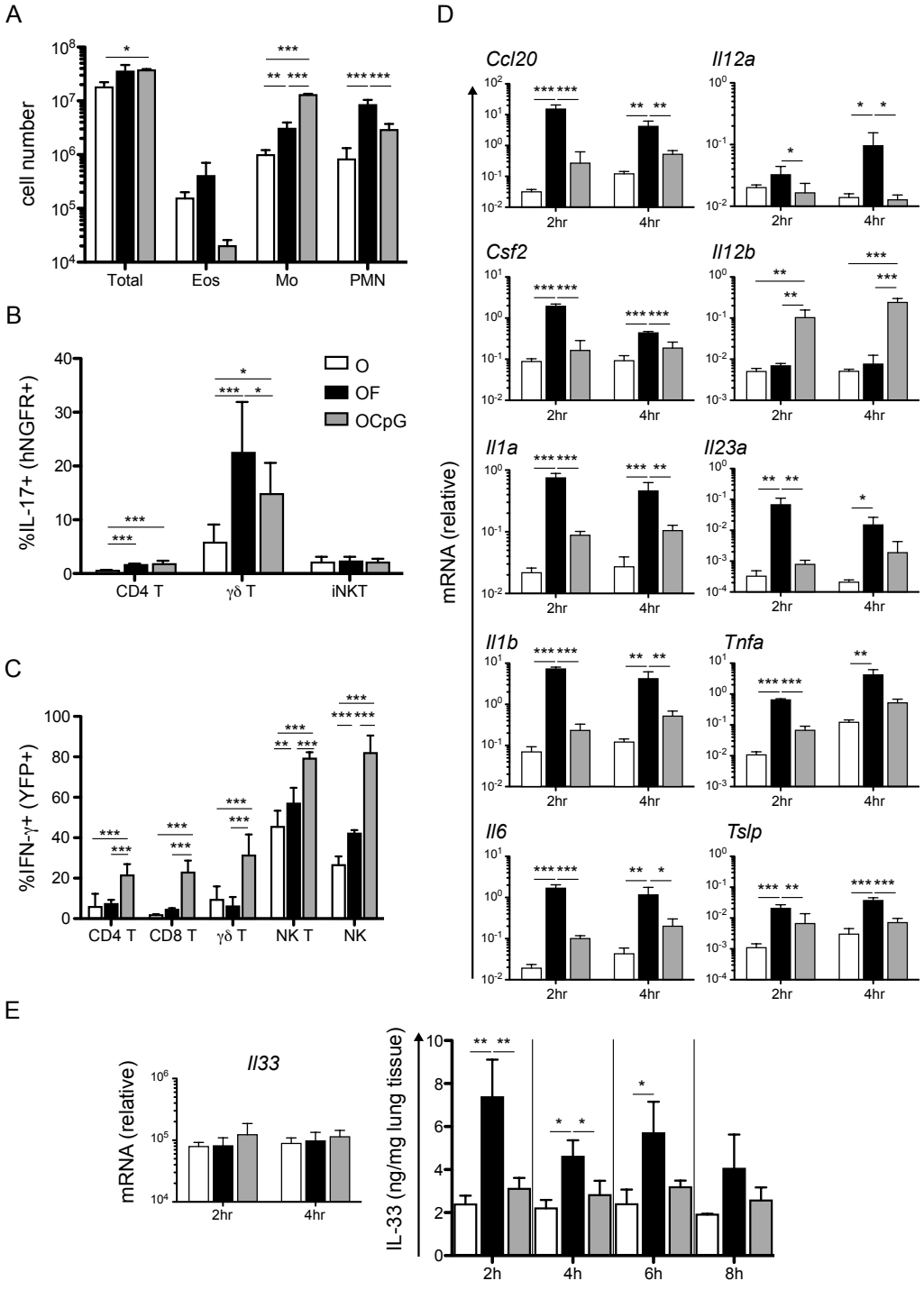

Figure 4.4 Flagellin induces a robust innate inflammatory response in the lungs.

(A) Innate inflammatory infiltrate in the lungs one day after third i.n. sensitization (d3) with OVA (white bars), OVA plus flagellin (1μg) (black bars), or OVA plus CpG (3μg)

(gray bars) as described in Fig. 4.1A. **(B)** Percentages of CD4 T cells, γδ T cells, and iNKT cells in SMART-17A reporter mice (*Il17a*$^{SMART/SMART}$) producing IL-17A (hNGFR^{+}) one day after third i.n. administration (d3) of OVA (white bars), OVA plus flagellin (1μg) (black bars), or OVA plus CpG (3μg) (gray bars) as described in Fig. 4.1A. **(C)** Percentage of CD4 T cells, CD8 T cells, γδ T cells, NKT cells, and NK cells in GREAT reporter mice (*Ifng*$^{GREAT/GREAT}$) producing IFN-γ (YFP^{+}) one day after third i.n. administration (d3) of OVA (white bars), OVA plus flagellin (5μg) (black bars), or OVA plus CpG (3μg) (gray bars) as described in Fig. 4.1A. **(D)** Inflammatory gene induction measured by quantitative PCR of RNA from whole lung tissue at indicated time points after i.n. administration of OVA (white bars), OVA plus flagellin (1 or 5μg) (black bars), or OVA plus CpG (3μg) (gray bars). Samples were normalized to *Hprt* mRNA. **(E)** *Il33* mRNA (left panel) and IL-33 protein (right panel) induction in whole lungs at the indicated time points after i.n. administration of OVA (white bars), OVA plus flagellin (black bars), or OVA plus CpG (gray bars) measured by quantitative PCR as described in (**D**) and by ELISA, respectively. Data in (**A**) are representative of three experiments with 3-4 mice per group, data in (**B**) are pooled from three independent experiments with combined totals of 10-12 mice per group, data in (**C**) are pooled from two independent experiments with combined totals of 7-8 mice per group, data in (**D**) are representative of two experiments with 4-5 mice per group, and data in (**E**) are representative of two experiments with 3 mice per group. Error bars indicate mean +SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

CpG ODN treatment also induced a robust increase in mRNA encoding the p40 subunit of IL-12 (*Il12b*), which may have contributed to the innate IFN-γ response seen with this stimulus (Fig 4.4D). In contrast, flagellin treatment did not induce increased amounts of *Il12b* and also induced many fewer lymphoid cells expressing the IFN-γ reporter from the GREAT mice (Fig 4.4C). Interestingly, mRNA encoding the p35 subunit of IL-12 (*Il12a*) was slightly elevated with flagellin treatment. However, previous studies have demonstrated that *Il12a* mRNA is present in many cell types, even those that do not eventually produce IL-12 (Trinchieri, 2003). Moreover, mRNA for IL-12 p35 may only be detected in activated cell populations, and not in whole tissue, like my experiments. Clearly, the inductions of inflammatory cytokine mRNAs seen in response to flagellin and to CpG ODN had some similarities, but also had some substantial differences (Fig. 4.4D). In general, flagellin induced somewhat higher levels of inflammatory cytokines such as mRNA encoding for TNF, IL-1β, and IL-6, but weaker induction of a few cytokines, including IL-12 p40.

Based on the observation that CpG ODN promoted IL-12 production whereas flagellin did not, I hypothesized that perhaps strong induction of IL-12 by i.n. sensitization with CpG ODN was responsible for the production of IFN-γ. To test this hypothesis, I injected mice with a blocking monoclonal antibody against IL-12, and then sensitized the mice i.n. with OVA plus CpG ODN as before. Innate IFN-γ production in response to CpG was substantially reduced by anti-IL-12 p40 antibody treatment (Chapter 3, Fig 3.4), demonstrating that the differential ability of CpG ODN treatment but not flagellin treatment to induce robust IL-12 production was a major reason for differential IFN-γ production in the innate phase of the response. Interestingly, the mice

treated with anti-IL-12 p40 antibody and then sensitized with OVA plus CpG ODN did not induced IL-4 production in CD4 T cells on day 6 in the draining LN, based on failure to express the KN2 reporter for IL-4 production (Fig 4.3B and C). These results argue against models in which the T_H2 response induced when flagellin was used as adjuvant was a default response that occurred in the presence of inflammatory cytokines and a low level of IFN-γ, and instead suggest that flagellin but not CpG ODN induced production of one or more cytokines that contributed positively to the induction of OVA-specific T_H2 cells. Therefore, I next examined the mRNA and protein expression of cytokines that have been implicated as being important in inducing type 2 immune responses in the lungs. Interestingly, flagellin induced the mRNA that encodes TSLP (Fig 4.4D), a cytokine that has been shown to condition DCs to polarize naïve T cell into T_H2 cells. Sensitization with i.n. flagellin also led to an increase in the mature form of IL-33 in lung tissue homogenates (Fig 4.4E). This appeared to be a post-transcriptional response, as there were only small changes in *Il33* mRNA. In contrast, CpG ODN induced little or no *Tslp* mRNA or IL-33 release. These data suggest that a flagellin-induced type 2 immune response may have resulted from the combination of multiple cytokines, particularly TSLP and IL-33, which are induced preferentially by flagellin.

The flagellin-induced type 2 immune response is partially dependent on MyD88 signaling in DCs

DCs are important for bridging innate and adaptive immune responses by sensing microbial ligands with their TLRs and other innate immune receptors, and by activating and polarizing CD4 T cells (Medzhitov, 2007). To begin to define the signals that caused

DCs to promote a T_H2 response, I used mice (*Myd88$^{fl/fl}$ CD11c-Cre*) that deleted selectively in DCs the gene encoding the signaling adaptor protein MyD88, which is required for signaling by most TLRs and by receptors for IL-1 family cytokines. I examined the ability of these mice to induce IL-4-producing CD4 T cells in the lung draining LN. Interestingly, deletion of MyD88 with the CD11c-Cre transgene resulted in a statistically significant partial decrease in the percentage of IL-4$^+$ CD4 T cells on d6 in lung-draining LN of mice sensitized with flagellin plus OVA (Fig 4.5A).

I next examined the effect of deletion of MyD88 in DCs on the phenotype of migratory DCs (CD11c$^+$I-A^{bhi}) in the lung-draining LN (Fig 4.6). Additionally, fluorescently-labeled OVA was used to make it possible to track the migratory DC that had taken up the antigen. Administration i.n. of OVA with flagellin or CpG ODN led to the upregulation of CD80 and CD86 on the surface of migratory DCs that had taken up fluorescently-labeled OVA, whereas only CpG ODN led to the upregulation of CD40 as well (Fig 4.6B, C). Interestingly, migratory DCs that had taken up antigen from *Myd88$^{fl/fl}$ CD11c-Cre* mice sensitized with OVA plus flagellin had a partial defect in CD80 upregulation compared to similarly treated control mice; in the mutant mice expression of CD86 showed a trend toward partially reduced expression that was not statistically significant. These data suggest that MyD88 signaling downstream of either TLRs or IL-1R family members in DCs was required for their full activation.

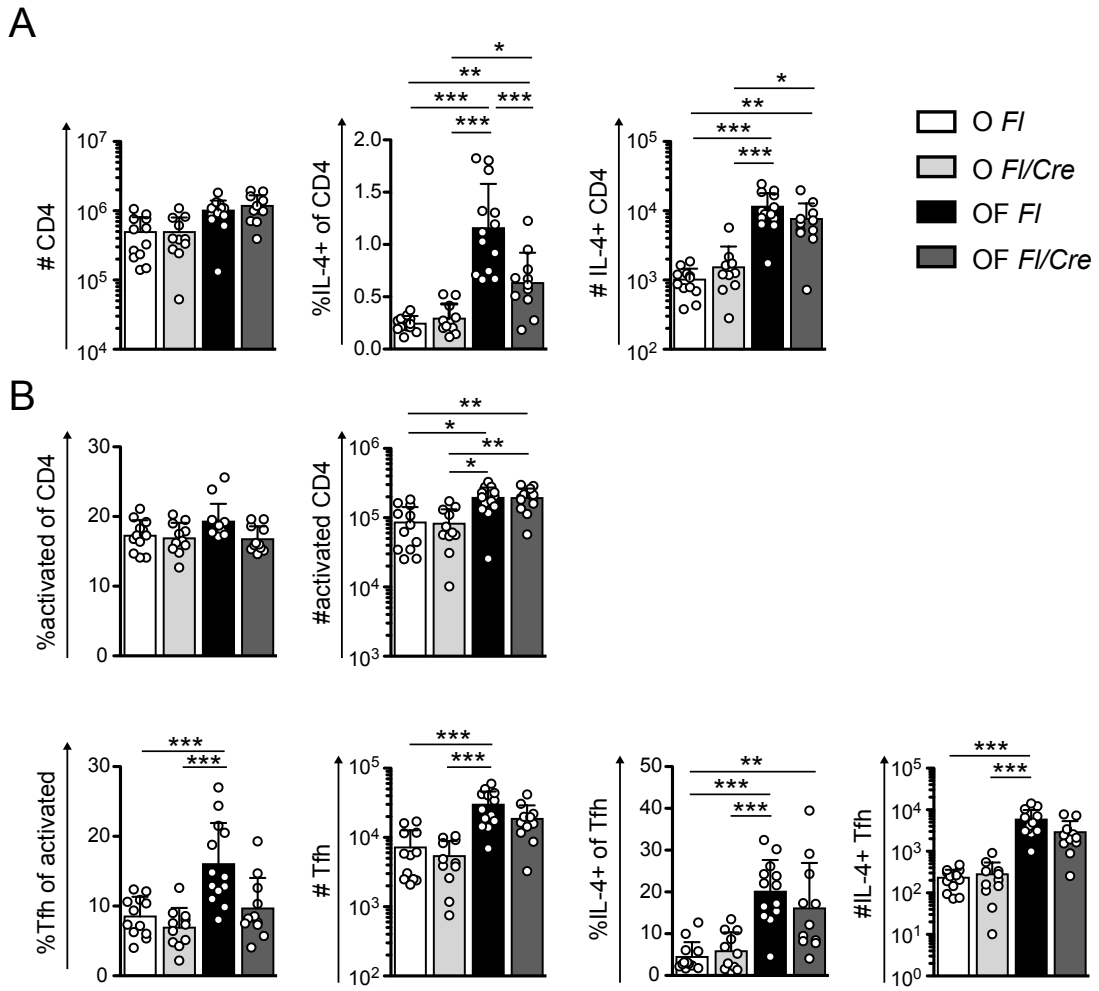

Figure 4.5 Flagellin-induced type 2 immune response of CD4 T cells is partially dependent on MyD88 signaling in DCs.

4get/KN2 reporter mice expressing MyD88 (*Myd88$^{fl/fl}$ Il4$^{4get/KN2}$ (Fl))* and reporter mice that are deficient in MyD88 selectively in DCs and alveolar macrophages (*Myd88$^{fl/fl}$ Il4$^{4get/KN2}$ CD11c-Cre (Fl/Cre)*) were administered i.n. OVA (*Fl* – white bar, *Fl/Cre* – light gray bar) or OVA plus flagellin (1μg) (*Fl* – black bar, *Fl/Cre* – dark gray bar) three times on successive days as described in Fig. 1A. On d6, expression of IL-4 (GFP^+hCD2^+) by CD4 T cells was examined in the mediastinal LN using the same gating

strategy as Fig. 4.3. **(A and B)** Shown are the numbers of total, activated, and T_{FH} phenotype CD4 T cells (as defined in Fig 4.3), and the percentages and numbers of these cells that were positive for the IL-4 reporter ($GFP^{+}hCD2^{+}$). Data are pooled from three independent experiments with combined totals of 11-13 mice per group. Each circle represents one individual mouse. Error bars indicate mean +SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

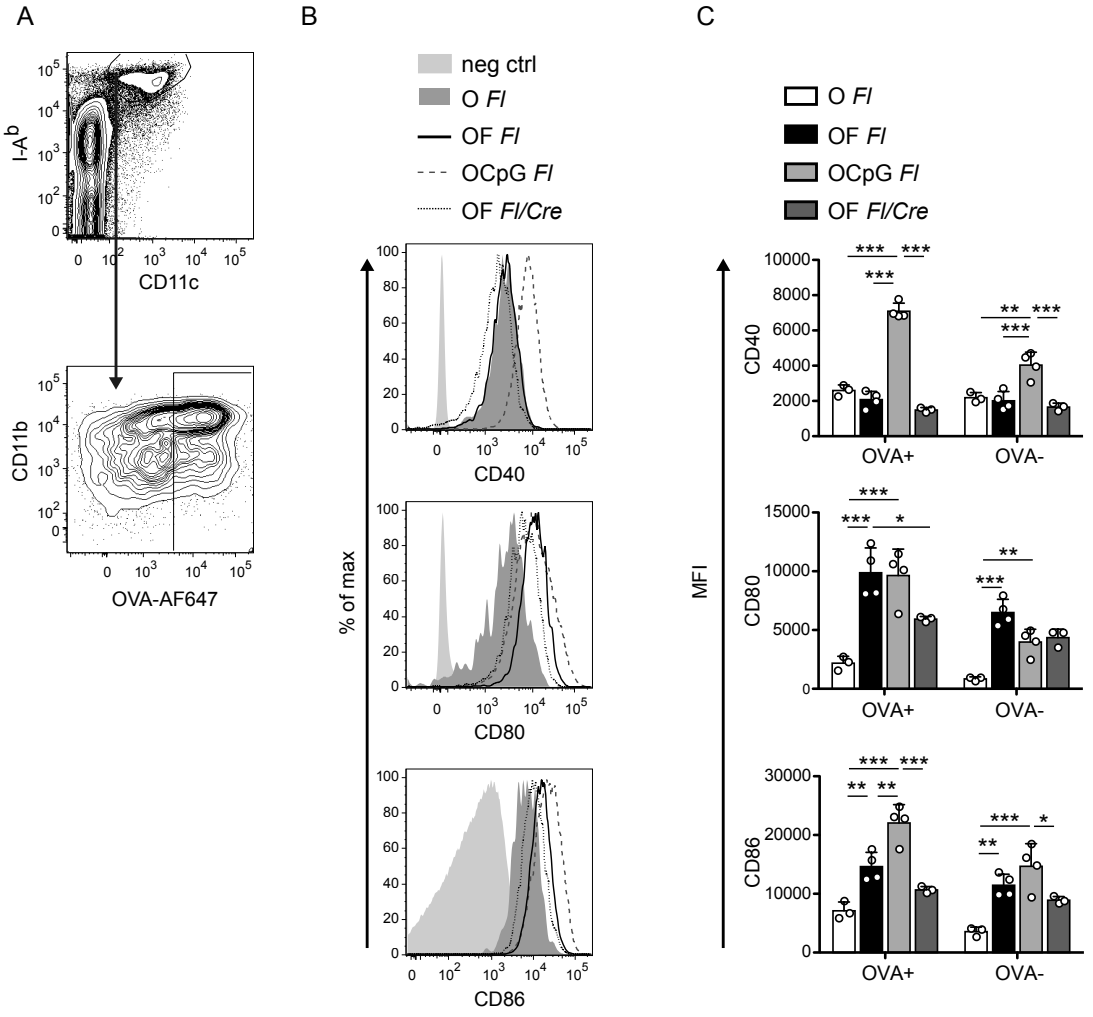

Figure 4.6 Inhalation of flagellin induces DC activation that is partially dependent on their MyD88 signaling.

Expression of activation markers on migratory dendritic cells in the lung-draining LNs of *Myd88*$^{fl/fl}$ (*Fl*) and *Myd88*$^{fl/fl}$ *CD11c-Cre* (*Fl/Cre*) mice one day after i.n. administration (d1) of OVA-AlexaFluor647 (AF647) (*Fl* - white bars), OVA-AF647 plus flagellin (1μg) (*Fl* – black bars, *Fl/Cre* - dark gray bars), or OVA-AF647 plus CpG (0.75 or 3μg) (*Fl* - light gray bars). (**A)** Representative flow cytometry plots showing gating strategy for migratory DCs ($CD11c^{+}I\text{-}A^{b\ hi}$) and OVA-AF647 antigen uptake. (**B)** Representative

histograms of different activation markers on OVA-AF647^{+} migratory DCs. Activation marker expressions on CD11c^{-}I-A^{b-} cells were used as the negative control histograms. (**C)** Level of expression (MFI) of activation markers on migratory DCs that did (OVA^{+} - left side) or did not (OVA^{-} - right side) take up fluorescent OVA. Data are representative of 3 independent experiments with 3-4 mice per group, and similar data were obtained from an experiment in which mice were treated with non-fluorescent OVA. Error bars indicate mean +SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ using one-way anova with Bonferroni post-test.

Discussion

In order to dissect how innate stimulation in the lungs via TLRs can promote polarization of adaptive immune responses, I used i.n. immunization with either of two TLR ligands, CpG ODN and flagellin, as adjuvants and found that these ligands promoted T_H1 and T_H2 responses in the lungs, respectively. Mice sensitized with CpG ODN plus OVA i.n. induced a predominant T_H1 response after OVA rechallenge in the lungs. This response was characterized by increased amounts of serum OVA-specific IgG2c, increased numbers of monocytes and CD8 T cells in the lung airspace, and increased numbers of IFN-γ-producing CD4 and CD8 T cells in the lung tissue. In contrast, mice sensitized with flagellin plus OVA i.n. induced a predominant T_H2 response in the lungs after OVA rechallenge. This response was characterized by increased amounts of serum OVA-specific IgE, increased numbers of eosinophils in the lung airspace, and increased numbers of IL-4-producing CD4 T cells and basophils in the lung tissue.

The early innate immune response induced by CpG ODN and flagellin were also dissimilar in some regards. Sensitization with CpG ODN, but not flagellin, rapidly induced mRNA encoding IL-12 p40, in agreement with a previous study which measured IL-12 mRNA in a microarray (Pesce et al., 2010), and the subsequent appearance of substantial numbers of IFN-γ-producing lymphocytes in the lungs. Both IL-12 and IFN-γ have been strongly implicated in T_H1 induction (Szabo et al., 2003; Trinchieri, 2003), suggesting that the ability of CpG ODN to induce a robust innate type 1 response is likely connected to its ability to promote T_H1 polarization of the CD4 T cell response to OVA. In contrast, i.n. sensitization with flagellin induced little mRNA for IL-12 p40, but

considerable mRNA encoding IL-1α, IL-1β, and TSLP, and also induced the cellular release of the IL-1 family member IL-33. Correlated with the low IL-12 p40 mRNA production seen after flagellin administration, the lymphocytes recruited to the lung in the first few days produced much less IFN-γ than lymphocytes recruited in response to CpG ODN. TSLP and IL-33 have been implicated in the induction of Th2 responses (Bartemes and Kita, 2012), suggesting that differences in the innate immune responses in the lungs to CpG ODN and flagellin likely underlie the divergent adaptive immune responses that were generated.

I based the sensitization and rechallenge regime on previous studies using the TLR4 ligand LPS as the adjuvant for inducing a T_H2 response to i.n. immunization (Eisenbarth et al., 2002; Herrick et al., 2000). In those studies, a low dose of LPS (0.008-0.12μg) as adjuvant led to robust T_H2 cytokine production from mediastinal LN and eosinophil infiltration into the lungs, characteristic of acute asthma. Conversely, a high dose of LPS (10-100μg) led to T_H1 cytokine production from mediastinal LN cells and infiltration of neutrophils into the lung. Thus, LPS was able to promote either a T_H2 or a T_H1 response, depending on the dose of LPS used. In contrast to this quantitative effect, flagellin induced a robust T_H2 response over a wide dose response range, whereas CpG ODN induced a T_H1 response at all doses tested (data not shown). The doses of flagellin and CpG ODN used in these experiments induced similar magnitudes of innate inflammatory responses on d3 (Fig. 4.4). Therefore, the model immunization used in these studies provides an opportunity to dissect robust qualitative differences between an adjuvant that promotes a lung T_H2 response and another adjuvant that promotes a lung T_H1 response.

It is possible that qualitative differences also underlie the divergent responses to low dose LPS vs. high dose LPS. Tan et. al. (2010) showed that mice deficient for TLR4 signaling selectively in the hematopoietic compartment no longer induced a T_H1 response when sensitized with a high dose of LPS, but instead induced a T_H2 response. Interestingly, both low and high dose LPS induced TSLP mRNA expression in the lungs. These studies suggest that perhaps low dose LPS stimulates lung epithelial cells to release T_H2 promoting cytokines, such as TSLP, whereas high dose LPS additionally stimulates DCs to produce IL-12, and this drives a T_H1 response in the lungs, which may inhibit the T_H2 response (Liu, 2007; MacDonald and Maizels, 2008).

In the studies reported here, I used cytokine reporter mice to assess which cytokines were made by effector CD4 T cells *in vivo* following challenge in the lung with antigen. This approach had the advantage over *in vitro* restimulation approaches because I was able to determine the fraction of lung infiltrating CD4 T cells that had made IL-4, IFN-γ or IL-17A *in vivo*. These results correlated well with the character of the inflammation seen in the airways at the same time. For example, the mice that had been immunized with OVA plus CpG ODN and rechallenged with OVA had increased numbers of IFN-γ-producing CD4 T cells in the lung and an inflammatory infiltrate that was especially enriched for monocytes and CD8 T cells. In contrast, the mice sensitized with OVA and flagellin and rechallenged subsequently with OVA had a strong increase in the numbers of IL-4-producing CD4 T cells and this was accompanied by an inflammation that was enriched strongly for eosinophils. My results are consistent with previous studies in which restimulated mediastinal LNs from mice sensitized with flagellin and OVA were found to express IL-5 and IL-13, two cytokines produced by T_H2

cells (Van Maele et al., 2014; Wilson et al., 2012). In addition, previous studies found that immunization with flagellin and antigen can also lead to T_H2 responses following subcutaneous (s.c.) immunization (Cunningham et al., 2004; Didierlaurent et al., 2004).

The use of cytokine reporter alleles also allowed us to assess which cell types produced these effector cytokines *in vivo* during both the innate and adaptive phases of the immune response. I was able to detect cytokine production by other lymphoid cell types such as γδ T cells and CD8 T cells, as well as by basophils, which may be responding via OVA-specific IgE bound to their FcεRI (Paul and Zhu, 2010). Thus, it was evident that CpG ODN induced a robust type 1 innate immune response in the lung. Moreover, during the innate phase of the response to OVA plus flagellin or OVA plus CpG ODN, there were increased numbers of IL-17-producing CD4 and γδ T cells in the lungs. These cells may have contributed to the substantial numbers of neutrophils present in the lungs on d3, since a feature of IL-17 is the ability to sustain recruitment of neutrophils (McKenzie et al., 2006). Flagellin also induced mRNA encoding inflammatory cytokines including IL-1α, IL-1β and TNF, which promote rapid recruitment of neutrophils. The relative contributions of IL-1, TNF, and IL-17 to the sustained neutrophil recruitment in the lungs after repeated flagellin administration are currently not clear. Flagellin also led to robust infiltration of neutrophils in lung airspace on d1, 24 hr after the initial i.n. administration of flagellin and OVA (data not shown), which is consistent with previous studies showing predominant neutrophil accumulation in the lung airspace as early as 10h (Feuillet et al., 2006; Honko and Mizel, 2004).

The low level of IL-12 production made in response to i.n. flagellin plus OVA treatment may have facilitated the robust T_H2 response that developed, since a developing

T_H1 response can inhibit the generation of a T_H2 response (Szabo et al., 2003). I propose that, in addition, flagellin promoted the T_H2 response by positive means. To address this issue, I pre-treated mice with neutralizing antibody to IL-12 p40 and then sensitized them with CpG ODN plus OVA and found that although innate IFN-γ production and the T_H1 response were strongly inhibited (Chapter 3), these mice did not induce increased numbers of IL-4-producing CD4 T cells on d6 in the draining LN (Fig. 3). These results demonstrate that inflammation in the lungs in the absence of IL-12 production does not automatically lead to a default T_H2 response, as proposed previously (Liu, 2007; MacDonald and Maizels, 2008), and lead me to hypothesize that flagellin in some way positively contributed to the induction of a T_H2 response.

Flagellin induced robust cytokine production and one or more of these cytokines may contribute to the T_H2 response induced after i.n. flagellin plus OVA immunization. Strikingly, flagellin, but not CpG ODN, led to the release of the mature form of IL-33 in whole lung homogenates and also induced mRNA encoding TSLP in whole lungs. In agreement with my findings, a previous study detected IL-33 and TSLP in the lung airspace after flagellin exposure (Wilson et al., 2012).

A variety of studies have shown that IL-33 and TSLP are each sufficient to induce T_H2 responses, and also may be necessary in some model systems (Bartemes and Kita, 2012; Saenz et al., 2008). For example, systemic administration of IL-33 led to upregulation of genes encoding IL-4, IL-5, and IL-13 in various tissues, led to increased numbers of eosiniphils in the spleen, blood, and lungs, and increased levels of serum IgE (Schmitz et al., 2005). Similarly, transgenic overexpression of TSLP in mice leads to an asthma-like phenotype (Zhou et al., 2005) and TSLP has been shown to be induced in

different allergen models, such as inhalation of HDM (Hammad et al., 2009). Blocking TSLP prevented the development of T_H2 cells in a s.c. model using papain as the allergen (Sokol et al., 2008), whereas blocking IL-33 by using the extracellular domain of ST2, one of the chains of the heterodimeric IL-33 receptor, was found to inhibit the T_H2 responses induced by HDM in another study (Willart et al., 2012), and in a chronic asthma model involving *Aspergillus*, *Alterneria*, and HDM (Iijima et al., 2014). While IL-33 and TSLP are attractive candidates for the cytokines induced by flagellin that contributed to T_H2 development, IL-1α and IL-1β have also been implicated in promoting T_H2 responses, and these cytokines were also robustly induced by flagellin. In one study, sensitization with IL-1α plus OVA induced T_H2 response in the lungs (Willart et al., 2012). Moreover, IL-1R signaling was shown to be necessary for the HDM-induced T_H2 response. Interestingly, human cultured airway epithelial cells exposed to IL-1β produced TSLP (Lee and Ziegler, 2007), and the HDM-induced production of IL-33, GM-CSF, and KC was diminished in *Ilr1*$^{-/-}$ mice (Willart et al., 2012). These findings suggest the possibility that the IL-33 released and TSLP produced in the lungs of mice treated with flagellin plus OVA were the result of responses to IL-1α and/or IL-1β , which were made directly in response to flagellin.

I found that *Tlr5*$^{-/-}$*Tlr11*$^{-/-}$ mice failed to respond to flagellin as adjuvant (data not shown), indicating that the adjuvant effects seen in this study require recognition by TLRs. I did not further address which cells had to express TLR5 and/or TLR11 to promote the responses reported here. Two previous studies used bone marrow chimeric mice lacking TLR5 either in hematopoietic cells or in non-hematopoietic cells to address which cells in the lung directly sense flagellin to induce T_H2 responses in the lungs (Van

Maele et al., 2014; Wilson et al., 2012). While both found a role for non-hematopoietic cells, presumably lung epithelial cells, in mediating the adjuvant effects of i.n. flagellin, one study found that there was also a role for TLR5 in hematopoietic cells, presumably DCs or alveolar macrophages (Wilson et al., 2012), whereas the other study found that the predominant role of TLR5 was in non-hematopoietic cells (Van Maele et al., 2014). In any case, lung epithelial cells are known to express TLR5 (Prince, 2006; Wilson et al., 2012) and they likely contributed to the responses reported here.

I tested the role of the signaling adaptor molecule MyD88, which is required for signaling by most TLRs and by receptors for IL-1 family cytokines, in DCs for the response to i.n. flagellin by using mice in which the CD11c-Cre transgene was used to delete a conditional allele of *Myd88*. After i.n. flagellin plus OVA sensitization, these mice exhibited a partial defect in upregulation of the costimulator CD80 on DCs that had taken up OVA and migrated to the draining LN (Fig. 4.6), and a partial decrease in the fraction of CD4 T cells producing IL-4 on d6 in the draining LN (Fig. 4.5). In the HDM-induced mouse model of asthma, DCs were shown to be essential for activating T_H2 responses in the lungs, as depletion of DCs using CD11c-DTR mice completely ablated the HDM-induced T_H2 response (Hammad et al., 2010).

MyD88 signaling is downstream of both TLR signaling and IL-1R family signaling, therefore, the effects of deleting MyD88 in DCs could reflect an important role for TLR5 and/or TLR11 recognition of flagellin by DCs or it could reflect an important response of these cells to IL-1α, IL-1β or IL-33, or a combination of these responses. Both *in vitro* and *in vivo* studies have shown that IL-1α, IL-1β, and IL-33 can have important effects on DCs. One study showed that IL-33-stimulated BMDCs upregulated

CD80 and MHCII, and had enhanced *in vitro* priming of naïve T cells to express the T_H2 cytokines IL-5 and IL-13 (Rank et al., 2009). In another study, mice genetically deficient for one chain of the IL-33R (ST2) had decreased CD40 and CD80 upregulation in lung and LN DCs after s.c. sensitization with OVA not depleted of endotoxin and i.n. challenge with OVA compared to control mice (Besnard et al., 2011). Similarly, i.n. administration of IL-1α and OVA led to upregulation of activation markers on LN DCs (Willart et al., 2012). The partial defect in T cell production of IL-4 in the *Myd88$^{fl/fl}$ CD11c-Cre* mice is consistent with a role for one or more IL-1 family members acting on lung DCs to promote the T_H2 response.

As there was only a partial defect in DC maturation and subsequent IL-4 production in CD4 T cells in the *Myd88$^{fl/fl}$ CD11c-Cre* mice, it is likely that lung epithelial cells responded to flagellin via their TLR5 and produced cytokines that act on DCs in addition to IL-1 family members. In agreement with this scenario, TSLP has been reported to condition DCs to promote T_H2 responses (Bartemes and Kita, 2012; Ito et al., 2005; Saenz et al., 2008). In murine studies using a OVA plus alum sensitization model of asthma, blocking TSLP signaling prevented maturation of lung DCs and their migration to the draining LN during the sensitization phase (Shi et al., 2008), and neutralizing TSLP prevented maturation of lung DCs after challenge (Zhang et al., 2011). Thus, it is possible that TSLP contributes to the IL-4 response in the draining LN on d6, along with either direct recognition of flagellin by lung DC or with IL-1 family members acting on lung DCs.

I have performed experiments to determine whether TSLP and/or IL-33 are necessary for flagellin-induced IL-4 production in CD4 T cells by neutralizing TSLP with

anti-TSLP or blocking IL-33R signaling with anti-ST2. The combined treatment with neutralizing TSLP and blocking ST2 in mice did not clearly decrease the fraction of CD4 T cells producing IL-4 on d6 in the draining LN of mice immunized with flagellin plus OVA (data not shown). It is possible that the lung epithelial cells responding directly to flagellin produced IL-1α as well as IL-33 and TSLP, which were all induced in the lung by flagellin treatment. Each of these cytokines may have contributed to the T_H2 response in a redundant fashion. To test this hypothesis, I pretreated *Myd88$^{fl/fl}$ CD11c-Cre* mice with anti-TSLP before i.n. sensitization with flagellin plus OVA. Interestingly, in a single preliminary experiment (data not shown), these mice had almost no increase in the percentage of IL-4-producing CD4 T cells in the draining LN compared to mice treated with OVA alone, whereas *Myd88$^{fl/fl}$ CD11c-Cre* mice pretreated with isotype control antibody and sensitized with flagellin plus OVA had a partial response, similar to what is shown in Fig. 4.5. This preliminary data supports a model in which lung DCs internalized antigen and responded to multiple IL-1 family members and to TSLP, migrated to the draining LN, and induced IL-4 production by CD4 T cells in the LN, leading to the observed IgE and T_H2 responses.

In summary, I found that CpG ODN used as an adjuvant in the lungs induced a T_H1 response, likely through early IL-12 and IFN-γ production. Conversely, flagellin used as an adjuvant in the lungs induced a T_H2 response, which may have been due to early induction of TSLP and IL-1 family members, combined with little limited induction of IL-12. Interestingly, another study suggested that flagellin's ability to induce a T_H2 response has relevance in human asthma. Wilson et. al. (2012) showed that about four out of seven house dust extract samples collected from households in North Carolina

contained flagellin. Mice sensitized and rechallenged with three of these flagellin-containing samples had eosinophil infiltration in the lung airspace after rechallenge, and this response was TLR5 dependent. In addition, asthmatics have slightly higher levels of anti-flagellin antibodies in the serum compared to non-asthmatics. Future studies should investigate how widespread flagellin is in house dust extract in a greater sampling of homes across a greater region, particularly in households with asthmatic members. House dust extract contains many complex allergens, such as ones from house dust mite (Der p1, Der p2) (Lambrecht and Hammad, 2014), cockroach Ag (Thomas et al., 2005), and chitin from fungi (Van Dyken et al., 2011). More careful analysis of how these multiple allergens may synergistically induce allergy or asthma and whether they all use common pathways to initiate type 2 immune responses, can hopefully lead to the design of better or more targeted treatments or prevention strategies.

Chapter 5 : Discussion

Summary

In my studies, I investigated how different innate immune stimulation in the lungs via Toll-like receptors (TLRs) can promote polarization of distinct adaptive immune responses. I found that intranasal (i.n.) immunization with either of two TLR ligands, CpG ODN and flagellin, as adjuvants promoted T_H1 and T_H2 responses in the lungs, respectively. I demonstrated that the CpG ODN-induced T_H1 response was driven by MyD88 signaling in dendritic cells (DCs), most likely through TLR9 signaling in DCs inducing IL-12 production (Chapter 3). I next demonstrated that the flagellin-induced T_H2 response was partially driven by MyD88 signaling in DCs, most likely through IL-1R family signaling in DCs (Chapter 4). The release of IL-33 and induction of mRNA encoding thymic stromal lymphopoietin (TSLP), but not IL-12 p40, may be the determining factors of the flagellin-induced T_H2 response. While these studies have provided more insight of how TLR stimulation can drive T_H1 and T_H2 responses in the lungs, they suggest future experiments and directions that can provide greater insight to these complex immune responses, which have important implications for understanding infectious diseases, asthma, and vaccine design.

Distinguishing the roles of TLR and IL-1R family signaling in CpG ODN- and flagellin-induced immune responses

Most TLRs and IL-1R family members signal through MyD88. Using *Myd88$^{fl/fl}$ CD11c-Cre* mice, I was able to demonstrate a role of TLR and/or IL-1 signaling in CpG

ODN- and flagellin-induced immune responses. I found that the CpG ODN-induced T_H1 response and a part of the flagellin-induced IL-4 production by CD4 T cells were dependent on MyD88 signaling in DCs. As discussed in Chapters 3 and 4, I attribute the defects in CpG ODN-induced immune response in the lungs to the inability of DCs in the *Myd88$^{fl/fl}$ CD11c-Cre* mice to sense CpG ODN through TLR9. In contrast, the partial defects in the flagellin-induced immune response in the lungs of *Myd88$^{fl/fl}$ CD11c-Cre* mice is likely due to the inability of DCs to sense IL-1 family members. However, these studies have not conclusively excluded the potential role of IL-1R family signaling in DCs after CpG ODN treatment or TLR5 and/or TLR11 signaling in DCs after flagellin treatment. Although there is strong evidence that direct CpG ODN sensing by TLR9 in DCs is important for promoting a T_H1 response when CpG ODN is systemically administered (Spörri and Reis e Sousa, 2005), it is less clear whether or not DCs express TLR5 and/TLR11 and can respond to flagellin (Chapter 1). *In vitro* studies have shown that bone-marrow derived DCs (BMDCs) treated with different TLR ligands, including flagellin and CpG ODN, can induce mRNA encoding TSLP (Kashyap et al., 2011). In addition, IL-33 can be produced by BMDCs stimulated with flagellin (Su et al., 2013). Thus, although the current thinking attributes the release or production of IL-33 and TSLP in the lungs to epithelial cells, DCs may also be capable of producing these cytokines.

The most definitive and ideal tools to address these questions would be generating mice with floxed alleles of *Tlr5, Tlr11,* and *Tlr9*, and crossing them to *CD11c-Cre*. To address these questions with the current tools available, it would be possible to examine whether there are any defects in early (2hr) cytokine induction in the lungs of *Myd88$^{fl/fl}$*

CD11c-Cre mice treated i.n. with flagellin or CpG ODN. Any defect in gene induction observed at this early time would most likely be due to the inability of DCs to sense directly the TLR ligand, and not due to defects in IL-1 family cytokine bystander activation because there would probably not be enough time for the DCs to respond to cytokines made by other cell types. Alternatively, lung DCs could be sorted from lungs of treated wild type mice and cytokine mRNA levels measured. A caveat of such an experiment would be the length of time this would require. This extended length of time may allow for cytokine production by other cell types to influence DC cytokine production, particularly during the lung digestion step, thus I favor using the genetic tools and examining early time points. Alternatively, it would be possible to address these questions using mixed bone marrow chimeras. Briefly, bone marrow from CD11c-DTR mice and *Tlr5*$^{-/-}$*Tlr11*$^{-/-}$ or *Tlr9*$^{-/-}$ mice at a 1:1 ratio would be transferred into lethally irradiated wild-type recipients. After reconstitution, wild-type DCs could be depleted by administering diphtheria toxin, so the only DCs remaining in the short term would be *Tlr5*$^{-/-}$*Tlr11*$^{-/-}$ or *Tlr9*$^{-/-}$. The mice could then be i.n. sensitized with flagellin or CpG ODN and inflammatory cells and antibody titers could be examined after OVA rechallenge.

Distinguishing the roles of MyD88 signaling in conventional DCs and alveolar macrophages in CpG ODN- and flagellin-induced immune responses

Another complication with the interpretation of the results from the *Myd88*$^{fl/fl}$ *CD11c-Cre* mice is that CD11c-Cre deletes efficiently not only in conventional DCs, but also in alveolar macrophages (Abram et al., 2014). As discussed in Chapter 3, I attribute the defects in the CpG ODN-induced immune responses in *Myd88*$^{fl/fl}$ *CD11c-Cre* mice to

defects in MyD88 signaling in DCs and not alveolar macrophages. In regards to flagellin, previous studies have shown that alveolar macrophages express TLR5 and *in vitro* stimulation with flagellin leads to TNF-α production (Hawn et al., 2007). The flagellin-induced release of IL-33 may be partially coming from alveolar macrophages, as previous studies have shown that alveolar macrophages can produce IL-33 during *Nippostrongylus brasiliensis* (Wills-Karp et al., 2012) and influenza infections (Chang et al., 2011) (Umetsu et al 2011). To more definitively address the potential role of alveolar macrophages in sensing TLR ligands and or IL-1 family members, it would be possible to examine CpG ODN-induced IFN-γ production in *Myd88*$^{fl/fl}$ *Ifng*$^{GREAT/+}$ *LysM-Cre* mice, which deletes mainly in macrophages (including alveolar macrophages) and in neutrophils (Abram et al., 2014). If no defect is seen, this would strongly suggest that MyD88 signaling in DCs is mostly required for the CpG ODN-induced IFN-γ production. However, if there is a partial inhibition or the phenotype completely recapitulates what is seen in *Myd88*$^{fl/fl}$ *CD11c-Cre* mice, this would suggest that alveolar macrophages may be involved. Likewise, examination of flagellin-induced IL-4 production in the draining LN of *Myd88*$^{fl/fl}$ *Il4*$^{4get/KN2}$ *LysM-Cre* mice could be used to address a possible role of alveolar macrophages in this response.

Role of lung epithelial cells in sensing TLR ligands

Future studies should also examine what cytokines lung epithelial cells are producing *in vivo* after TLR stimulation, and particularly whether or not they are producing TSLP. Using bone marrow chimeras, Van Maele et al. (2014) showed that the early gene expression induced by i.n. flagellin was mostly due to non-hematopoietic

cells, presumably lung epithelial cells. However, whether lung epithelial cells contribute to the CpG ODN-induced immune response is uncertain, as it has not been established that these cells express TLR9 at physiologically relevant levels. To address these questions, it would be possible to isolate lung epithelial cells shortly after i.n. administration of flagellin or CpG ODN, and examine cytokine mRNA.

Lung and migratory DC activation by TLR ligands and inflammatory cytokines

Activation of DCs is one of the prerequisites for their ability to activate and prime naïve T cells (Medzhitov, 2007). Interestingly, I found that MyD88 signaling in DCs and/or alveolar macrophages was important for upregulation of activation markers on lung DC and migratory DC in the draining lymph node (LN) in response to i.n. CpG ODN. Thus, it appears that direct TLR signaling in DCs is necessary for activation, and bystander cytokines, if any, are insufficient to activate DCs. In contrast, I found that MyD88 signaling in DCs and/or alveolar macrophages was only partially necessary for upregulation of CD80 on migratory DCs that had taken up antigen in response to i.n. flagellin. This result suggests that maturation of lung DCs in response to flagellin is due to sensing of cytokines that include both IL-1 family members and other cytokines, each of which contribute significantly. Flagellin also induces TSLP, which has been shown to condition DCs to prime T_H2 responses. To address whether TSLP may also be important for DC maturation, it would be possible to pre-treat mice with anti-TSLP antibody and examine flagellin-induced DC maturation. If a partial defect is seen, then it would be helpful to pre-treat *Myd88*$^{fl/fl}$ *CD11c-Cre* mice with anti-TSLP to see whether eliminating DC ability to sense IL-1 family members and blocking TSLP is sufficient in combination

to prevent DC maturation. However, the mechanism by which DCs become activated following i.n. flagellin administration may be complicated to investigate *in vivo* because, as shown in Figure 4.4, flagellin induces many inflammatory cytokines other than IL-1 family members and TSLP. For example, TNFα and IL-6 could potentially be involved in upregulation of activation markers on DCs. In addition, as previously discussed, it is unclear whether lung DCs can directly respond to flagellin administered i.n. If so, stimulation through TLR5 or TLR11 by flagellin on DCs could potentially promote DC maturation to some extent. Thus, the partial defect seen in *MyD88$^{fl/fl}$ CD11c-Cre* mice may also be due to DCs being unable to sense flagellin directly.

Potential roles of DC activation markers and DC subsets in the flagellin-induced T_H2 response

As reviewed in Chapter 1, there have been studies suggesting that some DC activation markers may be associated with the development of different T helper lineages. In my studies, migratory DCs from flagellin-treated mice expressed similar levels of CD40 compared to migratory DCs from OVA-treated mice. I.n. administration of flagellin was able to lead to the upregulation of CD80 and CD86 on migratory DCs that have taken up antigen. Interestingly, migratory DCs that have taken up antigen from *Myd88$^{fl/fl}$ CD11c-Cre* mice treated with flagellin had a partial defect in CD80 upregulation, and showed a trend toward a partial defect in CD86 upregulation compared to control mice. In contrast, migratory DCs from CpG ODN-treated mice upregulated CD40, CD80, and CD86, all of which were dependent in MyD88 signaling in DCs. Because CD40 stimulation by its cognate ligand CD40L induces transcription of *Il12a*

and/or *Il12b* (depending on the cell type) (Trinchieri, 2003), the lack of CD40 upregulation on DCs in mice treated with flagellin i.n. may help promote flagellin-induced T_H2 response by not inducing IL-12 production. In preliminary studies, migratory DCs from either flagellin- or CpG ODN- treated mice did not upregulate OX40L and ICOSL, both of which have been implicated as being important in driving T_H2 responses (Chapter 1). Whether the unique pattern of cell surface activation marker expression on migratory DCs induced by flagellin compared to CpG ODN has any relevance to flagellin's ability to induce a T_H2 response needs to be further explored.

Though I have not directly tested which DC subset is responsible for flagellin-induced T_H2 response, I have some correlative observations that suggest there may not be a preferential DC subset that primes the T_H2 response in the flagellin-sensitization model. Migratory $CD103^+$ and $CD11b^+$ DCs in the draining LN had similar uptake of fluorescent antigen and expression of activation markers (data not shown). In the case of i.n. administration of HDM, monocyte-derived DCs, which were identified as being $Fc\varepsilon RI^+CD11b^+$, were shown to be important in promoting T_H2 responses (Plantinga et al., 2013). In my experiments, this population was not detected in the LN, despite the fact that i.n. flagellin induced monocyte infiltration in the lungs. Further studies will be needed to examine whether a particular DC subset is responsible for the flagellin-induced T_H2 response. There are a variety of mouse models that can be used to delete specific lineages of DCs to address this question (Gao et al., 2013b; Hammad et al., 2010; Hildner et al., 2008; Kumamoto et al., 2013b; Nakano et al., 2012b).

Potential roles of type 2 innate immune cells in flagellin-induced T_H2 response

Despite the fact that i.n. OVA plus flagellin induced TSLP mRNA and released IL-33 protein, my preliminary studies showed that flagellin-treated mice did not have a greater percentage of IL-5-producing ILC2s compared to OVA-treated mice (data not shown). As previously discussed, these two cytokines are known to activate ILC2s to produce type 2 cytokines, IL-5 and IL-13. IL-5-producing ILC2s are necessary for chitin-induced lung eosinophilia (Van Dyken et al., 2014). The lack of increased IL-5 production by ILC2s in flagellin-treated mice is consistent with the fact that I did not observe increased numbers of eosinophils early after i.n. sensitization (Fig 4.4), in contrast to the rechallenge phase. However, I did observe that ILC2s from both flagellin-treated and CpG ODN-treated mice had increased expression of activation markers, CD69 and ICOS, compared to ILC2s from OVA-treated mice (data not shown). Strikingly, ILC2s from CpG ODN-treated mice had a greater upregulation of these activation markers than ILC2s from flagellin-treated mice. Taken together, these data suggest that either ILC2s upregulate CD69 and ICOS in response to cytokines other than TSLP and IL-33, or that other cytokines suppressed the ability of ILC2s to produce IL-5 in response to TSLP and IL-33. Some support for the latter hypothesis is that, despite the fact that i.n. CpG ODN induced ILC2 activation, it led to a decrease in the total number of ILC2s, in the percentage of ILC2s producing IL-5, and in the total number of eosinophils compared to flagellin-treated mice. Even though there was not an increase in the number of eosinophils with i.n. flagellin plus OVA, compared to OVA alone, this does not necessarily indicate that no IL-5 and IL-13 were produced. An alternative explanation for my preliminary results is that the methods of detecting IL-5 that I used

(culturing lung cells from treated mice with Brefeldin A, followed by intracellular cytokine staining for IL-5) may not have been adequate to detect any differences. Chang et al. (2011) found that influenza infection led to airway hyperresponsiveness and infiltration of neutrophils, but not eosinophils. They also found that IL-33 was produced, and there was an increased number of IL-13-producing ILC2. Thus, further studies are needed to more carefully analyze what ILC2s are producing in response to flagellin and CpG. This could be done by sorting cells and examining transcript expression. These cells may be producing other proteins, particularly proteins involved in epithelial wound repair, as was shown after influenza infection (Monticelli et al., 2011).

Interestingly, besides differences in cytokine production between flagellin and CpG ODN during the innate immune response, I also found that one day after the third i.n. sensitization, flagellin but not CpG ODN, led to an increase in the number of non-alveolar macrophages (data not shown). Since flagellin did not promote IFN-γ production and potentially could promote a type 2 environment based on adaptive immune response results, I examined whether these macrophages were activated by IL-4/IL-13 to become alternatively activated macrophages (AAMϕ) and express genes associated with this activation state, namely arginase I and mannose receptor (CD206) (Van Dyken and Locksley, 2013). Indeed, mice that received treatment with i.n. flagellin had increased percentage and numbers of macrophages ($F4/80^{+}CD11b^{+}Ly6C^{-}$) that were alternatively activated in that they expressed arginase I, as detected by the arginase I reporter mice (YARG) (Reese et al., 2007) and/or expressed CD206 on the cell surface (data not shown). In addition, mice treated with i.n. flagellin also had increased percentage and numbers of monocytes/macrophages ($F4/80^{+}CD11b^{+}Ly6C^{+}$) that were also arginase I^{+}

and/or $CD206^{+}$. Interesting, IL-4R$\alpha^{-/-}$ mice had a similar magnitude increase in flagellin-induced AAMϕ compared to what was seen in control mice, suggesting that other signaling pathways or cytokines were responsible for this alternative activation. Supporting this observation, in preliminary experiments, I was not able to detect IL-4 or IL-13 in the lungs on d3 after i.n. treatment with flagellin plus OVA using 4get/KN2 mice and YetCre13 IL-13 reporter mice (Price et al., 2010). Also, one previous study has shown that intracellular pathogens, such as *Mycobacterium tuberculosis*, can induce arginase 1 expression in macrophages through TLR signaling, not STAT6 signaling, which is downstream of IL-4Rα (El Kasmi et al., 2008). However, CpG ODN did not lead to increased arginase I nor CD206 expression on non-alveolar macrophages or monocytes, compared to OVA alone. Another explanation for the intact specialization of AAMϕ in the IL-4R$\alpha^{-/-}$ mice is that some of the genes induced in macrophages may indeed be dependent on IL-4/IL-13, but not arginase or CD206 expression. One way to address this question would be by sorting macrophages and monocytes from the lungs of i.n. flagellin treated mice and examining genes that are only upregulated by IL-4/IL-13 and/or by IL-10, which is also known to induce AAMϕ.

Possible reasons why flagellin, a TLR ligand expressed on bacteria, leads to a T_H2 response

The biological significance of why the TLR5 and TLR11 ligand flagellin induces a T_H2 response in the lungs is unknown. This is particularly puzzling because flagellated bacteria express multiple TLR ligands and other PAMPs that induce a T_H1 and/or T_H17 response (Medzhitov, 2007). Perhaps during these infections, there are concomitant T_H2

responses. Though T_H2 responses in the lungs can have negative consequences such as the development of asthma and allergy, T_H2 responses are thought to have evolved to provide protection to the host against the damaging effects of multicellular metazoan parasites (Allen and Sutherland, 2014). The potential T_H2 response mounted against flagellated bacteria may be important in strengthening lung epithelia to prevent dissemination to other sites of the body, to expel bacteria, and also to induce tissue remodeling, wound healing, and the anti-inflammatory response, which may be helpful particularly if bacteria infection induces immunopathology. Supporting this hypothesis, Van Maele et al. (2014) showed that flagellin induced not only pro-inflammatory cytokines, but also genes associated with tissue differentiation and repair in the lungs, including the gene encoding the protein amphiregulin. Arginase I is important in tissue remodeling and repair (Allen and Sutherland, 2014), and as discussed previously, I found that flagellin induced arginase expressing macrophages and monocytes. In addition, flagellin led to the activation of ILC2s, which may produce proteins important for tissue repair.

CpG ODN and flagellin as potential adjuvants in the lungs

CpG ODN and flagellin may be good candidates for vaccine adjuvants because most vaccines provide protection by eliciting humoral immunity (Coffman et al., 2010). CpG ODN, when used as an adjuvant, led to increased amounts in the serum of OVA-specific IgG, which included IgG1 (data not shown) and IgG2c (Fig 3.1). Flagellin, when used as an adjuvant, led to increased serum titers of OVA-specific IgG, which included IgG1 (data not shown) but not IgG2c, and also increased serum OVA-specific IgE (Fig

4.1). Similarly, Wilson et al. (2012) showed that flagellin induced antigen-specific IgE. Additionally, Van Maele et al. (2014) demonstrated that flagellin induced an increase in the levels of antigen-specific IgG1 in the serum, and increased antigen-specific IgA and IgG1in the lung airspace. Since nasal vaccines may be one of the most effective routes of immunization for providing protection locally, to other mucosal sites, and systemically, future studies should explore using CpG ODN and flagellin as adjuvants in the lungs. Indeed, one study has shown that mice immunized i.n. with flagellin plus the F1 antigen of *Yersinia pestis* had increased levels of F1-specific IgG, and had increased survival against lethal *Y. pestis* i.n. challenge (Honko et al., 2006). Similarly, another study has shown that i.n. immunization with flagellin plus inactivated influenza (PR8) increases serum IgA and IgG compared to immunization with PR8 alone (Skountzou et al., 2010). In that study, immunization with flagellin plus PR8 led to increased survival following influenza virus infection compared to PR8 immunization alone.

CpG ODN has been a prime vaccine adjuvant candidate, and is currently being clinically tested in vaccines for various infectious diseases and cancer (Scheiermann and Klinman, 2014). However, many of the current clinical trials, even for influenza virus, are being tested using intramuscular or subcutaneous (s.c.) routes of immunization. Also, although there have been numerous murine studies showing that s.c. or i.p. immunization with CpG ODN and pathogen antigen can provide protection against subsequent infections (Klinman, 2006), very few studies have addressed the efficacy of using CpG ODN as an adjuvant in the lungs. Whether i.n. administration of vaccines are more effective than systemic immunization for respiratory infections and other types of infection should be considered.

My results suggest that TLR9 expression in conventional DCs is important for mediating CpG ODN responses in the lungs. However, how relevant my murine studies will be in humans is unclear. Studies have shown that TLR9 expression is more restricted in human DCs compared to mouse DCs. From studies using human peripheral blood samples (Kadowaki et al., 2001) and human lung biopsy samples (Demedts et al., 2006), mainly pDCs express TLR9. However, these studies focused on secretion of IFNα upon CpG-A stimulation, and therefore they do not exclude the possibility the human lung cDCs may express low levels of TLR9 compared to pDCs and may make other cytokines in response to CpG ODN stimulation.

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