Characterization of Host Protective Immunity against Influenza Infection in Ferrets and Mice

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Immunology
University of Toronto

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2013

Abstract

Influenza virus infects the human population worldwide and causes acute respiratory disease. Currently, the primary strategy for preventing influenza is seasonal vaccination which is capable of providing protection in most populations. However, seasonal vaccines are less efficacious to immunize the elderly and poorly induce cross-protective immunity against the reassorted pandemic virus in the recipients. Neuraminidase (NA) inhibitors have also been widely utilized to limit disease outcome. The currently used NA inhibitors, nonetheless, generate the drug-resistant progeny viruses; moreover, they are unable to directly target the host immune responses which cause immunopathology in severe cases. Therefore, new strategies that provide more effective immunogenicity, cross-protection and therapies against influenza infection must be developed. In this thesis, the adjuvanticity of CpG oligodeoxynucleotide (ODN), type I interferon (IFN) and Complete Freund’s adjuvant (CFA) when coadministered with seasonal influenza vaccines in ferrets is presented. It has been found that the adjuvanted vaccines are efficacious to induce neutralizing antibody responses. Several common and distinguished signaling pathways leading to dendritic cell (DC) maturation and B cell activation have been discovered from their adjuvanticity. Furthermore, it was determined that seasonal H1N1 prior infection more effectively induces cross-protection against the newly emerged 2009 pandemic
H1N1 (H1N1pdm) virus in ferrets and mice than the seasonal vaccines. The prior infection-induced cross-reactive but non-neutralizing antibodies are capable of providing substantial protection in the H1N1pdm infected mice when CD8 T cells are absent. Lastly, function of different vaccine adjuvants for controlling H1N1pdm infection in mice has been investigated. Unlike other adjuvants, CFA is capable of protecting the mice from infection through enhancement of Treg cell suppressive molecules galectin-1 and CTLA-4 which downregulated DC costimulation and effector T cell responses. Overall, this thesis has provided novel mechanistic insights for developing protective strategies against influenza infection.
Acknowledgments

First, I would like to thank my supervisor Dr. David Kelvin for his advice, support and understanding throughout the years. He leads me to an amazing research field of infection and immunity. Also, I would like to thank my supervisory committee members Dr. Rupert Kaul and Dr. Mario Ostrowski for their guidance and suggestions.

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I would like to give special thanks to Mr. Washington Shao and Ms. Xiaonan Wang for their friendship, scientific suggestions and especially the introduction of new research tools.

Finally, I would like to thank my entire family, especially my wife Jessie Gong, for their support, love and encouragement over the years. It is to my family I dedicate this thesis.
Declaration of Work Performed

I declare that with the exception of the items indicated below, all the work reported in this thesis has been performed by me.

Dr. Luoling Xu performed the RNA extraction for the microarray analysis and processed the microarray chip hybridization and scanning. Dr. Longsi Ran collected the raw data of microarray analysis.

Ferret tissue samples were collected by Ms. Jean Flanagan, Mr. Roman Skybin and Ms. Maria Monroy, from the Animal Resources Centre at Toronto General Hospital (Toronto, Canada). Ferret cDNA products were sequenced at the International Institute of Infection and Immunity (Shantou, China). Histology staining of the lung tissue samples was performed by the Histology Laboratory at Toronto General Hospital (Toronto, Canada).
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<tr>
<td>α</td>
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<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>AICDA</td>
<td>Activation-induced cytidine deaminase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>Cdt1</td>
<td>Chromatin licensing and DNA replication factor 1</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
</tr>
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<td>CFI</td>
<td>Complement factor I</td>
</tr>
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<td>CK1</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>C1QL2</td>
<td>Complement component 1, q subcomponent-like 2</td>
</tr>
<tr>
<td>C1R</td>
<td>Complement component 1, r subcomponent</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>Dbf4</td>
<td>Activator of S-phase kinase-like protein 1</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FCN1</td>
<td>Ficolin 1</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>GNA15</td>
<td>Guanine nucleotide binding protein, alpha 15</td>
</tr>
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<td>GNB3</td>
<td>G protein, beta polypeptide 3</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GNG2</td>
<td>Guanine nucleotide binding protein (G protein), gamma 2</td>
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<td>HA</td>
<td>Hemagglutinin</td>
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<td>HPAI</td>
<td>High pathogenic avian influenza</td>
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<td>H1N1pdm</td>
<td>Pandemic H1N1</td>
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<td>IFI</td>
<td>Interferon induced genes</td>
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<td>Interferon</td>
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<td>Interleukin</td>
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<td>IPA</td>
<td>Ingenuity pathway analysis</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<td>ISG</td>
<td>Interferon stimulated gene</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LAIV</td>
<td>Live attenuated influenza vaccine</td>
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<td>MAP2K2</td>
<td>Mitogen-activated protein kinase kinase 2</td>
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<td>mDC</td>
<td>Myeloid dendritic cell</td>
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<td>MHC</td>
<td>Major histocompatibility antigen</td>
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<td>MCM</td>
<td>Minichromosome maintenance protein</td>
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<td>Nuclear factor-kappa B</td>
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<td>Mx</td>
<td>Myxovirus resistance</td>
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<tr>
<td>NCBI</td>
<td>National centre for Biotechnology Information</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T-cell</td>
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<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NOD2</td>
<td>Nucleotide-binding oligomerization domain containing 2</td>
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<td>NP</td>
<td>Nucleoprotein</td>
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<tr>
<td>OAS</td>
<td>Oligoadenylate Synthase</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>Oct2</td>
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<td>ODN</td>
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<tr>
<td>Orc6</td>
<td>Origin recognition complex, subunit 6</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
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<td>PEG-IFN</td>
<td>Pegylated-IFN</td>
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<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
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<td>Polyinosinic: polycytidylic acid</td>
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<td>RSAD2</td>
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<td>sH1N1</td>
<td>Seasonal H1N1</td>
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<tr>
<td>SOCS</td>
<td>Cytokine Signaling</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2</td>
</tr>
<tr>
<td>TIV</td>
<td>Trivalent inactivated influenza vaccine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
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<tr>
<td>USP18</td>
<td>Ubiquitin specific peptidase 18</td>
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Chapter 1

Introduction
1.1 Overview of influenza (A) virus

Circulation of influenza viruses is frequently observed among different hosts. Seasonal influenza viruses cause regional outbreaks in global human populations every year and result in millions of infection cases with associated influence on world economics (Molinari et al., 2007). Influenza viruses are enveloped and contain negative-sense single-stranded RNA. They are classified as type A, B and C viruses and, of these three types, Influenza A viruses are the most pathogenic and demonstrate the highest frequency of mutation (Wright et al., 2007). Generally, coinfection with two or more genetically distinguished influenza A viruses in a single host cell leads to generation or reassortment of a new virus which is considered to cause pandemic infection.

Influenza A virus (IAV) includes eight RNA segments which encode 11 to 12 viral proteins (Figure 1.1): receptor binding protein hemagglutinin (HA); sialic acid cleaving enzyme neuraminidase (NA); matrix protein (M1); ion channel protein (M2); nucleoprotein (NP); three RNA dependent RNA polymerases (PB1, PB2 and PA); non-structural proteins (NS1 and NS2); and recently identified N40 protein (Wise et al., 2009). IAV is categorized as different subtypes by the antigenic properties of HA (H1-H16) and NA (N1-N9). The subtypes of IAV are divided into two major groups, 1 and 2, which are further classified into 5 clades H1a (H1, H2, H5, H6), H1b (H11, H13, H16), H9 (H8, H9, H12), H3 (H3, H4, H14) and H7 (H7, H10, H15) according to the antigenic features of HA (Air, 1981; Fouchier et al., 2005; Nobusawa et al., 1991; Russell et al., 2004). HA establishes host tropism by binding to the host cell receptors which are distinguished as the α-2,3-linked or α-2,6-linked sialic acid (SA) moieties. Avian influenza virus HA preferentially binds to the α-2,3-linked SA receptor; in contrast, human strain HA binds to the α-2,6-linked SA receptor (Gagneux et al., 2003; Matrosovich et al., 1999). During fusion of the virus and host cells, the host cell protease is required to cleave a cleavage site on the HA. The amino acid sequence of this cleavage site determines the tissue tropism and severity of the disease (Skehel & Wiley, 2000). NA is essential for the newly produced virions to be released from host cells by damaging the SA containing receptors of the host cell and virus membranes. In general, group 1 of IAV comprises H1a, H1b and H9 clades which contain the 1918/2009 pandemic virus and human seasonal H1N1 virus (Shapshak et al., 2011), as well as the H5 subtype that includes the highly pathogenic avian influenza (HPAI) strain H5N1 (Matrosovich et al., 1999). In group 2, H3 and H7 clades include human H3N2 strains and HPAI H7N7 strains.
Antigenic evolution in seasonal influenza virus is commonly related to antigenic drift which makes a new strain with mutated amino acids in HA or NA that partially escapes from the pre-existing immunity in the host (Steel et al., 2010). In contrast, influenza A viruses that undergo antigenic shift demonstrate more dramatic changes in antigenicity and typically cause pandemic emergence (Wright et al., 2007).

**Figure 1.1 Structure of influenza A virus.**

The influenza A virus contains two major surface glycoprotein HA and NA. M2 is the ion channel which is embedded in a lipid bilayer. The M1 protein is underneath the virus envelope and interacts with the surface proteins and ribonucleoproteins (RNP). RNP includes 8 negative sense single RNA fragments, NP and polymerase complex heterotrimer (PA, PB1, PB2). The nonstructural protein (NS) 2 is contained within the virion, but the NS1 is not. (Adapted from Taubenberger and Kash, 2010).

### 1.1.1 Overview of seasonal influenza virus

Evolution of seasonal influenza virus causes epidemic outbreaks which lead to millions of infections and about 500,000 deaths annually (Wright et al., 2007). The yearly circulating human seasonal influenza viruses are H1N1 strains, H3N2 strains, and influenza B strains. With antigenic drift, new seasonal strains incorporate replication error in their genome RNA which encodes mutated amino acids in the surface antigens HA or NA (Arias et al., 2009). These partial changes in the antigenicity may allow the new strains to evade the host pre-existing immunity to some extent. Thus the seasonal influenza vaccines which include the circulating H1N1, H3N2 and influenza B strains in the following season are produced each year to immunize the global
population. Seasonal influenza virus causes mild to moderate symptoms in the adult population, whereas severe cases are usually seen in young children and elderly people (discussed below).

1.1.2 Overview of pandemic influenza virus

If a single host cell is coinfected with 2 or more different IAVs, the progeny virus contains the RNA fragments from parental viruses. Thus the newly reassorted virus with dramatically changed antigens is traditionally considered to be a potential pandemic strain which can escape the host’s pre-existing immunity and cause a large number of infections associated with an increased number of deaths. Influenza A viruses have caused approximately 14 pandemics since 1510. Over the last 120 years, six pandemic infections occurred in 1889, 1918, 1957, 1968, 1977 and 2009, respectively (Taubenberger & Morens, 2009). The 1918 pandemic resulted in the worst morbidity and mortality in recent history with approximately 500 million people infected and up to 50 million deaths (Johnson & Mueller, 2002). The pandemics in 1957 and 1968 caused about 1 to 2 million deaths worldwide (Guan et al., 2010). The novel reassorted H1N1 swine origin influenza virus emerged in 2009 to produce the first pandemic in the twenty-first century. The 2009 H1N1 pandemic virus infected millions of people globally and caused more than 18,000 deaths within the first year (Korteweg & Gu, 2010).

1.2 Pathogenesis of influenza virus

Influenza A and B viruses cause respiratory disease in humans with associated symptoms such as fever, coughing/sneezing and body aches. The symptoms usually improve in a few days; however, the pandemic viruses with increased pathogenesis cause more severe illness and prolong the recovery from the infection. Furthermore, each year seasonal influenza viruses cause acute respiratory infections in a large proportion of young children and elderly people who lack efficient immunogenicity. The aspects of indicating the pathogenesis of influenza virus are generally categorized by the virulence factors and host factors.

1.2.1 Pathogenesis of seasonal influenza

Influenza viral proteins contribute to pathogenesis in the lungs of humans. Among these proteins, HA is responsible for targeting the host cells for infection (Liu et al., 2010a; Tumpey et al., 2007). The HA of seasonal IAV binds to the α-2,6-linked SA receptors which are expressed on the epithelial cells of the human upper respiratory tract (Gagneux et al., 2003). Thus the
infection caused by seasonal IAV is restricted to the upper respiratory tract with associated mild symptoms. On the other hand, high titers of seasonal IVA shed in the upper respiratory tract favors transmission in human populations. HA also plays a role in pathogenicity through its susceptibility to proteases. To initiate infection, HA of influenza virus has to be cleaved into two subunits HA1 and HA2 (Chen et al., 1998). The cleavage of seasonal IAV HA is performed by a trypsin like protease which is mainly produced in respiratory and gastrointestinal cells (Klenk & Garten, 1994). Thus seasonal IAV infections are commonly unable to cause systemic inflammation or tissue damage in the host.

Typically, children are most frequently hospitalized as a result of seasonal influenza infection. The annual rates of hospitalization range from 10 to over 100 per 10,000 children according to the influenza season and geographic region (Neuzil et al., 2000; Poehling et al., 2006). Symptoms such as high fever, cough, sore throat and runny nose commonly observed in adults are also seen in children. In contrast to adults, children infected with influenza A or B virus develop gastro-intestinal symptoms (Peltola et al., 2003). Acute otitis media (AOM) is the most common complication observed in the respiratory tract of children infected with seasonal influenza. The rate of AOM ranges from 25% to 75% in infected children according to different clinical reports (Peltola et al., 2003). Furthermore, secondary infection with bacteria is commonly observed in children infected with seasonal influenza and is the main cause of mortality in pediatric patients (Kuiken & Taubenberger, 2008; Peltola et al., 2003). Generally, seasonal influenza infection causes more severe symptoms in young children and this may partially be due to their immature immune systems; however, mortality in infected children is limited (Dawood et al., 2010; Neuzil et al., 2000).

In the elderly population aged above 65, increased morbidity and mortality caused by seasonal influenza infection has been shown annually (Thompson et al., 2003; Thompson et al., 2004). Immunesenescence is one of the reasons that aged people fail to develop efficient immune responses to overcome seasonal influenza caused illness (McElhaney, 2011). Moreover, elderly individuals with chronic underlying medical circumstances such as neurological, cardiopulmonary and metabolic diseases, as well as those who are immunosuppressed, are more susceptible to influenza infection with associated severe symptoms (Anonymous, 2012). Given that increasing risk factors are usually associated with poor clinical outcome seen in elderly
people, seasonal vaccination is commonly recommended for them to control severity of the illness.

1.2.2 Pathogenesis of pandemic influenza

Pandemic influenza viruses spread worldwide quickly and cause a large number of infections with associated high mortality. The reassorted virus evades the pre-existing immunity and typically induces severe symptoms in the host. HA is one of the viral proteins that contribute to the disease outcome caused by the pandemic virus. As discussed above, HA of seasonal IAV binds to the 2,6-linked SA receptors and seasonal virus mostly infects the human upper respiratory tract with associated mild symptoms. In contrast, it has been shown that mutation in the HA of pandemic virus, particularly a D222G substitution in HA of 2009 H1N1, switches the binding specificity from α-2,6 to α-2,3-linked SA (Liu et al., 2010b). The α-2,3-linked SA expressing receptors are mostly found on the tracheobronchial epithelial cells in the human lower respiratory tract (Shinya et al., 2006). The binding to 2,3-linked SA receptors by pandemic virus changes the site of infection to the deeper respiratory tract and causes more severe pneumonia. Thus 2009 H1N1 virus with substitution D222G in HA is usually detected in severe or fatal cases (Mak et al., 2010). Moreover, HPAI H5N1, which specifically attaches to the α-2,3 receptors and infects type II pneumocytes in humans, causes high mortality in infected patients (van Riel et al., 2006). Therefore, diagnosis of any HA mutation that changes the host cell tropism should be considered in future surveillance to evaluate severity of the disease caused by the potential pandemic strains.

The viral RNA polymerase complex contains PA, PB1 and PB2, which control transcription and replication of the viral genome in infected host cells. It has been shown that several mutations in PA and PB2 increase the replication efficiency of avian influenza virus in mammalian cells (Bussey et al., 2010; Song et al., 2009). The replacement of viral RNA polymerase genes in HPAI H5N1 with the ones from low pathogenic H5N1 virus strongly decreased pathogenicity in the infected animals (Salomon et al., 2006). Also, it has been reported that RNA polymerase complex and NP significantly contribute to the pathogenicity of 1918 pandemic virus (Watanabe et al., 2009). Thus mutation of RNA polymerase genes may be closely associated with the pathogenesis of pandemic virus. Additionally, several other viral proteins such as PB1-F2, NA and NS1 have been found to play important roles in virus
pathogenicity. PB1-F2 with amino acid mutation N665S highly increases the pathogenesis of both 1918 pandemic virus and HPAI H5N1 by inducing more pro-inflammatory cytokine TNF-α (Conenello et al., 2007). Mutation of amino acids in NA supports more efficient virus replication, whereas mutated NS1 suppresses anti-viral interferon responses in host (Pappas et al., 2008).

The host immune system is vital for controlling influenza infection. Seasonal viruses commonly induce mild inflammation and normal host immune responses during infection, and symptoms are normally improved within a week. In contrast, pandemic viruses dramatically enhance inflammation in the respiratory tract and stimulate excessive host immune responses in most severe cases. The disease outcome is typically associated with immunopathology mediated pulmonary tissue damage that causes acute respiratory distress symptoms. Thus immune response is considered a host factor involved in the influenza caused pathogenesis (discussed below). Suppression of overactive host immune responses is proposed as a new strategy that may lead to therapeutic treatment for pathogenic influenza infection in humans.

1.3 Host immune responses to influenza

Host immunity to influenza virus plays crucial roles in the control of influenza caused illness. After infection, respiratory epithelial cells are first targeted by influenza virus and produce the progeny virus that subsequently infects alveolar macrophages and resident DCs in the respiratory tract. Then cytokines and chemokines that are expressed by the infected local macrophages/DCs attract innate immune cells such as neutrophils, monocytes and natural killer (NK) cells to the site of infection. Innate immune cells establish the first line of defense to influenza and also mediate activation of adaptive immunity in the host. Following the infection, the local and infiltrated innate immune cells express large amounts of the cytokines and chemokines which recruit B cells and T cells to generate influenza specific adaptive immunity that controls the current infection and also possibly provides protection against the next influenza.

Host immune responses against influenza virus have been studied for 70 years (Andrewes, 1939). The discovered important properties and new insights of innate and adaptive immunity against the influenza virus have significantly improved our understanding of the
correlation between immunity and illness. In this chapter, several crucial aspects of influenza immunity are reviewed.

### 1.3.1 Innate immunity to influenza

Innate immune responses outline the primary defense that prevents virus infection and replication in the respiratory epithelial cells upon influenza infection. Innate immunity includes the virus sensing system, acute innate cell responses, and innate cell mediated cell killing, which are specifically discussed below.

#### 1.3.1.1 Detection of influenza virus by innate immunity

Influenza virus initiates innate immune responses when recognized by the three major families of innate receptors which are the toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I like receptors (RLRs). TLR7, which is mainly expressed by pDCs, recognizes viral single-stranded (ss) RNA, whereas TLR3 and RIG-I bind to viral double-stranded (ds) RNA. Ligation of these receptors results in the expression of type I interferons and pro-inflammatory cytokines (Alexopoulou et al., 2001; Heil et al., 2004; Yoneyama et al., 2004). The first wave of type I IFN production is induced by the IFN regulatory factor IRF3 which is recruited following activation of TLR3 and RIG-I signaling pathways. Phosphorylated IRF3 homodimer moves into the nucleus and forms the transcription complex with transcriptional coactivator p300 and CREB-binding protein (CBP) to produce IFN-β (Suhara et al., 2002). Additionally, serine/threonine protein kinase (PKR) activated by the viral dsRNA can induce type I IFN mRNA expressions via NF-κB and AP-1 (Chu et al., 1999). Synergism of these signaling pathways contributes to the original accumulation of type I IFN, which further activates the expression of IRF7 (Sato et al., 1998). IRF7 has been known as a primary transcription factor for inducing type I IFN expressions, whereas IRF3 is likely to assist IRF7 in accomplishing its entire function (Honda et al., 2005). IRF7 is capable of triggering the expression of several IFN-α subtypes and establishing the second wave of type I IFN responses (Sato et al., 1998). Furthermore, viral ssRNA can activate MyD88-IRF7 mediated IFN-α expression directly by binding to TLR7/8 which is mainly expressed by pDC (Bowie & Haga, 2005). pDCs are considered the main sources for producing type I IFN due to their capacity of constitutively expressing IRF7 (Prakash et al., 2005). Also, type I IFN stimulates pDCs to express high levels
of IRF7 which form the positive loop for inducing expression of IFNs (Asselin-Paturel & Trinchieri, 2005).

Type I IFN demonstrates anti-viral activities by inhibiting protein synthesis in the infected cells. Furthermore, type I IFN stimulates the JAK/STAT signaling pathway to activate the expression of several anti-viral ISG molecules including OAS1/2, MxA, RNaseL and ISG15 which restrain influenza virus replications (Katze et al., 2002). Also, type I IFN promotes maturation of DCs which present antigens to CD4+ and CD8+ T cells and assist adaptive cellular responses against viral infection (Le Bon & Tough, 2002a). Recently, it has been demonstrated that influenza viral RNA can bind to the NOD-like receptor NLPR3 to induce inflammasome signaling that activates caspase-1 for cleaving pro-IL1β and IL18 via a viral ion channel (M2) dependent mechanism (Ichinohe et al., 2010). Another NLR NOD2, which commonly binds to bacterial protein or derived peptides, has also been found to recognize influenza ssRNA and activate type I IFN responses following influenza infection (Sabbah et al., 2009). These early anti-viral immune responses are mainly mediated by the respiratory epithelial cells, alveolar macrophages, and DCs. In particular, the activities of alveolar macrophages and DCs play crucial roles in the regulation of both innate and adaptive immunities against influenza infection (discussed below).

1.3.1.2 Innate immune cell responses

Alveolar macrophages become activated and phagocytic upon influenza virus infection in alveoli. The activated macrophages engulf the infected cells to inhibit virus replication. Alternatively, activated macrophages in the lungs produce nitric oxide sythtase 2 (NOS2) and several pro-inflammatory cytokines/chemokines which are associated with influenza caused pathogenesis (Jayasekera et al., 2006; Lin et al., 2008). Thus these two distinct activities of macrophages in the lungs closely regulate the balance between anti-viral and pathogenic responses in the host. It has been shown that HPAI virus can infect both alveolar macrophages and infiltrated peripheral macrophages and that the infected blood-derived macrophages express high levels of pro-inflammatory cytokines that cause immunopathology in the lungs. In contrast, the HPAI infected alveolar macrophages are less efficient at producing viral progeny and pro-inflammatory cytokines (van Riel et al., 2011). Additionally, alveolar macrophages and/or peripheral-derived CCR2+ macrophages play a role in the establishment of CD8+ T cell
responses and survival (Wijburg et al., 1997). These findings suggest that macrophages may regulate immune responses during influenza infection, in addition to their traditional function of phagocytosis.

DCs are the primary antigen presenting cells (APCs) in immune system. Resident DCs in the respiratory tract are located underneath the airway epithelium and on top of the basal membrane. By the solid interactions between their extended dendrites and the respiratory epithelial cells, resident DCs scrutinize the airway lumen and uptake non-self antigens (GeurtsvanKessel & Lambrecht, 2008). Virions and apoptotic bodies of infected cells are engulfed by the resident DCs to obtain viral antigens; and DCs can be infected by influenza virus to process antigen presentation. Once resident DCs acquire the viral antigen, they migrate to the draining lymph nodes with the homing receptor CCR7 and present the antigens to the circulating CD4+ and CD8+ T cells via MHC class I/II molecules (Hintzen et al., 2006). To process MHC class I mediated antigen presentation, influenza virus derived peptides are released into cytosol by proteasomes and subsequently assembled with the MHC class I molecule in the endoplasmatic reticulum (ER). Next viral peptide-MHC class I complexes are transported to the DC membrane through the Golgi apparatus and are recognized by CD8+ cytotoxic T cells. For MHC class II antigen presentation, viral peptides are derived from influenza virions in endosomes or lysosomes and are assembled with MHC class II molecules. The viral peptide-MHC class II complexes are then relocated to the cell membrane and bind to CD4+ T helper cells. In addition, mature DCs utilize multiple costimulation molecules such as CD40, CD80 and CD86 to assist MHC I/II antigen presentation for fully activating T cell responses (McGill et al., 2009).

NK cells play crucial roles in innate immunity. In influenza infection, type I IFN and cytokines produced by the macrophages activate NK cells with increased expression of IFN-γ which subsequently promotes Th1 responses (Welsh, 1986). NK cells can recognize antibody bound infected cells to lyse them via antibody dependent cell cytotoxicity (ADCC). Also, NK cells can directly bind to HA of the infected cells by the cytotoxicity receptors NKp44 and NKp46 that trigger lysis of the infected cells (Arnon et al., 2001; Mandelboim et al., 2001). Recently, invariant NKT (iNKT) cells expressing invariant TCR α and β chains, which are reactive to glycolipid antigens associated with CD1d on APCs, have been suggested to play
important roles in anti-influenza immunity and regulation of pro-inflammatory cytokine mediated pathology (Paget et al., 2011).

Characterization of innate immunity against influenza infection provides valuable information about the early stages of host immune responses, which may play crucial roles in protection and pathogenesis. Further studies in this field would lead to better understanding of disease progression in the initial phase, and may aid in the development of novel therapeutic strategies for controlling early infection. With the concern that innate immunity has tight interactions with adaptive immune responses, however, attention should be paid to the therapy that may limit activation of innate immunity and thus result in inefficient adaptive immune responses against influenza virus.

1.3.2 Adaptive immunity to influenza

Adaptive antibody and cellular responses form the second line of anti-influenza defense (Figure 1.3). Neutralizing antibodies are induced to directly block virion entry and restrain the following infection. Also, the non-neutralizing antibodies can bind to the viral antigens expressed on the infected cells to mediate ADCC and lyse these cells. On the other hand, CD8+ T cells activated by the DCs that present influenza viral antigen efficiently control infection by destroying the infected cells through cytotoxicity. In addition, CD4+ helper T cells assist both antibody and cytotoxic T cell responses to limit influenza virus infectivity. Adaptive immunity is specific and effective in controlling influenza infection. Failed or inadequate adaptive immune responses are commonly associated with severe or lethal cases in humans.

1.3.2.1 Antibody responses

Specific antibody responses are induced following influenza infection (Mancini et al., 2011). The antibodies recognize the localized regions of the antigen called epitopes on free influenza virions or surfaces of the infected cells. Early after primary influenza infection, B cells encounter viral antigens expressed by the DCs that migrate from the site of infection to the draining mediastinal lymph nodes (Sealy et al., 2003). With costimulation of CD40 from DCs, B cells proliferate vigorously and differentiate into plasma cells to produce antigen specific antibodies. This B cell response can contribute to long-lasting humoral immunity by the additional help from CD4+ T cells (Sealy et al., 2003). IgM, IgG and IgA are the main antibody isotypes raised in the host after influenza infection. IgM antibodies are first induced after
primary infection and assist complement mediated influenza virus neutralization (Fernandez et al., 2008). IgG antibodies are long-lived and comprised of different subtypes such as IgG1, IgG2a and IgG2b which predominantly mediate humoral immunity against influenza via neutralization or ADCC (Stanekova & Vareckova, 2010). The secretory IgA antibodies are produced locally and provide protection for the epithelial cells of the mucosal respiratory tract by transcytosis transport. The dimeric IgA antibodies that are transported into epithelial cells by the poly IgG receptor bind to the viral proteins and prevent viral assembling (Mazanec et al., 1995). Also, IgA antibodies are able to neutralize virion entry locally.

**Figure 1.2 Host adaptive immune responses to influenza virus infection.**

After the influenza virus invades host cells and activates innate immune responses, the host adaptive immune system (i) humoral immune responses and (ii) cellular immune responses are activated to limit virus infectivity. The B cells are activated through BCR which recognizes the viral antigens to become the plasma cells for producing antibodies. Also, B cells behave as antigen presenting cells to present antigens to T helper cells. The activated T cells limit viral infection by directly lysing the infected cells via cytotoxicity and indirectly helping the cytotoxic T cells (via Th1) or B cells (via Th2) to inhibit viral replication. (Adapted from Valkenburg et al., 2011).

Among the antibodies raised following influenza infection, the antibodies against virus surface antigen glycoprotein HA or NA are important indicators for protective immunity. HA is responsible for the binding of the influenza virus to receptors and entering into host cells. Anti-
HA antibodies can directly recognize the free influenza virions and neutralize their entry into host cells. Consequently, the viron-antibody complexes are engulfed by innate immune cells via Fc receptors. Furthermore, HA epitopes expressed on the infected cells are recognized by the anti-HA antibodies which mediate ADCC to lyse these cells. HA antibodies are the most protective agents in the humoral immunity and are the primary indicators for the efficacy of the currently used influenza vaccines. The anti-HA antibodies induced by infection or vaccination, however, are very specific to the matched strain and have limitations for providing broad protection against different viruses with antigenic variations. Compared to the changeable HA globular head, the HA stem region is more resistant to mutation or antigenic escape because it is restrictedly exposed to the immune system (Stanekova & Vareckova, 2010). Recently, several studies discovered that the antibodies against conserved epitopes of the HA stem region provided broad neutralizing protection against different subtypes of influenza virus (Ekiert et al., 2009; Ekiert et al., 2011). In fact, anti-HA stem region antibodies are naturally induced following influenza infection but in limited titers. Thus a strategy that aims to enhance the anti-HA stem region antibodies must be considered in our future vaccine development for providing broad protection in recipients.

Antibodies against NA also demonstrate protection against influenza infection. NA has the enzyme properties to cleave sialic acid residues on the cell surface and assist virus release from the infected cells. Anti-NA antibodies are unable to block viral entry or replication in host cells but play a role in controlling spread of the virus. In addition, NA antibodies are involved in ADCC to clean the virus infected cells and limit viral titers during infection (Mozdzanowska et al., 1999).

M2 is the third surface antigen expressed on influenza virus. It forms the ion channels of the virus and plays a role in acidification of the virion and PH neutralization of the Golgi complex following influenza infection. M2 antibodies are also naturally induced by influenza infection but to a limited extent. Given M2 is the most conserved antigen of the influenza virus, it has been considered as a potential candidate for the universal influenza vaccine. Anti-M2 antibodies raised from the M2 protein was first shown to provide protection in an influenza mouse model (Treanor et al., 1990). Several recent studies also demonstrated that M2 antibodies can provide cross-protection against different viral strains in mice (Shim et al., 2011; Song et al., 2011). However, M2 antibodies mediated protection is less efficacious in ferrets, which are
considered more appropriate for influenza research (Fan et al., 2004). Thus more work must be performed to evaluate the applicable value of M2 antibodies for preventing influenza infection.

NP is an internal protein of the influenza virus and known as an antigen mostly targeted by T cells. Interestingly, recent studies demonstrated that anti-NP antibodies also provide protection in the mouse model (Carragher et al., 2008; Lamere et al., 2011a). The combination of NP protein and adjuvant induced broad protection against variant strains in immunized mice and ADCC is the primary mechanism used by anti-NP antibodies for preventing influenza infection (Lamere et al., 2011a). Also, long-lived NP antibodies can provide cross-protective immunity in mice over several months (Lamere et al., 2011b). Because NP is a conserved influenza antigen, it is also considered a candidate for universal vaccines that generate longevity of cross-protective antibody responses.

1.3.2.2 Cellular responses

After influenza infection, antigen specific CD4+ T cells and CD8+ T cells are induced. CD4+ T cells are activated by the antigen-MHC II complex, as well as costimulation molecules expressed on the DCs. The main functions of CD4+ T cells are to help generate optimal antibody and cytotoxic CD8+ T cell responses against influenza virus, although some of them demonstrate cytotoxicity targeting the infected cells (Brown, 2010a). CD4+ T cells also called T helper cells can be divided by two groups which are Th1 cells and Th2 cells. After influenza virus invades the host cells, the viral antigens are subsequently taken up by the respiratory DCs which migrate to the draining lymph nodes and present the antigens to the naïve CD4 T cells (Kim & Braciale, 2009). Additionally, the antigen presenting cells (APCs) express several inflammatory cytokines in the draining lymph nodes and subsequently enhance the CD4 T cell responses. As a result, the CD4 T cells proliferate due to the induction of their autocrine growth factors and become highly differentiated (Jelley-Gibbs et al., 2000). Development of Th1 polarization is promoted by IL12 and IFN-γ, and IL4 inhibited Th1 cell differentiation. In contrast, Th2 cell polarization is promoted IL4 and inhibited by IFN-γ (Swain et al., 1991). The highly differentiated Th1 and Th2 cells are difficult to be reversed due to epigenetic changes of their chromatin (Fields et al., 2004; Schoenborn et al., 2007). Th2 cells typically express cytokine IL4, IL5 and IL13 and promote B cell responses when interactions between B cells and Th2 cells are established via antigen-MHCII-TCR manner (Wright et al., 2007). In contrast, Th1 cells mainly express cytokines IFN-
γ, IL2 and TNF-α which subsequently enhance cytotoxicity of the CD8⁺ T cells. Given that production of inflammatory cytokines are a major characteristic of Th1 cells, their overactive responses to the highly pathogenic influenza viruses may contribute to the immunopathology observed in the severe infections (La Gruta et al., 2007). CD4⁺ T cells are not essential for generating CD8⁺ T cell responses during the primary infection (Riberdy et al., 2000); nonetheless, the recall and size of memory CD8⁺ T cell responses following secondary infection are significantly diminished in the absence of CD4⁺ T cells (Belz et al., 2002). In addition, it has been found that CD4⁺ T cells play vital roles in cross-protective immunity induced by either LAIV or prior influenza infection (Alam & Sant, 2011; Sun et al., 2011).

Cytotoxicity is the primary function of CD8⁺ T cells which hence are called cytotoxic T lymphocytes (CTL). Upon activation by the antigen specific DCs at the draining lymph nodes, CD8⁺ T cells migrate to the site of infection and target the infected cells expressing viral antigens and MHC I. Following attachment, the infected cells are destroyed by the CTL lysis to prevent virus replication and spreading. The granzyme/perforin killing mechanism is primarily used by the CTLs. The membrane of the infected cells is permeabilized by perforin, and thus GzmA/B enters into the cells and induces apoptosis. GzmA is also able to induce pro-inflammatory cytokines and mediate noncytotoxic activities which are mainly associated with the inhibition of viral protein synthesis (Andrade, 2010). Interestingly, a recent study showed that virus specific CTL are also capable of lysing the infected cells in the absence of GzmA and GzmB (Regner et al., 2009), which suggests that other mechanisms such as Fas/FasL induced cell apoptosis is involved in CTL mediated cytotoxicity (Topham et al., 1997). In addition to cell lysis, CD8⁺ T cells can enhance anti-viral activity by expressing multiple cytokines. IFN-γ is first produced by the viral antigen stimulated CTL; subsequently, TNF and IL2 are expressed when extensive activation of CTL is achieved (La Gruta et al., 2004). The synergism of CTL expressed cytokines contribute to further activation of anti-viral T cell responses. Antigen specific memory CTLs are detected in lymphoid organs and in circulation post infection. These cells also exist at the site of infection and are maintained by IL17 expressing T cells and DCs (GeurtsvanKessel et al., 2009; Rangel-Moreno et al., 2011). Upon secondary infection, the memory CTLs respond to the infection immediately and their effectiveness is influenced by costimulation at the initial stage of differentiation (van Gisbergen et al., 2011). In humans, CTLs mainly target the epitopes of internal viral proteins NP, M1 and PA which are conserved influenza antigens (Wang et al.,
Therefore, CTLs are commonly considered to provide cross-protection against a heterologous influenza virus (discussed below).

T regulatory (Treg) cells are stimulated to regulate cellular responses following influenza infection. Both CD4+ and CD8+ T cell responses are regulated by Treg cells through multiple mechanisms (discussed below). This cell population was found to play crucial roles in the restriction of overactive host immune responses when mice were infected with respiratory syncytial virus (RSV) or West Neil virus (WNV) (Fulton et al., 2010; Lanteri et al., 2009). Also, a recent study has shown that Treg cells are capable of reducing effector T cell responses and cytokine expression in mice infected with non-lethal influenza virus (Betts et al., 2011). These results suggest that Treg cells may be vital for the host to regulate immune responses induced by viral infections. More studies are needed to precisely evaluate the function of Treg cells during influenza infection.

1.4 Typical public health strategies for controlling influenza infection

Influenza is one of the pathogen-caused diseases that substantially influence world health and economics. To control influenza infection, vaccines and anti-viral drugs are commonly used in prevention and post-exposure treatments, respectively. Generally, vaccination is the primary strategy that prevents infections by immunizing the populations with viral proteins (Cox & Subbarao, 1999; Nichol & Treanor, 2006). The seasonal influenza vaccines contain H1N1, H3N2 and influenza B strains which may be circulating in the next season. Seasonal influenza vaccines induce neutralizing antibodies which block viral entry into host cells to prevent infection. Up to 75% of the immunized populations are protected by seasonal vaccination (Edwards et al., 1994a), but the protective efficacy is about 30-50% in vaccinated elderly people (Jefferson et al., 2005). Due to antigenic shift, pandemic virus has dramatically different surface antigens compared to previously circulating seasonal strains. Thus seasonal vaccines are less likely to provide protection against pandemic virus. When pandemic virus emerges, the vaccines containing the specific pandemic strains need to be manufactured to immunize populations worldwide in the following pandemic year. However, the currently used technologies for producing influenza vaccines are unable to meet the requirement for immunizing global populations in a short time if the novel reassorted strain causes high mortality coupled with
efficient human-to-human transmission. Therefore, new techniques (e.g., cell culture based) and strategies (e.g., antigen sparing) are being developed to address this dilemma (Belsey et al., 2006).

Influenza infection is usually self-limiting and not required for aggressive treatment; however, respiratory complications such as severe pneumonia, exacerbation of asthma, and chronic bronchitis may occur. Specifically, severe symptoms have been shown in pandemic infected patients. In serious influenza infections, anti-viral drugs such as neuraminidase (NA) inhibitors oseltamivir and zanamivir have been used to treat children, adults and elderly people (Jefferson et al., 2009; Kaiser et al., 2003). The efficacy of NA inhibitors ranged from 61% to 73% in influenza-confirmed patients according to multiple clinical studies (Jefferson et al., 2009). However, increasing evidence has shown that anti-viral drug such as NA-inhibitor confers selective pressure on viruses which mutate with drug resistance (Anonymous, 2009b). Thus anti-viral drug treatment for influenza patients should be further evaluated.

1.5 Influenza vaccine

In general, there are two types of influenza vaccines: the trivalent inactivated influenza vaccine (TIV) and the cold-adapted live attenuated influenza vaccine (LAIV) (Belsey et al., 2006). Currently the most commonly used annual influenza vaccines are TIVs, which contain whole inactivated influenza particles, split virus, or purified influenza HA and NA which are commonly produced using an egg-based manufacturing system. LAIV also contains three viral strains, each of which is manufactured by combining the gene segments from an attenuated master donor virus and the HA and NA from a currently circulating virus. Then the replication of the combined virus is gradually adapted to the decreasing temperature on a cell culture based system. TIVs are licensed for use in the populations aged over 6 months, whereas LAIV can only be administered in people between the ages of 2 and 49 years. Safety is the main reason that LAIV is not licensed for the children younger than two years old, since over wheezing and excess hospitalizations may occur (Belshe et al., 2008). In addition, enhanced efficacy was not observed in the 50 to 64 years aged population who used LAIV (Ambrose et al., 2008).

Various results have been obtained when comparing the efficacy between TIV and LAIV because of the diverse populations and different end points investigated in the studies. A meta-analysis comparing all age populations demonstrated no dramatic overall differences in efficacy
between TIV and LAIV and no significant differences in protection against drifted viruses (Beyer et al., 2002). Controversially, another meta-analysis reported greater efficacy and longer duration of protection in young children using LAIV (Rhorer et al., 2009). Furthermore, it has been shown that LAIV is more efficacious in immunizing the population with no or fewer prior influenza vaccinations or infections, whereas TIV showed greater efficacy in the population showing pre-existing influenza antibodies (Eick et al., 2009). Given LAIV is less efficacious to induce serum HI titers (Beyer et al., 2002), the common indicator used to evaluate TIV efficacy is inappropriate for demonstrating the protection raised by LAIV. Thus other indicators (i.e., T cell responses) for evaluating LAIV efficacy need be considered. In general, LAIV and TIV have different advantages for raising anti-influenza immunity in specific populations.

1.5.1 Adjuvanted influenza vaccine

The goal of influenza vaccine studies is to provide immunity in recipients using the minimal amount of antigen. Although the currently used TIV can elicit protection in up to 75% of immunized populations, the efficacy is not satisfactory in certain populations such as young children and elderly people. LAIV has shown promising results in young children; however, the safety of LAIV for use in the aged population or immunocompromised people is still in debate. Furthermore, a limited supply of vaccines for global immunization is commonly seen when pandemic virus emerges because our currently used technology is inefficient at amplifying viral antigens. Thus an adjuvant that is capable of enhancing immunogenicity with a limited amount of antigen has been developed to be co-administered with the influenza vaccine to meet the requirement to prevent regional outbreaks or the next pandemic.

1.5.2 TLR independent adjuvant

The adjuvants currently licensed for use in human vaccines are mainly TLR independent adjuvants. Among the adjuvants, aluminum (alum) is the first adjuvant that has accepted safety properties for use in humans. Alum is well-known for forming a depot of antigens, and the adjuvanticity of alum that activates immune responses via dendritic cell (DC) interaction has been illustrated very recently (Marrack et al., 2009). Some studies demonstrated that treatment with alum in vitro increased expression of the costimulation molecules on DCs and enhanced antigen presentation (Sokolovska et al., 2007; Ulanova et al., 2001); however, one study has shown a controversial finding that alum did not alter DC maturation (Sun et al., 2003).
Furthermore, it has been shown that alum still induced strong antibody responses in the absence of TLR signaling in gene deleted mouse models (Gavin et al., 2006; Schnare et al., 2001). This finding suggests that alum activates immune responses via a TLR independent manner. Additional studies have shown that entry of alum salts by engulfment leads to lysosomal disruption and NLRP3 inflammasome activation, which mediates a rapid inflammatory response for recruiting innate cells including eosinophils to express IL4 (Wang & Weller, 2008). IL4 enriched environment facilitates a Th2 response that mediates IgG1 and IgE antibody production (McKee et al., 2008). Alum showed optimal adjuvanticity for bacterial toxoid based vaccines; however, it has limitations for enhancing immunity when coadministered with other immunogens including viral antigens. When combined with 15 µg of influenza HA, alum salts (AlPO₄) completely protected ferrets from lethal H5N1 challenge; however, moderate to severe clinical symptoms were observed (Brown, 2010b). It is suggested that vaccine doses are significantly reduced with alum, but disease symptoms are still active after virus challenge by using this simple antibody-induction adjuvant. Thus development of an adjuvant that activates more extensive immune responses is required for better controlling symptoms of influenza caused disease.

Water-in-oil emulsion adjuvant Complete Freund’s adjuvant (CFA) has been considered the gold standard for inducing antibody production in animal models. CFA contains minimal essential stimulus muramyl dipeptide (MDP) which binds to NLR receptor NOD2, as well as possible TLR2/4 ligands (Ellouz et al., 1974). The activity of this traditional adjuvant, however, has not been well characterized in previous studies. This thesis demonstrates that the synergism of CFA containing stimuli dramatically induces DC maturation and B cell responses but with very limited interferon responses, when coadministered with TIV (Chapter 2). Due to extreme toxicity, nonetheless, CFA is prohibited for use in humans. In the past 15 years, squalene (a natural organic compound) based oil-in-water emulsion MF59 has been developed. Clinical trials have shown that MF59 is safe and efficacious for enhancing humoral and cellular immunity against different antigens derived from influenza, HPV and HIV (Clark et al., 2009; Jenkins, 2008; Nitayaphan et al., 2000; Puig & Gonzalez, 2007). The licensed MF59 adjuvanted seasonal influenza vaccines have immunized 20 million people in the last decade, and significantly increased neutralizing antibody titers and cross-reactive immunity against drifted stains in the recipients (El Sahly, 2010). Also, evaluation of MF59 activity in potential pandemic vaccines
demonstrated that adjuvanted vaccine containing avian stain H5N3 provided cross-reactive immune responses against H5N1 virus (Nicholson et al., 2001). In the 2009 pandemic year, a single dose of the MF59 adjuvanted monovalent pandemic vaccine (HA of 3.75 µg or 7.5 µg) induced seroprotection in young adults (Clark et al., 2009). In the elderly population, MF59 combined with 7.5 µg pandemic HA showed significantly higher rates of seroconversion than 3.75 µg HA after the first vaccination, and the HI titer was increased by at least two-fold post secondary immunization (Cheong et al., 2011). All these findings suggest that MF59 is a potent adjuvant for influenza vaccines in humans. The mechanism of MF59 activity has been investigated recently. It has been shown that MF59 significantly increased the expression of chemokines CCL2, CCL3, CCL4 and CXCL8 after stimulating human macrophages, monocytes and granulocytes in vitro (Seubert et al., 2008). Also, MF59 induced monocyte differentiation into DCs by decreasing CD14 and upregulating costimulation molecule CD86. In vivo experiments have shown that, at the site of injection, MF59 combined immunization recruited mature macrophages which engulfed antigens and differentiated into DCs at draining lymph nodes (Dupuis et al., 2001). In addition, MF59 assisted DC maturation by targeting skeletal muscle at the site of injection to induce the expression of pro-inflammatory cytokines and chemokines, which created local immune responses for indirectly activating DCs via a TLR independent manner (Mosca et al., 2008).

TLR independent adjuvants alum and MF59 activate host immune responses via multiple mechanisms including the stimulation of innate immune responses, the increase of antigen presentation, and the accumulation of immune cells. Further studies are required to explore the crucial mediators and pathways that are involved in each mechanism for illustrating the interactions among the different arms of immune function, thereby providing new insights for vaccine design.

1.5.3 TLR dependent adjuvant

It is known that TLR ligands are capable of triggering innate immune responses and driving strong adaptive immunogenicity, both of which are crucial to prevent infectious disease (Beutler et al., 2006). A variety of immune stimuli such as protein, lipopeptides, carbohydrates and nucleic acids, which have been well characterized as TLR ligands, are currently being examined as vaccine adjuvants in experimental animal models or clinical studies (Duthie et al.,
Binding of TLRs leads to activation of signaling pathways initiated from the toll/IL1 receptor (TIR) domain which recruits adaptors including MyD88, TIRAP and TRIF (Figure 1.3). Most TLRs activate signaling pathways via Myd88 in a dependent manner, except TLR3 and TLR4, which can also utilize TRIF (Hoebe et al., 2003). MyD88 is essential to induce the expression of pro-inflammatory cytokines by activated NF-κB or JNK, whereas TRIF is needed for activating IFN-β expression via IRF3 (Akira et al., 2001). TLR agonist induced pro-inflammatory cytokines and type I interferon play crucial roles in the maturation of DCs, which are needed to efficiently trigger adaptive immune responses. Therefore, TLR adjuvants have the potential to assist weak vaccines to generate optimal immunogenicity against pathogens.

MyD88 is required for all TIR initiated TLR signaling for producing pro-inflammatory cytokines. In TLR3 and TLR4 signaling pathways, the MyD88 independent pathway is mediated by TRIF, which leads to activation of IRF3 and induction of IFN-β. (Adapted from Takeda and Akira, 2004).
Localization of TLRs is distinguished between subfamilies. TLR 1, 2, 4, 5, 6 and possibly 11 and 12 of mice and TLR 10 of human are expressed on the cell surface, whereas TLR 3, 7, 8 and 9 are expressed intracellularly on endoplasmic reticulum or endosomes (Kawai and Akira, 2009). Generally, surface expressed TLR 1, 2, 6 recognize lipoprotein, TLR4 detects lipopolysaccharide (LPS), and TLR5 binds to a bacterial cell wall component flagellin. In contrast, cytoplasmic TLR 3, 7, 8 and 9 recognize nucleic acids (Ishii et al., 2005). The TLR ligands that have been evaluated in context of influenza vaccination are generally divided into two groups that trigger cell surface TLRs and intracellular TLRs.

1.5.4 Cell surface TLR adjuvant

Each TLR has its own distinct profile for being expressed on different cell types. In human and mice, TLR 2/4 is expressed on a variety of immune cell populations such as macrophages, NK cells, DCs, B cells and T cells, as well as non-immune cells including fibroblasts and epithelial cells. The TLR4 agonist LPS showed very promising adjuvanticity for vaccines; however, extreme toxicity prohibits its use in humans (Alving, 1993). To limit the toxicity of lipid A, another TLR4 agonist monophosphoryl lipid A (MPL) has been developed and it has been shown to promote T helper (Th) 1 cell responses in conjunction with vaccines in animal models (Mbawuike et al., 1996). Additionally, MPL-based adjuvant (“Ribi” adjuvant) has been proved as a safe and efficacious adjuvant when used in human clinical trials to prevent infectious disease and seasonal allergic rhinitis (Evans et al., 2003; Mothes et al., 2003). To improve the efficiency of influenza vaccination in the elderly population, a TLR4 agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) has been evaluated in a preclinical study for enhancing T cell responses which are commonly defect in older people. It has been demonstrated that GLA-SE activated myeloid DCs (mDCs) and significantly enhanced Th1 cell responses when peripheral blood mononuclear cells (PBMC) collected from the elderly cohort were stimulated with GLA-SE combined with TIV (Behzad et al., 2012). Furthermore, coadministration of GLA-SE and currently used TIV in both mice and nonhuman primates showed GLA-SE adjuvanted vaccine strikingly increased HI titers against the vaccine strain and drifted virus (Coler et al., 2010).

TLR2 ligand lipoprotein or related compounds have shown potential adjuvanticity for enhancing DC maturation and T cell responses in vivo. The electrostatic complex containing a
charged lipopeptide Pam(2)Cys and an opposite charged influenza antigen released antigen and adjuvant to the same TLR2 expressing DC and efficiently enhanced CD8\(^+\) T cell and antibody responses in mice (Chua et al., 2011). Furthermore, the synthetic peptide vaccine including the influenza CD8\(^+\) T cell epitopes and lipopeptide elicited DC maturation and cytotoxic T cell responses in a mouse model (Jackson et al., 2004). These results suggest that synthetic TLR2/4 adjuvants enhance the amount and quality of protective immunity induced by influenza vaccines.

TLR5 is expressed by epithelial cells in the lungs and gut of human, and is also detected in resident DCs in the lamina propria of the intestines in mice (Uematsu et al., 2006). TLR5 binds to bacterial protein flagellin which is an effective immune stimulus to activate both innate and adaptive responses. As an adjuvant, flagellin is typically expressed together with influenza viral antigen as a fusion protein for immunization via the intramuscular or intranasal route in animal model and human clinical study (Skountzou et al., 2010b; Turley et al., 2011). The flagellin fused M2e influenza vaccine significantly increased immunogenicity in immunized healthy adults (Turley et al., 2011). Also, the flagellin fused HA influenza vaccine highly enhanced the rates of seroconversion and seroprotection in immunized elderly people (Taylor et al., 2011). Thus the influenza-flagellin fusion vaccine is a promising candidate for providing protection in both young and elderly populations. On the other hand, flagellin can recognize other receptors in addition to TLR5. It has been shown that intracellular NOD leucine-rich repeat (NOD-LRR) family member neuronal apoptosis inhibitory protein 5 (NAIP5) binds to flagellin in the cytoplasm of macrophages to activate caspase-1 signaling for controlling Legionella pneumoniphila infection (Franchi et al., 2006). Furthermore, ICE protease activating factor (IPFA), a similar CARD containing NOD-LRR, also interacts with flagellin in cytosol to activate the caspase-1 involved pathway (Miao et al., 2006). It is possible that NAIP5 or IPFA contributes to the flagellin mediated adjuvanticity besides TLR5 ligation. Further studies are needed to clarify the role that each receptor plays in the flagellin fused influenza vaccination.

**1.5.5 Intracellular TLR adjuvant**

TLR3 recognizes double-stranded RNA in cytosol. The classical TLR3 ligand poly i:c, which is a synthetic double-stranded RNA, has been characterized as a powerful agent for treating or preventing viral infection in animal models (Wong et al., 2009); however, its use in humans was discontinued because of its toxicity (Robinson et al., 1976). To reduce the toxicity,
several analogue poly i:c agents have been synthesized and evaluated as adjuvants for influenza vaccine. It has been shown that analogue poly i:c molecule dsRNA oligonucleotides with a minimal length of 90 bp were sufficient to induce DC maturation that specifically activated CD8+ T cell responses in mice when administered with influenza vaccine (Jelinek et al., 2011). The efficacy of poly i:c adjuvanted vaccines has also been tested in aged animals, showing that synthetic dsRNA significantly promoted survival of older mice from lethal influenza challenge (Schneider-Ohrum et al., 2011). The protection mediated by the adjuvanted vaccines was due to the increased number of mature DCs in the lungs of aged animals but independent of HI titers or HA specific CD8+ T cell responses. Moreover, dsRNA with limited toxicity has been used as a mucosal adjuvant coadministered with TIV to mimic natural infection in the mucosal respiratory tract, and thus provides more broad protection against different viral strains and potentiates the mucosal immunity against pandemic infection (Ainai et al., 2011). However, it has been shown that two receptors, retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5), also recognize different features of viral genome RNA or dsRNA products to activate TLR independent signaling pathways for inhibiting virus replication. Interestingly, MDA5 but not RIG-I is indispensible to induce poly i:c mediated innate immune responses and stimulate type I IFN expression in the absence of TLR3 (Gitlin et al., 2006). This suggests that not only TLR3, but also MDA5 is likely involved in the adjuvanticity of poly i:c or its analogue molecule during influenza vaccination.

Synthetic single-stranded RNA imidazoquinolins are recognized by both TLR7 and TLR8 in humans, but only by TLR7 in mice (Heil et al., 2004). Ligation of TLR7 and 8 initiates type I IFN expression in multiple types of cells and activates cellular immune responses. Human plasmacytoid DCs (pDCs), which are the main source of type I IFN, highly express TLR7 but not TLR8, whereas TLR8 is mainly expressed by monocytes (Gorden et al., 2005). Thus TLR7 ligation in pDCs activates type I IFN expression and binding of TLR8 initiates pro-inflammatory cytokine production. Several synthetic TLR7 agonists have been examined for inhibiting hepatitis B/C virus infection or treating cancer in phase I/II trials; however, the role of the TLR7 ligand is not well characterized in influenza vaccination. Recently, a study has shown that nanoparticles containing influenza antigen and synthesized TLR4 and TLR7 ligands significantly promoted survival in mice challenged with a lethal avian virus and also induced robust immunity against 2009 pandemic H1N1 in nonhuman primates (Kasturi et al., 2011). The combination of
TLR4 and TLR7 adjuvanticity was synergistic in inducing antigen specific neutralizing antibodies compared to a single TLR ligand, and the increased antibody responses are a result of direct activation of TLRs in B cells, DCs and also T helper cells. This result suggests that TLR7 adjuvant assists vaccines in the presence of another adjuvant to significantly improve the efficacy of the immunization.

The adjuvanticity of TLR9 ligand CpG oligonucleotide (CpG ODN) for influenza vaccines has been well studied in both animals and humans. Synthetic unmethylated CpG ODN binds to TLR9 in macrophages, pDCs and B cells to trigger signaling pathways for producing pro-inflammatory cytokines, interferons, and antibodies (Hartmann et al., 1999; Krieg et al., 1995b; Krieg, 2002a). There are three types of synthesized CpG ODN, namely A, B and C for the different species including human, mice and non-human primates. Type A CpG ODN preferentially induces interferon responses and vigorously activates expression of interferon stimulated genes (ISG) which play crucial roles in anti-viral activity and cellular responses, whereas type B CpG ODN specifically targets TLR9 in B cells for enhancing antibody responses with limited interferon expression (Abel et al., 2005). Type C CpG ODN has the properties observed in both types A and B. Animal studies have shown that inactivated influenza particles combined with type B CpG ODN significantly increased neutralizing antibodies and promoted survival after challenge (Moldoveanu et al., 1998). Human clinical studies also demonstrated that CpG ODN adjuvanted vaccines dramatically increased the seroconversion and seroprotection rates in immunized populations (Cooper et al., 2004b). One study showed that synthesized CpG ODN-antigen particles taken up via B cell receptors (BCR) led to significantly increased B cell proliferation and differentiation into antigen specific plasma cells in mice (Eckl-Dorna & Batista, 2009a). On the other hand, repeated administration with high doses of CpG altered the morphology and function of lymphoid organs. Increased daily treatment in mice with 60 µg CpG suppressed the function of follicle DCs and germinal center B cells, then strongly reduced humoral responses and antibody class switching (Heikenwalder et al., 2004). Thus clinical use of CpG ODN as an adjuvant must be further evaluated to address the possibility of adverse events after administration.

In summary, TLR dependent adjuvants are efficacious for enhancing antigen specific immunity when coadministered with influenza vaccines or antigens via intramuscular or intranasal routes. TLR adjuvant dramatically induces DC maturation, T cell activation, and/or B
cell responses in the presence of vaccines. However, toxicity and safety are still major considerations that prevent the use of TLR adjuvants in humans. A chemical modified TLR agonist with limited toxicity may potentially enhance the efficacy of future vaccines.

1.6 Cross-protective immunity to influenza

The generation of broad protection against different influenza strains is the primary goal of the influenza vaccinology. The current seasonal TIVs induce neutralizing anti-HA antibodies that can prevent infection from the matched viral strain or those that share similar surface antigens; however, it has been shown that seasonal influenza vaccines have little value in providing cross-protection against the reassorted virus that emerged in the 2009 pandemic (Anonymous, 2009a). Although seasonal LAIV decreased morbidity and mortality caused by 2009 H1N1 in a mouse model, lack of supportive evidence was observed in human clinical studies (Sun et al., 2011). Thus specific vaccines were manufactured during the 2009 pandemic year to immunize the global population. On the other hand, prior infection with influenza virus has been found to provide cross-protective immunity, also called heterosubtypic immunity, against unrelated viruses in several animal studies (Ellebedy et al., 2010; Kash et al., 2010; Laurie et al., 2010). It is suggested that prior infection induces strong T cell responses which are typically absent following seasonal vaccination, and thus memory T cells target the conserved antigen epitopes for conferring cross-protection (Tu et al., 2010). In addition, several recent studies also demonstrated that non-neutralizing but cross-reactive antibodies raised by prior infection against NP and M2 provided cross-protection against heterologous viruses (Carragher et al., 2008; El Bakkouri et al., 2011). Therefore, prior infection is capable of inducing heterosubtypic immunity via both cellular and antibody responses.

1.6.1 Cross-reactive antibody responses

Anti-HA antibodies are mainly induced in recipients following seasonal influenza vaccination. These anti-HA antibodies target the variable HA global head and are very specific against the matched influenza strain. Thus it is unlikely that seasonal vaccine induced antibodies can provide protection against a newly reassorted virus with a distinct HA. Recently, several antibodies against the conserved HA stem region have been discovered to provide broad protection against different subtype viruses. Some of these antibodies bind to the group 1 influenza viruses and the others are reactive to the group 2 influenza viruses (Mancini et al.,
It has been shown that prophylactic or therapeutic treatment with these antibodies prevents infection and reduces replication of different influenza viral strains, specifically the HPAI H5N1 virus (Corti et al., 2011; Eick et al., 2009). Additionally, a recent study has reported that monoclonal antibodies are capable of neutralizing the HA of influenza A viruses from both group 1 and group 2 (Corti et al., 2011). It has been suggested that the anti-HA stem region antibodies have the potential to treat influenza patients with associated severe symptoms via cocktail therapy.

During the 2009 pandemic year, people who were born before 1950 had cross-reactive anti-HA antibodies against the 2009 pandemic H1N1 (H1N1pdm) (Hancock et al., 2009). This may partially explain that the clinical outcomes observed in the elderly population were mild during the pandemic year (Chen et al., 2010; Miller et al., 2010). It has been found that the old viruses circulating between 1930 and 1950 share similar surface antigenicity with the 2009 H1N1pdm virus (Skountzou et al., 2010a). Also, an animal study has confirmed that prior infection with old influenza virus, in particular the 1918 Spanish flu, induced cross-neutralizing anti-HA antibodies against H1N1pdm virus and promoted survival in mice against reinfection (Manicassamy et al., 2010). In contrast, neither the recent seasonal vaccines nor the prior infection with recent seasonal virus is able to raise cross-neutralizing antibodies against H1N1pdm (Valkenburg et al., 2011). These findings imply that cross-neutralizing antibodies can only be induced by the viruses showing similar antigenicity on the viral surface. Interestingly, several recent studies have demonstrated that non-neutralizing antibodies against conserved influenza internal antigens NP and M2 were cross-reactive with different influenza strains. Transfer of anti-NP or anti-M2 antibodies conferred substantial protection against heterologous infection (Carragher et al., 2008; El Bakkouri et al., 2011). Furthermore, the anti-NA antibodies induced by human seasonal H1N1 virus infection were able to provide partial protection from H5N1 lethal infection in mice (Sandbulte et al., 2007). Given that influenza infection raises anti-NA, anti-NP and anti-M2 antibodies to some extent, it is suggested that non-neutralizing but cross-reactive antibodies may also contribute to heterosubtypic immunity. In some circumstances, cross-reactive antibodies even played more crucial roles than CTLs in cross-protection (Nguyen et al., 2001). In this thesis, cross-reactive non-HA antibodies induced by seasonal H1N1 infection have been well characterized in the context of heterotypic protection against 2009 H1N1pdm virus.
1.6.2 Cross-reactive cellular responses

Heterosubtypic immunity has been studied in influenza research since the 1960s (Schulman & Kilbourne, 1965). In several studies showing that prior infection provided protection against different subtype viruses, cross-responsive CTLs have been considered as crucial effectors for the control of heterologous infections (Guo et al., 2010; Hillaire et al., 2011; Kreijtz et al., 2008). The cross-reactive CTLs induced by the influenza virus usually target the influenza internal antigens which exhibit less frequent mutation when compared to the surface antigens. After prior infection, these CTLs exist in multiple lymph organs, peripheral blood and also in the lungs where the cross-reactive CTLs respond to the secondary infection immediately (Flynn et al., 1998). Also, adoptive transfer of the CTLs obtained from the previously infected mice successfully promoted survival of the recipient mice from heterologous challenge (Hillaire et al., 2011). Prior infection in ferrets and pigs also provided heterosubtypic protection by enhancing secretion of IFN-γ which is an indicator of CTL responses during secondary infection (Ellebedy et al., 2010; Van Reeth et al., 2009). In humans, substantial evidence of CTL mediated heterosubtypic immunity has been observed based on analysis of the 1957 pandemic. It was shown that patients infected by the H1N1 virus before 1957 displayed mild to moderate clinical symptoms caused by the pandemic H2N2 virus in that year (Epstein, 2006). Furthermore, human PBMCs infected with seasonal influenza virus have demonstrated cytotoxicity targeting the HPAI or H1N1pdm infected cells in vitro (Lee et al., 2008; Tu et al., 2010).

By comparing the conservation between seasonal virus and H1N1pdm, numerous conserved CTL epitopes were found (Greenbaum et al., 2009). PBMCs collected from healthy donors before 2009 pandemic expressed large amounts of IFN-γ by CTL after stimulation with conserved antigens identified between the seasonal virus and 2009 H1N1pdm. This finding is further confirmed by a study showing that the CTLs collected from the seasonal virus infected donors demonstrated lytic activity on H1N1pdm infected monocyte-derived macrophages with associated secretion of IFN-γ and TNF-α (Tu et al., 2010). In animal models, several studies have shown that prior infections with multiple seasonal H1N1 or H3N2 viruses in mice, ferrets, pigs, and nonhuman primates all raised cross-responsive CTLs that mediate protection against H1N1pdm reinfection (Ellebedy et al., 2010; Hillaire et al., 2011; Van Reeth et al., 2009; Weinfurter et al., 2011). In these studies, it has been shown that depletion of CD8+ CTLs in mice significantly compromised the H3N2 infection-induced heterosubtypic immunity against
H1N1pdm (Hillaire et al., 2011). In contrast, lack of CD8\(^+\) T cells in ferrets only partially influenced cross-protection (Ellebedy et al., 2010). Furthermore, the results presented in this thesis demonstrate that cross-responsive CD8\(^+\) T cells primed by seasonal H1N1 infection were not essential for providing cross-protective immunity against H1N1pdm. Therefore, CD8\(^+\) CTLs demonstrated different capacities for providing heterosubtypic immunity.

Cross-responsive CD4\(^+\) T cells also showed protective immunity against heterologous infection. The analysis of 2009 H1N1pdm epitopes targeted by the CD4\(^+\) T cells showed about 41% similarity when compared to seasonal influenza viruses (Greenbaum et al., 2009). By stimulation with the conserved epitopes of the H1N1pdm virus, the CD4\(^+\) T cells isolated from PBMCs of the healthy donors previously exposed to seasonal virus produced IFN-\(\gamma\) with associated memory phenotype in vitro (Greenbaum et al., 2009). It is widely accepted that the memory cross-reactive CD4\(^+\) T cells were capable of helping host CTL responses against the heterologous strain. Interestingly, cross-responsive CD4\(^+\) T cells may also assist B cell activation for producing neutralizing antibodies against the unrelated virus. A recent study has shown that cross-reactive CD4\(^+\) T cells induced by the prior seasonal H1N1 infection potentiate neutralizing antibody production during H1N1pdm reinfection and thus promote survival in CD8\(^+\) T cell depleted mice (Alam & Sant, 2011). In future studies, the interactions between CD4\(^+\) T cells and B cells for generating heterosubtypic immunity should be further clarified.

1.7 Aberrant host immune responses to pathogenic influenza

Host immune responses are induced to limit viral replication and control subsequent illness after influenza infection. Human seasonal influenza viruses are self-limiting in replication and activate innate immune responses soon after infection. Expression of type I IFN, cytokines and chemokines are transient and limited to a small part of the respiratory tract (Loo & Gale, Jr., 2007). The levels of cytokine and chemokine expression are directly correlated with viral titers in the infected tissue. In addition, seasonal influenza viruses usually induce robust expression of type I IFN and activate large numbers of ISGs which have anti-viral activities for limiting viral replication (Loo & Gale, Jr., 2007). Therefore, seasonal influenza virus activates transient but normal innate immune responses which facilitate the control of virus replication. In contrast, pathogenic influenza viruses have more virulent properties that are associated with high levels of replication and spreading in the respiratory tracts. Several studies have shown that highly
The pathogenic 1918 Spanish influenza virus stimulates abnormal innate immune responses by expressing excessive pro-inflammatory cytokines and chemokines throughout the respiratory tract (Kash et al., 2006; Kobasa et al., 2007; Tumpey et al., 2005), whereas expressions of type I IFN and anti-viral ISGs are strikingly suppressed by the viral protein NS1 following infection (Kobasa et al., 2007). This aberrant immune response is also observed in patients infected with HPAI H5N1 which causes severe lung damage associated with a large number of infiltrated immune cells (Korteweg & Gu, 2010). The excessive pro-inflammatory cytokines and chemokines, also called cytokine storms, are largely produced by the blood derived macrophages which are infected by the pathogenic viruses when migrating to the site of infection (Osterholm, 2005). These findings demonstrate that abnormal activation of innate immune responses is a prominent property of pathogenic virus infection.

Adaptive T cells are activated to target infected cells for cleaning viral reservoirs in infected tissue following influenza infection. Seasonal viruses typically induce moderate T cell responses associated with limited expression of cytokines and chemokines during infection because they are mostly restricted to the upper respiratory tract and only cause short-term illness (Loo & Gale, Jr., 2007). On the other hand, pathogenic virus infection results in overactive anti-viral T cell responses associated with the excessive secretion of pro-inflammatory cytokines and chemokines which lead to pulmonary tissue damage and respiratory failure in the host (Osterholm, 2005). Expression of cytokine TNF-α, which is identified as a pathogenic factor contributing to pulmonary immunopathology, is also associated with activated T cells following pathogenic influenza infection. Genetic deletion of CD8+ T cell expressing TNF-α improved immunopathology in mouse lungs in the context of influenza antigen stimulation (Xu et al., 2004). In addition, the restricted CD8+ T cell expressing chemokine CCL3, which plays a role in inflammatory responses, promoted survival in mice infected with pathogenic 2009 H1N1pdm (Walsh et al., 2011). These findings suggest that effector T cells are also involved in influenza pathogenesis in addition to their anti-viral activities.

Apart from the protective roles played by innate and adaptive immunity against influenza, their abnormal responses result in immunopathology during pathogenic virus infection (Figure 1.4). Thus control of host immune responses is considered as an alternative strategy for treating patients with severe clinical symptoms. Because pathogenic virus replicates efficiently and causes systemic infection, anti-viral drugs have been used to control their infectivity and the
subsequent immunopathology. However, anti-viral drugs such as NA-inhibitors confer selective pressure on a virus that may produce progeny virus with drug resistance (Anonymous, 2009b). Also, NA inhibitors indirectly target host immunity to control pulmonary injury. Hence new therapies that are less susceptible to virus selection as well as capable of limiting excessive host immune responses must be developed.

Figure 1.4 Overactive host immune responses to pathogenic influenza.

Pathogenic influenza virus infected local and infiltrated innate immune cells (e.g., macrophages) which produce a large number of pro-inflammatory cytokines and chemokines. Additionally, the activated macrophages and DCs induce overactive effector T cell responses that produce excessive pro-inflammatory cytokines/chemokines and exacerbate inflammation in the infected tissue. (Adapted from Osterholm et al., 2005)

1.7.1 Treatment with immunomodulator to severe influenza

To suppress detrimental inflammation caused by pathogenic influenza infection, immunomodulators which are less susceptible to virus resistance have been tested. However, treatments with corticosteroids and Cox-2 inhibitors have shown limited efficacy for controlling
influenza caused illness (Anonymous, 2009b; Carter, 2007; Falagas et al., 2010). Furthermore, one study reported that immunosuppressant $\Delta^9$-tetrahydrocannabinol caused increased viral titers and decreased cell infiltration in the lungs when administered prior to or during influenza infection (Buchweitz et al., 2007). Interestingly, a recent study has shown that prophylactic treatment with the bacterially derived immunomodulator cholera toxin (CT) protected mice from a lethal influenza challenge. CT induced an initial inflammatory process and augmented immune response to reduce virus replication; moreover, it coincidently prevented pulmonary chemokine expression and downregulated immunopathology in the lungs (Norton et al., 2010). Due to toxicity, however, the bacteria derived compound may have limitations for use in humans.

Recently, a blood derived lipid mediator sphingosin-1-phosphate (S1P) has been discovered to play crucial roles in the regulation of immune responses (Rosen et al., 2008). S1P is a signaling lysophospholipid that binds to five high affinity G protein coupled receptors named S1P1-5 to modulate multiple cellular functions, mainly including cell proliferation, adhesion, migration and cytokine production (Rivera et al., 2008). It is suggested that activation of S1P receptor signaling leads to the regulation of different immunological processes including inflammatory responses. The synthetic immunomodulators FTY720 and AAL-R, which are the S1P analog pro-drugs, have demonstrated beneficial effects in the control of autoimmune disease, toxic shock syndrome, and viral infection (Marsolais et al., 2009; Niessen et al., 2008). Currently, AAL-R is being examined in preclinical models to treat influenza caused immunopathology. It has been demonstrated that AAL-R downregulates innate immune responses by limiting the number of infiltrated macrophages, neutrophils and NK cells in the lungs. In particular, post 2009 H1N1pdm infection, AAL-R significantly inhibited hyper-activation of macrophages and NK cells which showed reduced levels of CD69 (Walsh et al., 2011). Furthermore, S1P analog compounds downregulate maturation and costimulation of DCs which subsequently influence T cell responses (Marsolais et al., 2008). It has been shown that AAL-R treated mice had reduced T cell responses associated with restricted amounts of cytokine/chemokine TNF-$\alpha$ and CCL3 after 2009 H1N1pdm infection (Walsh et al., 2011). Taken together, these findings suggest that S1P analog downregulates host immune responses in the context of hyper-inflammation caused by pathogenic influenza; thus it may serve as a therapeutic agent in severe human infections.
1.7.2 Treg cell function in immune responses to viral infection

Treg cells are identified as a small population of CD4\(^+\) T cells that express surface molecule CD25 and transcription factor Foxp3 (Sakaguchi et al., 1995). This cell population, which is also called Tr1, develops in the thymus and periphery and stands for the major Treg cells in both humans and mice (Fontenot & Rudensky, 2005). Numerous studies have been performed to illustrate the mechanisms that Treg cells utilize to suppress immune responses. In general, Treg cells mainly target DCs and responder T cells to reduce their activities involved in the immunological process, including inflammatory responses (Shevach, 2009). It has been found that dysfunction or deficiency of Treg cells may result in the pro-inflammatory milieu observed in autoimmune diseases (Sakaguchi et al., 1995). Since CD4\(^+\) Treg cells are induced post pathogen infection (Betts et al., 2011; Fulton et al., 2010; Lanteri et al., 2009), this cell population may play regulatory roles in anti-viral host immune responses. It has been shown that Treg cells are crucial for the control of inflammatory responses caused by RSV or WNV infection. Partial depletion of Treg cells during RSV infection potentiated inflammation and innate immune responses by increasing the expression of cytokines and chemokines in mice (Fulton et al., 2010). Also, Treg cell deficient mice infected with WNV showed severe infection outcome and excessive inflammatory responses (Lanteri et al., 2009). Therefore, it is proposed that optimal manipulation of Treg cell responses benefits host immune responses for controlling the pathogenic virus infections. The Treg cell suppressive mechanisms and the potential role of Treg cells for suppressing immunopathology caused by the pathogenic influenza are discussed below.

1.7.3 Restriction of responding T cells by Treg cells

There are several mechanisms that are used by Treg cells to suppress responder T cell activities. In most studies, Treg cells have been found to restrict T cell responses by inhibiting transcription of cytokine IL2, which is in favor of Th1 differentiation (Oberle et al., 2007; Takahashi et al., 1998; Thornton & Shevach, 1998). Furthermore, one study has shown that Treg cells that express high affinity IL2 receptors CD25, CD122 and CD132 competed with effector T cell by consuming IL2 (Pandiyan et al., 2007). Controversial findings of another study, however, argue that consumption of IL2 is not a major mechanism of Treg suppression by showing that blockage of CD25 had no effect on Treg cell function in vitro (Tran et al., 2009). Nonetheless, restriction of IL2 activity is one of the major suppressive functions of Treg cells.
Recently, suppressive cytokines that mediate the role of Treg cells have been discovered. TGF-β and IL10 are the two cytokines that are extensively studied for their functions involved in Treg cell suppression. TGF-β is vital for activating and maintaining Foxp3+ Treg cells *in vitro* and *in vivo* (Chen et al., 2003; Liu et al., 2008); however, the suppressive function of this molecule is still controversial. Some studies have shown anti-TGF-β antibodies did not reverse the suppressive function of Treg cells *in vitro*, which leads to the proposition that TGF-β is bound to the cell surface of Treg cells and becomes an active form when released to the responder T cells (Nakamura et al., 2001). It has been shown that latency-associated peptide (LAP), which may play a role in the production of the latent form of TGF-β, is highly expressed by activated Treg cells (Andersson et al., 2008). This may explain why high concentrations of anti-TGF-β antibodies or soluble TGF-βII were unable to inhibit the suppressive function of the Treg cells (Piccirillo et al., 2002). Controversially, an *in vivo* study has shown that TGF-β^−/^− Treg cells still inhibited inflammatory responses in context of inflammatory bowel disease (IBD) caused by Foxp3+ T cells in mice (Fahlen et al., 2005). Suppression of TGF-β^−/^− Treg cells, however, may be maintained by TGF-β secreted from non-Treg cells since anti-TGF-β significantly block the function of TGF-β^−/^− Treg cells for controlling IBD. These results indicate that TGF-β is crucial for Treg cells to control autoimmune disease. The function of IL10 associated with Treg suppression has been well-characterized by *in vivo* studies. It has been reported that high expression of IL10 is observed in chronic mycobacteria tuberculosis (MTB) infection which restricted effector T cell responses in patients (Guyot-Revol et al., 2006). Also, deficiency of Treg cell expression of IL10 results in enhanced inflammation throughout the intestinal environment in the late-onset colitis model (Rubtsov et al., 2008). In contrast, *in vitro* studies have failed to demonstrate the role that IL10 plays in Treg cell function. Recently, IL35 has been identified as a new cytokine expressed by Treg cells to directly suppress responder T cells (Collison et al., 2007).

Treg cells also demonstrate suppressive activity by directly lysing responder T cells. A combination of anti-CD3 and anti-CD46 antibodies activated expression of Gzm A in human Treg cells which killed effector T cells via a perforin/granzyme dependent, Fas-FasL independent mechanism (Grossman et al., 2004). The activated murine Treg cells expressed high levels of GzmB, which are essential to mediate responder T cell cytolysis (Gondek et al., 2005). Furthermore, Treg cells expressing GzmB and perforin play crucial roles in cytolyis targeting
NK cells and CTLs in a microenvironment favoring tumorigenesis (Cao et al., 2007). Hence Treg cells are able to convert into cytotoxic suppressors in some situations.

Treg cells can directly contact responder T cells or DCs by expressing galectin-1 on their surface. Galectin-1, a member of the highly conserved β-galactoside binding protein family, is preferentially expressed by Treg cells (Garin et al., 2007). Secreted or cell membrane bound galectin-1 can recognize several glycoproteins such as CD7, CD43 and CD45. Interaction between galectin-1 on Treg cells and glycoprotein receptors on responder cells results in cell cycle arrest, apoptosis and inhibition of pro-inflammatory cytokines in the responder cells. Neutralization of galectin-1 strikingly inhibited the suppressive activity of human and murine Treg cells, and Treg cells from galectin-1 deficient mice showed limited suppressive capacity (Garin et al., 2007). Thus galectin-1 is one of the vital mediators that contribute to Treg cell suppression.

1.7.4 Restriction of DCs by Treg cells

DCs are the major APCs that activate CD4+ and CD8+ T cells by presenting antigens and providing costimulation signaling. Treg cells can also directly target DCs to restrain their activity and consequently inhibit pro-inflammatory T cell responses. CTLA-4 mediated suppression is the primary mechanism that is used by Treg cells to downregulate DC maturation and costimulation (Serra et al., 2003). CTLA-4 is constitutively expressed by Treg cells and is first identified in vitro to downregulate DC costimulation by showing a similar role to that of the reagents which reduce CD80 and CD86 on DCs (Misra et al., 2004). Mice with selectively deleted CTLA-4 on Treg cells demonstrate uncontrolled inflammation and develop systemic autoimmune disease at 7 weeks of age, suggesting this molecule is essential for Treg cells to control immune homeostasis (Wing et al., 2008). It is known that CTLA-4 can directly bind to CD80 and CD86 on DCs to downregulate or inhibit increase of their expression induced by several DC maturation stimuli (Onishi et al., 2008). Treg cells from CTLA-4 deficient mice lost the ability to preventing the increase of CD80 and CD86 on DCs when compared to wild-type Treg cells. Also, blockage of CTLA-4 on Treg cells partially inhibited suppression of CD80 and CD86 expression on DCs that were cocultured in vitro (Wing et al., 2008). These findings support the view that Treg cells reduce DC costimulation molecules CD80/CD86 and subsequently downregulate the CD28 mediated T cell responses. The biochemical process that
CTLA-4 applies to reduce the expression of CD80/CD86 on DCs is still under investigation. One study has reported a mechanism by which Treg cells extract the surface molecules from the DCs when forming the immunological synapse (Joly and Hudrisier, 2003).

Treg cells also use other molecules to restrain DC maturation and costimulation. LAG-3 (CD223), a CD4 homolog molecule, is another cell surface antigen expressed by Treg cells. LAG-3 binds to MHC II on immature DCs with very high affinity to initiate negative signaling via ITAM to inhibit DC maturation and stimulatory activity (Liang et al., 2008). Given that activated human T cells express MHC II, LAG-3 may also suppress effector T cell responses directly. CD39 is the major ectoenzyme expressed by all mouse Treg cells and 50% of human Treg cells (Borsellino et al., 2007). CD39 can hydrolyze ATP and ADP to AMP to reduce the concentration of ATP which is largely involved in pro-inflammatory responses and upregulation of CD86 on DCs (Borsellino et al., 2007; Idzko et al., 2002). CD39 mediated downregulation of ATP is another anti-inflammatory mechanism used by Treg cells. Foxp3 directly upregulates expression of CD39 after Treg cells are activated. Additionally, CD39 insufficient Treg cells are dysfunctional and unable to activate and proliferate in response to stimulation with anti-CD3 and anti-CD28 (Ring et al., 2009). It is suggested that consumption of ATP by CD39 in an extracellular environment may make Treg cells move to the inflamed region to reduce the ATP mediated pro-inflammatory responses. Furthermore, Neuropilin (Nrp-1) is another molecule used by Treg cells to interact with DCs. Nrp-1 is preferentially expressed by Treg cells and increases the duration of interaction with immature DCs (Sarris et al., 2008). In the context of a limited amount of antigens, it is proposed that Nrp-1 confers a privilege for Treg cells to compete with naïve responder T cells for interacting with DCs.

1.7.5 Anti-influenza activity of TLR agonists and their function for enhancing Treg cell suppressive capacity

As discussed earlier, TLR agonists have been commonly used as vaccine adjuvants to enhance vaccine efficacy for controlling influenza infection in animal models. On the other hand, some studies have also characterized the anti-influenza activity of TLR agonists in influenza infection. It has been shown that prophylactic treatment with the TLR3 agonist poly i:c or TLR9 ligand CpG ODN protects the mice from lethal H1N1 infection (Wong et al., 2009). This study suggests that the TLR agonist induced the innate immune cells to express type I IFN responses.
and subsequently stimulated anti-viral ISG molecules, resulting in anti-influenza immunity. Recently, another study has reported that combination treatment of TLR2/6 and TLR9 agonists provide substantial protection from H3N2 and H1N1pdm infections in a mouse model (Tuvim et al., 2012). Controversially, this finding argues against the view that type I IFN responses are required in TLR agonist mediated protection. Given that adaptive immune cells also express TLRs (Sutmuller et al., 2007), TLR agonists have the potential to target and activate these cell populations in addition to innate immune cells. Further studies are needed to characterize the capacity of TLR agonists for directly enhancing adaptive immune cell activity in the context of anti-influenza responses.

In the studies that investigate the regulation of Treg cell activity, TLR agonists have been discovered to enhance their suppressive capacity in vitro. It has been demonstrated that TLR1, 2, 4, 5, 7 and 8 are expressed by Treg cells, and stimulation with TLR ligands directly upregulates Treg cell function (Caramalho et al., 2003; Crellin et al., 2005; Komai-Koma et al., 2004). In particular, the TLR5 ligand flagellin has been found to increase Foxp3 expression in Treg cells and promote immune suppression on responder T cells in vitro (Carel et al., 2005). Also, LPS, which binds to TLR4, enhanced the suppressive capacity of Treg cells in certain circumstances (Caramalho et al., 2003). In contrast, some studies showed that ligation of TLR2 on mouse Treg cells reversed suppressive activity (Liu et al., 2006; Sutmuller et al., 2006). Recently, NOD2 has been identified as another innate immune receptor expressed by Treg cells. Engagement of NOD2 on Treg cells promoted Treg cell survival by expressing elevated anti-apoptotic molecules (Rahman et al., 2010). These findings indicate that the suppressive capacity of Treg cells can be enhanced via innate immune receptors. Given that Treg cell expansion and activation have been detected during influenza infection, Treg cells have the potential to restrict overactive host immune responses caused by pathogenic influenza virus. In this thesis, the function of TLR agonists for enhancing Treg cell suppression and controlling excessive host immune responses in severe influenza infection has been investigated in a mouse model.

1.8 Animal models in influenza research

To fully understand the pathogenesis of influenza virus and host immune responses, animal models have been used to study the viral and host factors that contribute to virulence and infection caused illness. Also, animal models are utilized in preclinical studies to evaluate the
safety and efficacy of potential vaccines and therapeutic agents for use in humans. Several mammalian models including mouse, ferret, guinea pig and nonhuman primate are available for influenza research. Different models are chosen based on the research questions addressed in the study. Ferrets and mice are broadly used in current influenza studies because the costs are relatively lower than those of other models and the reagents are readily available. The advantages and restrictions of the ferret and mouse models are compared and discussed below.

1.8.1 Ferret model

Ferrets were used to study influenza caused rhinitis in 1933 (Smith and Sweet, 1988). Since then, they have been extensively utilized for studying different characteristics of human influenza infection. In general, the understanding of anti-influenza immunity is examined by using the ferret model (Renegar, 1992). Ferrets are widely used in influenza research because influenza viruses, including human and avian viruses, can naturally infect ferrets and replicate efficiently in the lung without prior adaptation. In addition, the physical response of ferrets to infection intimately resemble the clinical symptoms that are observed in humans after influenza infection (Renegar, 1992; Smith and Sweet, 1988). High fever is usually observed in ferrets in the early days (day 1 or 2) post infection and is mostly persistent for one to two days; however, increased temperature can last for several days when ferrets are infected with more virulent virus (Govorkova et al., 2005). Decreased activity is also observed in early days post infection, and correlates with loss of weight. Sneezing, coughing and nasal discharge are commonly seen in the first week after infection. Additionally, similar patterns of illness are observed between humans and ferrets that are infected with the same type A strain, and the virus that induces pneumonitis in ferrets also causes pneumonitis in humans (Renegar, 1992; Smith and Sweet, 1988). Given that influenza caused illness is acute in ferrets, transmission of the virus from sick ferrets to uninfected animals can be studied to evaluate viral transmissibility. Ferret transmission models are established by direct contact or by spreading aerosol droplets without contact (Herlocher et al., 2001), and clinical signs of the uninfected ferrets following transmission determine the transmissibility of the virus. Thus evaluation of pathogenicity and transmissibility in this animal model provides the opportunity to fully understand virulence of the influenza virus, and closely link the experimental results and the human clinical data.
In vaccine development, ferrets are used for evaluating the safety and efficacy of inactivated and live attenuated influenza vaccines (Bodewes et al., 2010). Immunogenicity is primarily tested for novel vaccines. Several studies have demonstrated the effectiveness of vaccines that induce neutralizing antibodies to prevent virus replication and transmission in the ferret model (Baras et al., 2008; Ellebedy et al., 2010). Furthermore, ferrets are monitored after vaccination with the new live attenuated strains to verify the safety and attenuation phenotype of the vaccines (Maassab and DeBorde, 1985). By inoculation with a live attenuated or parent strain, daily clinical signs of fever, weight loss and activity are evaluated; additionally, viral titers in the upper respiratory tract and lungs are measured (Eichelberger & Green, 2011). The new strains are attenuated if no illness and limited viral replication are induced after inoculation. Also, the ferret model is used to demonstrate the impact of drug-resistant viruses which are generated from the treatment of NA inhibitors, and determine virus suitability and capability of spreading to naïve animals (Herlocher et al., 2004).

Immune responses in ferrets are similar to those in humans after influenza infection (Barber and Small, Jr., 1978). Cytokine expressions as well as humoral and cellular responses in ferrets can be assessed to demonstrate immune function in the host post infection, and provide parallel information to determine the pattern of immune responses in humans. Moreover, assessment of host immune responses in ferrets after vaccination assists in predicting the immunogenicity of novel vaccines and understanding the mechanisms involved in immunization. Due to the paucity of ferret reagents, investigation of immune responses in ferrets is still limited. Recently, several studies have utilized canine reagents to study the regulation of gene expression in ferrets post influenza infection and vaccination by microarray analysis, as high homology between ferret and canine genomes are recognized (Cameron et al., 2008; Danesh et al., 2011). Furthermore, human monoclonal antibodies that are cross-reactive to ferret lymphocytes have been used to determine cellular responses in ferret influenza research (Ellebedy et al., 2010). With the development of the ferret reagents, more broad investigations of host immune responses in ferrets can be performed to benefit the understanding of influenza infection in humans.

The ferret model is an excellent research tool for investigating virulence and host immune responses in influenza studies. Given numerous advantages of this small mammalian model, ferrets provide an excellent platform for understanding the pathogenesis and transmissibility of various influenza viruses, and determining the efficacy and safety of novel
influenza vaccines. The enriched information offered by ferret studies is crucial for public health to generate optimal strategies to prevent influenza infection and treat influenza caused disease.

1.8.2 Mouse model

Mice are typically used to study the mechanisms of immune protection and viral pathogenesis in influenza research. With the broad availability of reagents, numerous aspects of innate and adaptive immune responses can be investigated in the mouse model to discover the vital mediators involved in host immune function or virus mediated pathogenesis. Moreover, the functions of the identified crucial molecules can be validated by using gene deleted mouse models. Mice are useful to explore the viral or host factors that contribute to pathogenesis (Peiris et al., 2010). The results obtained in studies of AVI H5N1 and 2009 H1N1pdm virulence in mice showed that excessive expressions of cytokines and chemokines play a pathogenic role during infection in the mouse model (Aldridge, Jr. et al., 2009; Itoh et al., 2009). Furthermore, the efficacy of the novel vaccines was evaluated in mice. In one preclinical study, adjuvanted vaccines were tested in aged mice to evaluate immunogenicity (Bodewes et al., 2010). Also, the immunization mechanism(s) identified in the vaccinated mice facilitate the design of effective vaccines that induce robust host immunogenicity or persistent memory responses against influenza viruses.

The main disadvantage of the mouse model in influenza research is that the mouse is not a natural host for human seasonal virus. It has been found that the 2,3-α-SA receptor is predominantly expressed in the lungs of most laboratory mouse strains (Ibricevic et al., 2006); thus the human influenza virus is unable to infect mice without prior adaption. Consequently, limited viral strains can be studied in mice except for several avian influenza viruses which bind to the 2,3-α-SA receptor. Furthermore, mice display neither the clinical signs that are commonly seen in humans following influenza infection nor the sneeze reflex; therefore, the mouse model precludes the investigation of virus transmissibility (Schulman and Kilbourne, 1963). In vaccine studies, limited clinical data except lethality and weight loss can be collected from vaccinated mice following infection to determine efficacy of novel vaccines. Hence the lack of clinical signs may limit comparison of the clinical data between mouse studies and human trials.

Inexpensive costs, broad availability of reagents, and well-characterized genetics make the laboratory mouse strain very useful for investigating the mechanisms involved in
immunogenicity and pathogenesis in influenza research. Given that the mouse model lacks clinical signs and the mouse is less susceptible to human influenza infection, the results of mouse studies may have limitations when comparing or explaining the observations found in human clinical studies. Therefore, in recent studies, both ferret and mouse models have been used together to fully characterize the clinical outcomes and mechanisms involved in influenza infection or vaccination.
1.9 Thesis rationale

Anti-influenza strategies are divided into two major categories, prevention and therapeutic treatment. The currently used influenza vaccines provide up to 75% protection in the recipients, but the effects are limited in populations with less favorable immune systems, such as the very young and the elderly. Furthermore, the vaccines only prevent infection from viruses similar to those of the vaccine strains and have little value for broad protection against viruses with distinct surface antigens. Regarding to therapeutic treatment, to date the available anti-viral drugs including the NA inhibitors cause pressure on the virus to reproduce more resistant influenza progeny that can escape the treatment. Therefore, further studies are needed to improve our understanding of the vaccinology and therapeutic strategies for benefitting the host immune responses during influenza infection. In this thesis, the potential approaches that enhance the efficacy of prevention and treatment against influenza viruses have been extensively investigated. The first experimental chapter characterizes the activity of different adjuvant vaccinations in ferrets to illustrate the common and differing mediators and signaling pathways that lead to successful vaccination. During the outbreak of the 2009 pandemic, seasonal influenza vaccines were found to be ineffective at preventing infection by the reassorted strains. Thus the second part of this thesis focuses on cross-protective immunity that is acquired from prior influenza exposure to demonstrate the mechanisms that provide broad protection against different subtype strains. Furthermore, severe influenza cases showed pulmonary damage with associated immunopathology in the 2009 pandemic year. It is suggested that control of overactive host immune responses is needed for limiting serious infection. Given that Treg cells are suppressive, increase of their responses may be beneficial to the host infected with the pathogenic influenza virus. In the final experimental chapter, the therapeutic strategies for attenuation of host immune responses via enhancement of Treg cell suppression are evaluated in the resolution of immunopathology caused by the 2009 H1N1pdm infection.

1.10 Hypothesis

The general hypothesis is that successful control of influenza infection is closely related to the generation of effective and cross-responsive, but not hyperactive host immune responses against the virus.
Specifically, in the first experimental chapter investigating different adjuvant activity during vaccination, the hypothesis is that different adjuvant vaccinations induce common mediators and signaling pathways to generate effective immunity that provide protection against influenza infection. In the second study of accessing cross-protection induced by prior influenza infection, it is hypothesized that prior infection-induced cross-reactive but non-neutralizing antibodies confer protection against pandemic viral strains. In the third experimental chapter evaluating the protective effect of adjuvant CFA, the hypothesis is that adjuvant CFA-augmented Treg cell suppression downregulates overactive host immune responses to promote disease resolution during severe influenza infection.

1.11 Objectives

The objectives of this thesis is to uncover the mechanisms that are involved in adjuvanted vaccination, prior infection-induced cross-protection, and adjuvant induced resolution of immunopathology to improve our understanding of the requirements for reforming the strategies of control and prevention of influenza infection.

In particular, the first objective of this thesis is to compare the activated signaling pathways involved in different adjuvant vaccinations by comprehensive expression array to uncover the common and distinguished mediators that play crucial roles in the generation of effective anti-influenza immunity. Thus these molecules and signaling pathways can be targeted and evaluated in future vaccine designs for effective vaccines. Although seasonal vaccines provide effective protection against similar virus infection, they fail to prevent infection with the reassorted viruses. In contrast, prior infection has been known to induce cross-protective immunity against different subtype strains. The second objective of this thesis is to investigate the cross-protective immunity induced by prior infection with seasonal virus and explore the mechanisms that are associated with successful cross-protection against H1N1pdm. Therefore, the findings may improve our current understanding of what is needed to produce “universal influenza vaccines”. Besides the role that adjuvants play in assisting generation of vaccine-induced immunity, they also demonstrate potential capability in directly controlling influenza infection. The third objective of this thesis is to investigate the mechanism of adjuvant CFA mediated immune suppression during the severe H1N1pdm infection. The findings are valuable
for developing potential treatments to control immunopathology caused by aberrant host immune responses.

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Chapter 2

Molecular Characterization of in vivo Adjuvant Activity in Influenza-Vaccinated Ferrets

A version of this chapter is published in:

2.1 Summary

The 2009 H1N1 influenza pandemic has prompted a significant need for the development of efficient, single-dose adjuvanted vaccines. Here we investigated the adjuvant potential of CpG Oligodeoxynucleotide (ODN) when used with human seasonal flu vaccine in ferrets. We found that CpG ODN-adjuvanted vaccine effectively increased antibody production and activated type I interferon (IFN) responses, compared to vaccine alone. Based on these findings, pegylated IFN-α2b (PEG-IFN) was also evaluated as an adjuvant in comparison to CpG ODN and Complete Freund’s Adjuvant (CFA). Our results showed that all three adjuvant-added vaccines prevented seasonal human H1N1 Brisbane59 virus replication more effectively than vaccine alone. Gene expression profiles indicated that, as well as up-regulating IFN-stimulated genes (ISGs), CpG ODN enhanced B cell activation and increased TLR4 and IRF4 expression, whereas PEG-IFN augmented adaptive immunity by inducing MHC transcription and Ras signaling. In contrast, using CFA as an adjuvant induced limited ISG expression but increased the transcription of MHC, cell adhesion molecules, and B cell activation markers. Taken together, our results better characterize the specific molecular pathways leading to adjuvant activity in different adjuvants-mediated flu vaccination.

2.2 Introduction

Influenza infection is a prominent threat to human health around the world and can cause severe morbidity and mortality in susceptible individuals due to acute respiratory disease. Among the approaches to limit severe illness caused by influenza, vaccination is a critical component in the prevention of the spread of infection. The human seasonal influenza vaccine usually includes antigens from different influenza subtypes, H1N1, H3N2 and influenza type B, which are predicted to circulate in the following flu season. However, this vaccine provides protection to only 75% of the vaccinated population (Edwards et al., 1994), and the protection efficacy in immunized elderly individuals is lower than 50% (Jefferson et al., 2005).

For over 70 years, adjuvants have been used to enhance antigen-specific immune responses. CpG ODN and type I IFN have been evaluated for their efficacy in commercial influenza vaccines (Cooper et al., 2004; Proietti et al., 2002) with the general conclusion that adjuvant-mediated vaccines induce stronger antibody responses and elevated cytotoxic T
lymphocyte (CTL) activity. CpG ODN is a ligand of toll-like receptor (TLR) 9, which is mainly expressed by plasmacytoid dendritic cells (pDC), B lymphocytes and monocyte/macrophages. TLR9 stimulation by CpG can effectively induce type I IFN responses and augment humoral responses (Krieg et al., 1995; Roman et al., 1997). Type I IFN signaling is thought to be critical to the initiation of innate immune responses to viral infections, and the IFN stimulated genes (ISG), which include a variety of transcription factors, cytokines and chemokines, appear to be involved in stimulating adaptive immunity and eliminating the virus from the host (Takaoka & Yanai, 2006). As an adjuvant, type I IFN has been shown to induce higher CTL proliferation and antibody secretion than alum, and was equal to Complete Freund’s Adjuvant (CFA), considered to be the gold standard adjuvant for use animal models (Proietti et al., 2002). However, the connection between gene regulated immune protection and adjuvant-mediated vaccination are still unknown.

In this study, the domestic ferret (Mustela putorius furo), a well-established model to study the pathogenicity of influenza virus (Maher & DeStefano, 2004), was used to better characterize adjuvant activity following influenza vaccination. CpG-adjuvanted influenza vaccination resulted in an increased antibody response compared to vaccine alone in ferrets. In addition, elevated ISG mRNA levels were observed at an early stage post immunization, presumably in part due to IFN-α signaling activation. Based on these results and the known activities of type I IFN (Le Bon & Tough, 2002; Takaoka & Yanai, 2006), it was expected that type I IFN could also be an effective adjuvant in influenza vaccination. Pegylated IFN-α2b (PEG-IFN), which has been approved for use in humans since 2001, was therefore tested for its adjuvant potential in flu vaccination in ferrets along with CFA, which activates innate immune responses partly through NOD-like receptor family member NOD2 (Girardin et al., 2003; Ishii & Akira, 2007), to compare its effects on immune outcome and gene regulation to those of CpG and PEG-IFN.

2.3 Materials and Methods

2.3.1 Animals

Male ferrets 4-6 months old were purchased from Marshall Bioresources (New York city, NY, USA) and maintained at the Animal Resources Centre (University Health Network, Toronto, Canada). Ferrets were routinely screened for influenza infection.
2.3.2 Immunization

The 2007-2008 seasonal human flu vaccine, Fluviral (ID Biomedical Corporation of Quebec), which contains 15 μg HA of each inactivated influenza virus strain, A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004 in 0.5ml, was used. Fluviral vaccine (100 μl) was mixed with 100 μl of total 500 μg class B CpG ODN (10104, Coleypharma, USA) four hours before injection. Each group of ferrets was injected with either 200 μl PBS, 100 μl PBS plus 100 μl Fluviral or 200 μl CpG added Vaccine. At Day 35 following primary immunization, all ferret groups were boosted with Fluviral vaccine alone. All the animals were vaccinated through intramuscular (i.m.) injection. Fluviral vaccine was abbreviated as V\textsuperscript{2007} and the Fluviral vaccine administered with PBS and CpG was abbreviated as V\textsuperscript{2007}-Alone and V\textsuperscript{2007} + CpG in the text.

The 2008-2009 seasonal human flu vaccine Vaxigrip (Sanofi Pasteur Limited), which contains A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B/Florida/4/2006, was mixed with 100 μl PBS containing 1 μg PEG-IFN-α2b and 100 μl CFA before the injection. Ferrets were also vaccinated intramuscularly with CpG added vaccine, vaccine alone, and PBS. The abbreviations of the vaccinations were recorded as V\textsuperscript{2008}-Alone, V\textsuperscript{2008} + CpG, V\textsuperscript{2008} + IFN and V\textsuperscript{2008} + CFA. The amount of CpG and CFA were chosen based on the previous studies which showed optimal adjuvanticity with various antigens (Wang et al., 2008; O'Hagan et al., 2002). The dose of PEG-IFN (1μg/kg) was chosen according to the manufacture’s recommendations for use of PEG-IFN in humans (UNITRON PEG, Schering-Plough).

2.3.3 ELISA for anti-influenza antibodies

Serum from PBS, V\textsuperscript{2007}-Alone and V\textsuperscript{2007} + CpG injected ferrets was collected at Days 0, 14, 21, 28, and 35 after primary immunization and at Day 7 post second vaccine injection. Antibody responses were assessed by ELISA assay. Briefly, ELISA plates were directly coated with 2007-2008 FLUVIRAL vaccine at 5 μg/ml overnight at room temperature. Plates were washed with PBS containing 0.05% Tween 20 (T-PBS) and blocked with 1% bovine serum albumin (BSA) for one hour at 37°C. Antigen-coated plates were washed with T-PBS and incubated with 1:1,000 diluted serum samples overnight at 4°C. After washing with T-PBS,
plates were incubated with goat anti-ferret immunoglobulin (IgM and IgG) HRP conjugates (Rockland Immunochemicals, Gilbertsville, USA) in a 1:10,000 dilution for 2 hours at 37°C. The reaction was developed by o-phenylenediamine for 30 minutes and the optical density was read at 450nm.

2.3.4 Haemagglutination inhibition (HI) Test

Ferret serum samples were treated with receptor destroying enzyme (RDE) at 37°C overnight. Fresh turkey red blood cells (TRBC) were washed and diluted in PBS to a concentration of 0.5% (v/v). The non-immunized and immunized ferret sera were serially diluted in PBS in 96-well V bottom cell culture plates. The serial diluted sera from PBS, V2007 + PBS and V2007 + CpG groups were incubated with 25 μl (8 HA units/50 μl) of A/Solomon Islands/3, A/Wisconsin/67/2005 and B/Malaysia/2507/2004 strains (CDC at Atlanta, Georgia, USA) separately for 15 minutes. Then, 50 μl of 0.5% TRBC was added and the plates were incubated at room temperature for 30 minutes. The HI titer was the reciprocal of the highest serum dilution to completely prevent agglutination. The same assay was applied on the 2008-2009 vaccine immunized ferret serum by using 8 HA units/50 μl of A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 viruses.

2.3.5 Microneutralization assay

The serum neutralizing antibodies of the Fluviral and Vaxigrip immunized ferrets were determined using the viruses A/Solomon Islands/3/2006 (H1N1) and A/Brisbane/59/2007 (H1N1) respectively for analysis by microneutralization (MN) assay described in Rowe et al., 1999 (Rowe et al., 1999). Briefly, the 50% tissue culture infectious dose (TCID\textsubscript{50}) of each virus was determined by titration in MDCK cells under biosafety level 2 conditions. The serially 2-fold diluted RDE treated serum at a starting dilution of 1:10 was tested for neutralizing the 100 TCID\textsubscript{50}/50 μl of each virus in MDCK cell monolayer. The cytopathic effect was read after incubation for 20 hours.
2.3.6 Infection and monitoring of ferrets

All the immunized ferrets and PBS control group animals were moved at least 4 days prior to infection to the BSL-2 animal holding area, where they were housed in cages contained in bioclean portable laminar flow clean room enclosures (Lab Products, Seaford, Del.). Prior to infection, baseline temperatures were measured twice daily for at least 3 days. Ferrets were anesthetized with ketamine (25mg/kg), xylazine (2mg/kg), and atropine (0.05mg/kg) by the intramuscular route and infected intranasally (i.n.) with a total of 1ml of $10^6$ EID$_{50}$ of virus/ml in phosphate buffered saline (PBS) delivered to the nostrils. Temperatures were measured every day by using a subcutaneous implantable temperature transponder (BioMedic Data Systems, Inc., Seaford, Del.). Pre-infection values were averaged to obtain a baseline temperature for each ferret. The change in temperature ($^\circ$C) was calculated at each time point for each animal. Clinical signs of sneezing (before anesthesia), inappetence, dyspnea, and level of activity were assessed once daily. A scoring system based on Reuman et al.’s work (Reuman et al., 1989) was used to assess the activity level as follows: 0, alert and playful; 1, alert but playful only when stimulated; 2, alert but not playful when stimulated; and 3, neither alert nor playful when stimulated. Observation of the daily activity score was assessed blinded to the different treatment groups. A relative inactivity index was calculated as follows: $\Sigma_{(day \ 1 \ to \ day \ 5)} \{score + 1\}/\Sigma_{(day \ 1 \ to \ day \ 5)} n$, where $n$ equals the total number of observations. A value of 1 was added to each base score so that a score of 0 could be divided by a denominator, resulting in an index value of 1.0.

All the infected animals were euthanatized by intracardiac injection of Euthanasia V solution (1ml/kg body weight) at Day 5 p.i. Tissues from nasal turbinates and major organs including lung and spleen were collected either in Trizol or in formalin for later analyses.

2.3.7 Collection of nasal wash samples and virus titration

Nasal washes were collected on Day 1, 2, 3 and 5 post infection (p.i.). Ferrets were anesthetized as described above, and 0.5 ml of sterile PBS containing 1% bovine serum albumin and penicillin (100U/ml), streptomycin (100µg/ml), and gentamicin (50µg/ml) was injected into each nostril and collected in a petri dish when expelled by the ferret. The volume was brought up to 1 ml with cold sterile PBS plus antibiotics. Sedated ferrets were weighed on Day 0 and Days 1, 2, 3, 4, 5 p.i. To determine the viral load in the nasal washes, 20 µl supernatant of nasal wash was added to the 180 µl vDMEM (DMEM, 1% BSA, 50µg/ml gentamicin, 100 U/ml penicillin,
100 µg/ml streptomycin, 2.5µg/ml amphotericin B) cultured MDCK cells (2×10^5/ml) and followed by a 10-fold dilution on a 96-well cell culture plate. After incubation for 2 hours, the media was replaced with the 200 µl/well fresh vDMEM and the cells were cultured for 6 days. On Day Six, 50 µl of the cultured media from each well was transferred to the V bottom 96-well plate and 50 µl 0.5% TRBC was added to run a HI test. The virus titers expressed as TCID₅₀/ml from each nasal wash sample was calculated by Reed-Muench method.

2.3.8 Primer design and synthesis

Each gene primer was designed based on the conserved gene sequences obtained through the alignments of the coding sequences from the various species of dog (*Canis lupus*), domestic cat (*Felis catus*), human (*Homo sapiens*), cattle (*Bos taurus*) and/or pig (*Sus scrofa*). Primers were designed specifically for detecting the ferret target genes including the paralog genes of each family to produce a PCR product in the range of 60~250 base pairs (bp) using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers’ sequences (see supplementary material in Appendix 2.1) have GC content between 40-60% and penalties were set against self and pair complements to deter primer-dimer formation. The primers were synthesized desalted under HPLC purity by Operon (Huntsville, AL) and Invitrogen (Shanghai, China). The ferret specific gene primers available at ATCC BEI Resources (http://www.beiresources.org) are listed in Appendix 2.1.

2.3.9 Cloning and sequencing of partial coding sequences of ferret Immune genes

Polymerase chain reaction (PCR)-amplified products of target genes derived from mitogen-activated ferret (*Mustela putorius furo*) PBMCs, splenocytes and lung tissue. Amplified PCR products were cloned using the TOPO-TA cloning kit (Invitrogen, Burlington, ON) per the manufacturer’s instructions. Sequencing was done by the laboratory at International Institute of Infection and Immunity (Shantou, China). Sequences were analyzed using the BLASTN program of the National Center for Biotechnology Information portal and deposited to the public database GenBank (NCBI accession numbers are listed in Appendix 2.1).
2.3.10 Whole blood collection, RNA extraction and cDNA synthesis

Whole blood (1.5 ml) from each ferret was collected into a Paxgene tube (Qiagen, Mississauga) at Day 1, 3 and 5 after first vaccination and Day 7 post second immunization. RNA was extracted and purified by Qiagen Paxgene blood RNA kit according to the manufacturer’s instructions. RNA quality and concentration were determined by a spectrophotometer (Eppendorf, Mississauga, Canada). 500 ng of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Burlington, ON) in a 20 μl reaction under the following conditions: 6.25 μM random hexamers primer (Applied Biosystems), 50 mM Tris-HCl pH 8.3, 3 mM MgCl2, 75 mM KCl, 0.5 mM of dATP, dGTP, dTTP, and dCTP, 10 mM DTT, 40 U RNase Inhibitor (Applied Biosystems) and 200 U SuperScript II RNase H- reverse transcriptase at 42º C for 1 hr.

2.3.11 Real-time PCR

Quantitative real time PCR (QRT-PCR) was performed using an ABI-PRISM 7900HT Sequence Detection System and SYBR green PCR Master Mix (Applied Biosystems, Foster City, California). Each primer pair was tested with serially diluted concentrations of a control cDNA to generate a standard curve. Samples and standards were analyzed in triplicate. Each QRT-PCR reaction was performed in a 10 μl reaction volume with 0.25 μl of cDNA, 1 μl primers (500 nM each primer) and 5 μl of SYBR green PCR MasterMix in ABI-PRISM optical 384-well plates. β-actin was used as the house-keeping gene for sample normalization.

2.3.12 Microarray analysis

Peripheral blood RNA isolated from each animal at day 1 post vaccination was analyzed by microarrays in the 2008-2009 vaccine study (n=3/group). Briefly, cRNA was prepared from 500 ng total whole blood RNA by two-cycle cRNA synthesis according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA). cRNA samples (20 μg) were labeled and hybridized to Affymetrix Canine 2.0 oligonucleotide arrays to monitor gene expression of over 18,000 Canis familiaris mRNA/EST-based transcripts and over 20,000 non-redundant predicted genes. To minimize batch effects, RNA extraction and cRNA preparation of all samples were performed at the same time. Also, cRNA samples were hybridized on the same batch of microarray chips. As we have established in previous studies (Cameron et al., 2008; Rowe et al., 2010), canine arrays
were used due to the high levels of homology between canine and ferret nucleotide sequences (see the homology analysis for the current study in supplemental Table S1).

The arrays were scanned during the same session using an Affymetrix GCS3000 7G system according to standard Affymetrix protocols. Probe-level analysis was performed using Probe Logarithmic Error Intensity Estimate (PLIER). The raw intensity values for each individual target on the Affymetrix chips were pre-processed with variance stabilization, log2 transformation and were normalized against the PBS control group datasets with ArrayAssist V 5.5.1 (Stratagene, USA). T-test was performed with Benjamini-Hochberg false discovery rate (FDR) correction. Genes with a significant difference ($p \leq 0.05$; fold change $\geq 1.5$ or $\leq -1.5$) were selected for agglomerative hierarchical clustering with Pearson distance metrics and average linkage distance measurements between clusters using MultiExperimental Viewer 4.1 (Saeed et al., 2003).

Ingenuity Pathway Analysis (IPA) 5.0 software (Ingenuity Systems Inc., Redwood City, CA) was used to annotate and organize the gene expression data into networks and pathways.

2.3.13 Statistics

The number of neutralizing antibody titer and HI titer was equal to the last dilution of titration of the samples. The log transformed data were assessed for normal distribution by the Kolmogorov-Smirnov test. The neutralizing antibody titers and HI titers were found to be log-normal distributions before parametric analysis ($t$ test and ANOVA) in the study. One-way ANOVA was used for statistical analysis of the results represented in Fig. 2.4A and Fig. 2.6. Other analyses used the student’s $t$ test for comparing two independent populations.

Quantitative PCR results from 50 ferret immune-related genes were represented as heat map charts generated by the software MultiExperimental Viewer 4.1. Bar graphs were generated by SigmaPlot 8.0.

2.4 RESULTS

2.4.1 Humoral responses of CpG ODN-mediated immunization

We first investigated the effects of CpG ODN as an adjuvant for the 2007-2008 seasonal human flu vaccine, Fluviral ($V^{2007}$) in ferrets. Fluviral contains 15$\mu$g of HA protein from the
following influenza virus strains: A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004. Ferret serum samples were collected at time points following the primary immunization and vaccine alone boost. Immune activation was determined by anti-vaccine IgM and IgG antibody serum levels based on ELISA assays. Serum samples from V\textsuperscript{2007} + CpG treated animals had significantly higher IgM levels at Day 14 after primary immunization (Fig. 2.1 A, left panel), when compared to the IgM level of V\textsuperscript{2007}-Alone group. Interestingly, there were also statistically higher levels of antigen specific IgG in V\textsuperscript{2007} + CpG sera than V\textsuperscript{2007}-Alone treated animals at all the time points after primary vaccination and also at Day 7 post boost (Fig. 2.1 A, right panel). These results suggest that CpG ODN mediated influenza vaccination increases humoral immunity by inducing antibody production and promoting faster antibody class switching.

![Graphs showing antibody levels over time](image-url)

- **A**
  - Ferret Antigen Specific IgM
  - Ferret Antigen Specific IgG

**Graph Details**
- OD\textsubscript{450} values are plotted against days for each treatment group.
- Significant differences are indicated by asterisks: ** for p < 0.01, * for p < 0.05.
Influenza virus-specific antibody levels in serum from immunized ferrets were assessed by (A) ELISA, (B) HA inhibition and (C) microneutralization assays. (A) Serum IgM (left panel) and IgG (right panel) antibody levels against the commercial vaccine FLUVIRAL were measured at Days 0, 14, 21, 28, 35 and Day 7 post boost. The average relative absorbance density read at 450 nm from three individual samples were plotted graphically. (B) HI titers were measured from ferret sera against inactivated 2007-2008 seasonal Solomon Island A/Solomon Islands/3 H1N1, A/Wisconsin/67/2005 H3N2 and B/Malaysia/2507/2004 viruses. (C) Neutralizing antibody titers for blocking the live A/Solomon Islands/3 H1N1 virus were measure by microneutralization assays. Three independent experiments were performed. The number of HI titers and neutralizing antibody titers shown in the figure was log2 transformed from the original data. Error bars indicate standard deviation. Statistical analysis between adjuvant added vaccine and vaccine alone treated group animals were performed by the student t test. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. 

Figure 2.1 CpG ODN-assisted vaccination increased influenza virus-specific antibody levels in serum from immunized ferrets.
To confirm the results of ELISA assays, the antibody titers were also assessed by haemagglutination inhibition (HI) and microneutralization (MN) assays. HI assays indicated that the sera from V2007 + CpG treated animals contained significantly higher levels of antibody titers than the V2007-Alone treated animals (Fig. 2.1 B) after primary vaccination to Day 7 post boost. The MN assay also showed that the serum antibody from V2007 + CpG treated animals had significantly higher titers against the A/Solomon Islands/3/2006 (H1N1) virus than the animals treated with vaccine alone at all the time points (Fig. 2.1 C). These data further indicate that using CpG as adjuvant is able to augment the humoral immune response to influenza vaccinations.

2.4.2 Regulation of ISGs by CpG adjuvanted vaccination

It has been shown that the addition of CpG ODN to V2007 upregulated humoral responses after vaccination, we next assessed gene expression activity associated with increased immunogenicity at mRNA level. Quantitative PCR (Q-PCR) was used to generate a 50-gene expression profile from the whole blood RNA samples collected at Days 1, 3, and 5 after primary immunization and Day 7 post boost in the vaccination groups V2007 + CpG, V2007-Alone and PBS V2007 Boost. The PBS treated group was used as control for normalization at Days 1, 3 and 5 after primary vaccination and at Day 7 post boost. The identical gene expression pattern was observed in CpG adjuvanted and non-adjuvanted vaccination groups following normalization to PBS group at day 0. Since most of the immune related genes were significantly regulated at Day 1 in the V2007 + CpG group compared to the control group, the Day 1 Q-PCR data of 50 genes was illustrated by heat map (Fig. 2.2 A). The expression levels of the 12 immunologically relevant genes, from Day 1 after primary vaccination to Day 7 post boost, were statistically analyzed and represented by bar graphs (Fig. 2.2 B; Fig. 2.3).

ISG molecules, such as the anti-viral genes OAS1, RIG-I, Mx-1, ISG15, ISG20; transcription factors IRF1, IRF7, STAT1, STAT2; and the Th1 cell chemokine CXCL10 (Qian et al., 2007), which are induced by type I IFN during the virus infection, were significantly elevated at Day 1 after primary vaccination (Fig. 2.2 A). However, at later time points the expression level of most ISGs decreased to basal level or became downregulated, such as OAS1, IRF7 and RIG-I. Only CXCL10 and STAT1 showed sustained increased expression levels at Day 7 after boost (Fig. 2.2 B). Although the ISGs were modulated similarly in the group without
adjuvant, at Day 3 and Day 5 post primary vaccination the induced level of genes such as OAS1, RIG-I, ISG15 and STAT2 were markedly lower than with CpG (Fig. 2.2 B). This observation suggested that CpG stimulated a faster and stronger type I IFN response than that in the animals treated with vaccine alone.

Given that CpG mediated vaccination strongly induced ISGs, we then determined whether IFN regulated ISG expression in vaccinated animals. We examined the mRNA level of IFN-α and IFN-γ by Q-PCR. IFN-α was elevated in the \( V^{2007} + \) CpG group at Day 1 after primary vaccination and Day 7 post boost, whereas, IFN-γ levels were not highly increased by vaccination plus CpG (Fig. 2.3 A). Even though \( V^{2007} \)-Alone immunization increased the IFN-α level at Day 3 and Day 5, it was not statically significant when compared to the \( V^{2007} + \) CpG group (Fig. 2.3 A). Several studies have reported that IFN-stimulated response repressors, such as IRF-2, ICSBP and IRF4/PIP, likely terminate ISG transcription following IFN induction (Friedman et al., 1984; Nelson et al., 1993; Yamagata et al., 1996). In this study, IRF4 was highly upregulated in the \( V^{2007} + \) CpG group at Day 5 after primary vaccination and Day 7 post boost (Fig. 2.3 B). Since ISG expression was low during these time points, these results implied a negative role for IRF4 in the regulation of IFN-stimulated genes during adjuvant-mediated vaccination. Since IRF4 is also involved in the B cell class switch recombination and plasma cell differentiation (Klein et al., 2006; Lohoff et al., 2002; Lu, 2008), the elevated IRF4 expression in \( V^{2007} + \) CpG immunized animals may indicate a role in humoral immunogenicity regulation.
Figure 2.2 (legend is on next page)
Figure 2.2 CpG ODN vaccine activated ISGs expression early post immunization.

mRNA expression profiles from immunized ferrets with and without CpG ODN were determined by Q-PCR. (A) The heat map represents the mRNA expression profile of 50 ferret immune-related genes in V2007 + CpG (V + CpG) and V2007-Alone (V-Alone) group animals (n = 3/group) at Day 1 post vaccination. The heat map was generated by Multiexperiment Viewer software version 4.1 from the β-actin normalized real-time PCR data relative to PBS control group. Genes were listed by descending mRNA-level (red, upregulation; green, downregulation). V represents the V2007. (B) Transcription of nine ISG genes (OAS1, RIG-I, Mx-1, CXCL10/IP10, ISG15, IRF1, IRF7, STAT1 and STAT2) in ferret whole blood was quantified by Q-PCR and displayed graphically. The PBS V2007 boost group animals were treated with PBS at Day 0 and boosted with commercial vaccine at Day 35 after injection. The RNA samples used for expression analysis were extracted from the peripheral blood of three ferrets (n = 3) in each group at every time point. mRNA levels were normalized to β-actin and then to PBS control groups. The data was averaged from three independent experiments and the error bars represent standard deviation. Horizontal bars indicate the student t test performed between the selected two groups. *: p ≤ 0.05, **: p ≤ 0.01.

2.4.3 IFN-α and CFA-mediated vaccination

We next evaluated IFN-α as an adjuvant to human influenza vaccination to determine if the CpG-induced IFN response was responsible for the induced humoral response. Pegylated IFN-α (PEG-IFN) was tested as an adjuvant for the 2008-2009 human flu vaccine (V2008), Vaxigrip, in ferrets. The activity of pegylated IFN-α was evaluated by antibody titer quantification, and compared to the response of CpG ODN (V2008 + CpG), CFA (V2008 + CFA), vaccine alone (V2008-Alone) and PBS control groups. To determine antibody titers, HI and microneutralization assays were run using serum samples collected on Days 14, 21, 28 and 35 post V2008 immunizations. Quantification of HI titers for the live influenza A viruses Brisbane/59/2007 H1N1 and Brisbane/10/2007 H3N2 determined that CpG, PEG-IFN and CFA mediated vaccinations induced significantly higher titers than vaccine alone (Fig. 2.4 A). Furthermore, the microneutralization assay showed that V2008 + CpG, V2008 + IFN and V2008 + CFA immunized ferrets had higher titers of Brisbane/59/2007 H1N1 neutralizing antibody than V2008-Alone at Day 14 after vaccination (Fig. 2.4 B). Later time points showed only CpG and CFA mediated vaccinations stimulated statistically stronger antibody responses. In a further comparison of antibody induction among the adjuvanted immunizations, both CpG and CFA adjuvanted vaccines stimulated significantly higher antibody titers than the PEG-IFN adjuvanted vaccine. However, significant differences were not observed between the CpG and CFA groups.
These results indicate that adjuvant addition increases humoral responses and that V\textsuperscript{2008} + CFA produces the strongest responses of the three adjuvant-mediated vaccinations.

Figure 2.3 The levels of ISG regulatory genes in CpG ODN adjuvant-mediated immunization.

The average transcription levels of (A) IFN-\(\alpha\) and IFN-\(\gamma\) and (B) IRF4 in CpG ODN mediated immunization were determined by Q-PCR and plotted graphically from various time points following immunization. Increases in mRNA levels were relative to \(\beta\)-actin and then normalized to PBS control groups. Average was obtained from three independent experiments and the error bars indicate standard deviation. Horizontal bars show the student \(t\) test performed between the selected two groups. *: \(p \leq 0.05\).
Figure 2.4  IFN-α and CFA-mediated vaccination induced influenza virus-specific antibody production.

Antibody titers in vaccinated ferret serum against 2008-2009 seasonal live A/Brisbane/59/2007 H1N1 virus and A/Brisbane/10/2007 H3N2 virus were measured by HI inhibition assays. (B) Neutralizing antibody titers for blocking the live A/Brisbane/59/2007 H1N1 virus were measured from immunized ferrets by microneutralization assays. Data represents triplicate measurements of sera collected from three animals. The number of HI titers and neutralizing antibody titers shown in the figure was log2 transformed from the original data. Error bars indicate standard deviation. Statistical analysis between adjuvant added vaccine and vaccine alone treated group animals were performed by either one-way ANOVA in figure (A) or student t test in figure (B). *:  p ≤ 0.05, **:  p ≤ 0.01, ***:  p ≤ 0.001.
2.4.4 Virus infectivity in adjuvant-treated animals

We next determined whether an increase in humoral immunity led to increased protection from viral infection. Ferrets were immunized with the following combinations: PBS, V<sup>2008</sup>-Alone, V<sup>2008</sup> + CpG, V<sup>2008</sup> + IFN and V<sup>2008</sup> + CFA. At day 42 following immunization, the ferrets were infected with 10<sup>6</sup> EID<sub>50</sub> Brisbane/59/2007 H1N1 virus to evaluate the protection efficacy of adjuvant-mediated vaccinations. Brisbane/59/2007 H1N1 virus has been found to replicate only in the nasal cavity of ferrets (Munster et al., 2009; Rowe et al., 2010). From Day 2 p.i., 50-75% of the animals in each group exhibited clinical signs of respiratory disease, including nasal discharge and sneezing. The symptoms lasted for 2-3 days and decreased at Day 5 p.i. All animals exhibited increased temperature at Day 2 p.i., which diminished by Day 3, except the groups of V<sup>2008</sup>-Alone, V<sup>2008</sup> + IFN and PBS, which showed a second increase in temperature on Day 4 p.i.. In addition, the animals displayed a decrease in activity by Day 3 p.i., except the animals immunized with V<sup>2008</sup> + CFA. The relative inactivity index (Table 2.1) showed that V<sup>2008</sup> + CFA was 1.1, V<sup>2008</sup> + CpG and V<sup>2008</sup> + IFN were both 1.3, and V<sup>2008</sup>-Alone and PBS control groups were 2.0 and 1.6, respectively. These results suggest that adjuvant-mediated vaccination in ferrets dramatically reduces influenza severity compared to receiving the vaccine alone.

To evaluate infectivity, nasal washes were collected from animals on each day post infection and viral loads (TCID<sub>50</sub>/ml) were calculated. Although viral loads in each group were elevated at Day 1 and Day 2 p.i., the virus replication in the nasal turbinates of the V<sup>2008</sup> + CFA group was significantly lower than in the PBS control group. Furthermore, the Day 3 p.i. virus

### Table 2.1 Inactivity of Infected Ferrets

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Relative Inactivity Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1.6</td>
<td>0±0</td>
<td>1.5</td>
<td>0.6</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>V&lt;sup&gt;2008&lt;/sup&gt; + CFA</td>
<td>1.1</td>
<td>0±0</td>
<td>0.0</td>
<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>V + CpG</td>
<td>1.3</td>
<td>0±0</td>
<td>0.6</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>V + IFN</td>
<td>1.3</td>
<td>0±0</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>V + PBS</td>
<td>2.0</td>
<td>0.5</td>
<td>1.4</td>
<td>1.4</td>
<td>0.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative inactivity index was calculated from daily activity scores as described in 2.3.6

<sup>b</sup> Average of daily activity score was obtained from 3 animals in each group

<sup>c</sup> 2008-2009 human flu vaccine
titer measured in nasal washes of adjuvant groups were markedly diminished compared to those of the PBS control (Fig. 2.5), whereas no significant decrease was observed in the ferrets that received vaccine alone. By Day 5 p.i., the virus titer was not detectable in the majority of animals. Interestingly, the assessment of antibody titer in serum at Day 5 p.i. showed that adjuvant-immunized animals had significantly higher antibody levels than animals treated with vaccine alone, as determined by HI and neutralization assays (Fig. 2.6 A, B). Additionally, in nasal washes collected at day 5 p.i., the anti-Brisbane/59 IgG levels in the CpG and CFA adjuvanted vaccination groups were significantly higher than the vaccine alone. Given the clinical results and the decreased viral loads and increased antibody production observed in animals vaccinated with adjuvant compared to animals receiving the vaccine alone, we suggest that adjuvant induces a faster and stronger memory antibody response upon infection.

Figure 2.5 Adjuvant-mediated vaccination decreased viral load following influenza infection.

Viral load in nasal wash at Day 3 post H1N1 influenza challenge was determined. Nasal wash from each group animals (n = 3) was collected post infection, cultured and titrated. The virus titer was calculated by Reed-Muench method and expressed as TCID₅₀/ml. Error bars demonstrate standard error of the mean. Statistical analysis was performed between each adjuvant added vaccination group and PBS control group by student t test. V represents the V²⁰₀⁸. *, p ≤ 0.05, **: p ≤ 0.01.
Figure 2.6 Memory antibody response at Day 5 post H1N1 infection was increased in adjuvant-mediated vaccinated animals.

(A) Antibody titers from ferret sera against live seasonal A/Brisbane/59/2007 H1N1 virus was determined by HA inhibition assays at Day 5 post infection for adjuvant and non-adjuvant assisted vaccinations. Student t test was utilized for statistical analysis. (B) Neutralizing antibody titers for blocking the A/Brisbane/59/2007 H1N1 virus were assessed by microneutralization assays. The statistical analysis determined between Day 35 after vaccination and Day 5 post infection is shown by horizontal bar. The differences between adjuvant groups and vaccine alone group at Day 5 p.i. were analyzed by one-way ANOVA. Data represents the average of triplicate measurements of sera collected from three animals. The number of HI titers and neutralizing antibody titers shown in the figure was log2 transformed from the original data. Error bars indicate standard deviation. V represents the $V^{2008}$. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

2.4.5 Microarray analysis of CpG adjuvant immunization

Above we showed that the expression of our selected immune related genes changed most significantly at Day 1 after vaccination with CpG compared to control (Fig. 2.2 A). We next investigated the large scale gene expression profile of Day 1 adjuvant vaccinations by microarray analysis. The heat map (Fig. 2.7) represents the expression of immune-related genes significantly altered at Day1 post vaccination from adjuvant-mediated $V^{2008}$ vaccinations (one ferret per column). A similar expression pattern was observed by Q-PCR for three genes selected from the antigen presentation, adaptive immunity and complement clusters shown in Fig. 2.7 (see supplementary material in Appendix 2.2). Fold gene expression changes for each adjuvant-
treated group compared to PBS controls are listed in Table 2.2. Also, we used vaccine alone as a comparator and observed a similar expression pattern to that of the PBS group.

Consistent with our Q-PCR results of $V^{2007}$ + CpG vaccination, which showed upregulation of 10 ISGs, the microarray data determined that 15 ISG members were increased by at least 1.5 fold in $V^{2008}$ + CpG immunization (Fig. 2.8 A, Table 2.2). The Ingenuity Pathway Analysis (IPA), combining the Q-PCR and microarray data, suggests that CpG-adjuvanted vaccination stimulates type I IFN signaling and activates ISG members through STAT1 and STAT2 (Fig. 2.8 B upper panel). Also, toll-like receptor 4 (TLR4), which specifically recognizes bacterial lipopolysaccharides (LPS) and mediates innate immunity (Jin & Lee, 2008), was induced following CpG-adjuvanted vaccination (Table 2.2). Transcription analyses showed that co-stimulatory molecules and MHC genes were not elevated by vaccination with CpG, except for the upregulation of cathepsin family antigen processing genes, CTSB and CTSS. Similarly, neither microarray nor Q-PCR showed increased IFN-γ levels after CpG-mediated vaccination when compared to the vaccine alone. Interestingly, the immune regulator SOCS1 (Table 2.2), which negatively regulates IFN-γ signaling (Dalpke et al., 2001), was found to be upregulated in $V^{2008}$ + CpG immunized animals by microarray analysis.

2.4.6 Microarray analysis of IFN-α adjuvant immunization

Given that CpG-mediated vaccination significantly increased ISGs, we tested IFN-α for its direct adjuvant potential during immunization against influenza. We have found that treatment with PEG-IFN alone resulted in significantly increased mRNA levels of ISGs in ferrets.
Figure 2.7 Adjuvant-mediated vaccination activated differing arms of innate and adaptive immunity.

The heat maps represent RNA expression determined by microarray analysis Day 1 post vaccination. The heat map generated from the gene expression profile of the four different vaccination groups (n = 3/group) shows the innate, adaptive, Ag processing/presentation and complement immune related genes, which were significantly regulated by at least 1.5 fold change (p ≤ 0.05) in one of the adjuvant additive groups. The fold change of the gene expression is shown in Table 2.2 (red, upregulation; blue, downregulation). V represents the V2008.
Figure 2.8 CpG ODN and PEG-IFN-adjuvanted vaccine-regulated IFN signaling pathways.

(A) IFN-responsive gene expression was determined by microarray analysis from the RNA of vaccinated ferrets at Day 1 post vaccination (red, upregulation; blue, downregulation) and plotted by heat map. (B) A schematic was created from the gene expression profiling data obtained Day 1 post immunization by IPA: the upper panel shows the IPA canonical pathway of IFN signaling using the Q-PCR of 2007 vaccine study and microarray data of the 2008 vaccine study from CpG-mediated immunized ferrets; the lower panel exhibits the IFN signaling pathway activated by PEG-IFN-adjuvanted vaccination by using the microarray data of the 2008 vaccine study (red, upregulation). (C) Gene expression heat map of molecules involved in Ras signaling at Day 1 post-vaccination (upper panel; red, upregulation), and IPA modeling of JAK/STAT pathway-mediated Ras signaling using the microarray data from the whole blood RNA of PEG-IFN-mediated immunized ferrets at Day 1 post vaccination (lower panel; red, upregulation).

at early time points. Here, microarray analysis revealed that 345 genes were highly upregulated following $V^{2008} +$ IFN vaccination. Similar to the gene profile of CpG mediated immunization,
V\textsuperscript{2008} + IFN stimulated the expression of ISGs, including STAT1, IRF2, OAS1/2, ISG15, USP18, IFIT2, RSAD2, IFI44 and IFI44L (Fig. 2.8 A), although to a lesser extent than the V\textsuperscript{2008} + CpG group, shown by the IPA analysis (Fig. 2.8 B lower panel).

V\textsuperscript{2008} + IFN immunization also upregulated antigen processing and presentation genes, such as CTSD and MHC class Ib, as well as the T cell adhesion molecule, CD84 (Tangye et al., 2003) (Table 2). We also found the MHC enhanceosome member RFX5, which regulates MHC during antigen presentation (Reith et al., 2005), was significantly increased following PEG-IFN mediated vaccination. Interestingly, PEG-IFN mediated vaccination also induced the gene expression of intracellular signaling molecules Ras and the MEK member MAP2K2 (Fig. 2.8 C), which are known mediators of B cell proliferation and B cell memory after CD40 stimulation (Coughlin et al., 2005; Coughlin et al., 2006). JUNB, which is downstream of Ras following BCR stimulation (Yin et al., 2008), was also up-regulated in V\textsuperscript{2008} + IFN vaccination. Additionally, the expression of the guanine nucleotide activating protein (GAP), RGS1, involved in B cell activation (Hong et al., 1993), which was elevated in the V\textsuperscript{2008} + CpG group, was also increased in V\textsuperscript{2008} + IFN immunized ferrets (Table 2).

2.4.7 Microarray analysis of CFA adjuvant immunization

The precise molecular mechanism of CFA-mediated influenza immunization is not completely understood. We therefore investigated the gene profile of CFA-adjuvanted influenza vaccination in ferrets. Microarray analysis revealed that a total of 1,255 genes were regulated at Day 1 post V\textsuperscript{2008} + CFA immunization. Since CFA is thought to contain the NOD2 agonist MDP, which induces NF-κB and MAPKs to initiate proinflammatory cytokine expression in innate immune cells (Kufer, 2008), we examined the expression of NOD2 signaling genes. The expression of the MAP Kinase family member ERK was induced at Day 1 after CFA mediated vaccination (Table 2.2), but proinflammatory cytokines were not induced.
Table 2.2 *In vivo* differential microarray gene expression analysis on whole blood of ferrets immunized with adjuvanted 2008-2009 vaccines (n=3) versus PBS controls (n=3) on day 1 post vaccination.

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*a* Gene symbol for each gene

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\(^a\) The gene symbol is compatible to the human ortholog

\(^b\) The mean fold change is normalized to the corresponding PBS control group. Boldface indicates the gene expression is significantly induced by at least 1.5 fold change (p < 0.05) versus controls.

\(^c\) 2008-2009 human flu vaccine
CFA may also contain a TLR ligand, which could synergistically activate DCs with MDP (Fritz et al., 2005; Ishii & Akira, 2007). Similar to CpG- and IFN-mediated vaccination, the expression of antigen processing genes, namely CTSD, MHC class I DLA-64, MHC class II HLA-DMA/CLIP (CD74) and MHC transcription enhancesome RFX5 (Fig. 2.7, Table 2.2) were significantly increased in CFA-mediated immunization. However, the MHC class II transactivator (CIITA), which regulates MHC class II gene transcription, was significantly down-regulated (Table 2.2). We also found that CFA activated the expression of several adhesion molecules, including Integrin-β, VCAM1, BCAM, CD36, CD84 and CD44 (Fig. 2.9 A), and genes such as Zyxin, Talin 1, CRKL, RAP2A and Rho family members involved in integrin signaling and mediating cell adhesion and motility (Fig. 2.9 B). Moreover, expression of CXCL14, a chemokine involved in B cell migration and activation (Sleeman et al., 2000), was also found elevated (Table 2.2).

B cell intracellular signaling molecules, such as Lyn, Syk, and PI3K, were significantly up-regulated by CFA at Day 1 (Fig. 2.9 A, C). In addition, increased mRNA level of B cell proliferation regulator Bam32 (Han et al., 2003; Marshall et al., 2000) was observed in CFA-mediated vaccination. In contrast to the PEG-IFN-induced transcription of MEK member MAP2K2, involved in Ras mediated BCR signaling, CFA activated the expression of Ras downstream effectors, c-Raf and MAP Kinase ERK (Fig. 2.9 C). Following BCR engagement, Ras activation is one result of Ca^{2+} signaling (Cullen & Lockyer, 2002) which leads to gene induction through the calcineurin-NFAT pathway to stimulate B cell proliferation (Healy et al., 1997). Indeed, the calcineurin family members PPP3R1, NFAT molecule NFATC3, and the calcineurin-NFAT activation regulators G protein complex (GNA15, GNB3, GNG2 and GNG11) and CK1 (CSNK1D and CSNK1G2), were all highly induced following CFA-adjuvanted vaccination (Fig. 2.9 D). Moreover, we found that the small G protein Rac, another factor which activates NF-κB and JNK signaling (Coso et al., 1995; Hill et al., 1995; Minden et al., 1995), was induced after vaccination with CFA (Fig. 2.9 C) and AICDA (AID), essential for initiating the immunoglobulin gene hypermutation (SHM) and class switch recombination (CSR) in B cells (Chaudhuri et al., 2003), was increased by 2.6 fold in CFA mediated vaccination (Table 2.2). Although AID was induced, only one of its activator molecules, Oct2 (Park et al., 2009), was significantly increased whereas the other, HoxC4, was downregulated (Fig. 2.9 C).
Lastly, SOCS family member, SOCS3, was induced by $V^{2008} + CFA$ treatment (Table 2.2). High SOCS3 expression may favour Th2 cell differentiation and inhibit IL12-mediated Th1 development (Seki et al., 2003), which is consistent with our finding that IL12A (Table 2.2) was
Figure 2.9 Cell adhesion and B cell signaling pathways were activated in ferrets immunized with CFA-mediated vaccines.

(A) Heat map of cell adhesion molecule gene expression and genes involved in B cell activation/calcineurin-NFAT signaling at Day 1 post vaccination (red, upregulation; blue, downregulation). (B), (C) and (D) IPA canonical pathways of integrin signaling, B cell activation, and calcineurin-NFAT created by using the microarray data of CFA immunized ferret peripheral blood RNA Day 1 post vaccination, (red, upregulation; green, downregulation). V represents the $V_{2008}$ significantly down-regulated in CFA-adjuvanted vaccination. Also, more complement components, such as C1QL2, C1R, C5AR1 and CFI (Fig. 2.7, Table 2.2), were activated in $V_{2008}$ + CFA immunization compared with the other adjuvanted immunizations which upregulated only FCN1 and C3 precursor. However, $V_{2008}$ + CFA stimulated few ISGs, such as transcription regulator IRF1, IRF2, and antiviral gene RSAD2 (Table 2.2), when compared to CpG and PEG-IFN adjuvanted vaccinations.

2.5 Discussion

Here we investigated the adjuvant potential of CpG ODN when added to the seasonal human influenza vaccine, and subsequently characterized the molecular gene signatures of the induced immune responses. Since CpG ODN initiated robust IFN responses following vaccination, we evaluated type I IFN as an adjuvant by using PEG-IFN in combination with the human influenza vaccine, Vaxigrip. We also compared the effect of PEG-IFN to the standard adjuvant, CFA. To demonstrate the activity of each adjuvant during immunization, gene expression profiling was performed by microarray.

Gene expression profile generated by Affymetrix canine arrays was used to expand on the gene signatures identified with Q-PCR analysis (Fig. 2.2). At this time, it is not known whether microvariation in the canine probes affects detection of ferret orthologs. Our group has established the utility of canine platform to assess ferret gene expression using a homology analysis of the limited publicly available ferret and canine cDNA sequences and a ferret-specific Q-PCR validation strategy (Cameron et al., 2008; Rowe et al., 2010). Cross-species microarray analyses are supported by previous studies (Dillman, III & Phillips, 2005; Ji et al., 2004; Nieto-Diaz et al., 2007), however, a lack of publicly available canine whole blood microarray datasets has not allowed us to make the same comparisons. Nonetheless, high homology was identified
between numerous ferret and canine genes derived from the current study (see supplementary material in Appendix 2.3) and extensive ferret-specific Q-PCR validation on surrogate genes was performed.

The CpG experiment showed that our genes of interest changed most significantly at day 1 following vaccination (Fig. 2.2), therefore this time point was chosen for more extensive microarray analyses. We demonstrated that although CpG ODN led to the activation of ISGs and subsequent stimulation of humoral responses, when PEG-IFN was used it was unable to reproduce the humoral activation to the same extent as that of CpG ODN. Furthermore, CpG ODN as well as IFN and CFA adjuvant-mediated immunizations stimulated stronger antibody responses than the vaccine alone and gave better protection for animals following seasonal H1N1 infection. The microarray analysis for each adjuvant showed differing molecular signatures indicating that distinct molecular pathways were activated depending on the adjuvant used.

Based on microarray profiling and Q-PCR analysis, the CpG-adjuvanted immunization activated canonical type I IFN signaling responses. The upregulation of ISGs included the virus sensing RIG-I and PKR, the virus replication inhibitors OAS1, 2 and L, the transcription factors STAT1, STAT2, the IFN-regulating molecules IRF1, IRF7, and the Th1 cell chemoattractant CXCL10. In plasmacytoid DC (pDC), CpG binds to the intracellular receptor TLR9 to activate IFN-α expression, which initiates the ISG stimulation (Haller et al., 2006). In our study, the ISG induction was transient after vaccination and the relatively increased IFN-α expression was only observed at 24 hours post vaccination. Typically, pDCs are thought to be the primary producers of type I IFN and that production of IFN following CpG stimulation leads to maturation of the conventional DCs (cDCs), which in turn augments the B cell response toward a Th1-like phenotype by inducing IFN-γ and IL12 (Krieg, 2002). Our gene profiling did not support this mechanism for CpG-induced pDC and cDC-mediated enhancement of humoral response through Th1 regulation; since molecular signatures associated with DC maturation and/or Th1 signaling were not observed. Moreover, we found that SOCS1, the suppressor of IFN-γ signaling (Dalpke et al., 2001), was significantly up-regulated. Therefore, CpG may function as a non-Th1 biasing adjuvant during immunization, a possibility we are studying further.

Several recent studies indicate that CpG can directly activate B cells through TLR9 and MyD88 to promote class switching toward a Th1 phenotype (Jegerlehner et al., 2007; Lin et al.,
In addition, TLR9 signaling has been implicated in plasma cell proliferation and differentiation after immunization with antigen and non-soluble CpG, which leads to IL-6 and IgM secretion (Eckl-Dorna & Batista, 2009). Here we found that IRF4, which is involved in antibody class-switching and plasma cell differentiation (Fillatreau & Radbruch, 2006; Klein et al., 2006; Lu, 2008), was upregulated in CpG-mediated vaccination. Since both ligation of TLR9 and intracellular activation of IRF4 regulate antibody class switching, we postulated that the IRF4 could be induced downstream of TLR9 engagement in B cells. In this scenario, it is possible that downstream activation of IRF4 may occur via MyD88-mediated NF-κB and/or AP-1 activation, which has been a proposed route for B cell activation, proliferation and Ig production after TLR9 anchoring (Peng, 2005). High levels of IRF4 occurring after the antigen boost also suggests that IRF4 could play a role in the memory plasma cell differentiation, which is consistent with the findings in the study by Klein et al. (Klein et al., 2006). Taken together, our data suggests that CpG-adjuvanted vaccination activates B cells via TLR9-mediated expression of genes such as IRF4, involved in plasma cell differentiation and antibody class switching.

Interestingly, the microarray profiling of CpG mediated vaccination indicated that TLR9 activation correlated with TLR4 upregulation. TLR4 cooperates in BCR signaling to enhance the antibody response through LPS ligation (Dye et al., 2007; Minguet et al., 2008). Although LPS is the classic antigen for TLR4, an updated list asserts that other pathogens, such as viral protein and parasitic heat shock proteins, also bind to this innate receptor (Aosai et al., 2002). Thus, TLR4 may be also involved in immune activation by recognizing the vaccine antigen in conjunction with TLR9-mediated vaccine responses.

As discussed above, type I IFN induces adaptive immunity by stimulating DC cell surface co-stimulatory molecules and MHC antigens which enable DCs to activate B cells (Le Bon et al., 2001). Since type I IFN is an inducer of adaptive immunity, we used pegylated IFN-α2b as an adjuvant to compare with CpG-adjuvanted immunization. PEG-IFN induced similar ISG expression to CpG-adjuvanted vaccination as determined by microarray analysis (Fig. 8 b). The MHC class I gene was also induced possibly as a result of increased levels of the MHC transcription enhancesome RFX5. An increase of the MHC class I molecule on the surface of antigen presenting cells (APCs) can then activate T cells to express the adhesion molecule CD84. Since RFX5 was also up-regulated in CFA adjuvanted vaccination, it is possible that RFX5 was
activated by the same signaling pathway and may represent as a common mediator of adjuvant activity in both PEG-IFN and CFA mediated vaccination. In the future, it will be important to elucidate the mechanism of RFX5 induction since MHC upregulation in our adjuvant-mediated studies are important components of adaptive immunity. Interestingly, PEG-IFN stimulated Ras and MEK genes involved in Ras-MEK-ERK signaling. Ras-MEK-ERK signaling in B cell plays an important role in generating the high affinity antibody (Sanjo et al., 2007). It is therefore likely that type I IFNs can directly activate B cells through type I IFN receptor to initiate the Ras-MEK-ERK pathway, as type I IFNs can stimulate B cells directly to produce antibody and express IFIT2/3 early after influenza infection (Coro et al., 2006; Sanjo et al., 2007).

Our microarray data suggest that IFN-α2b activates MHC class I expression which may play a role in activating CD8+ T cells post-vaccination. Furthermore, the data implies that IFN-α activates antibody generation by B cells through Ras signaling. However, our HI and microneutralization assays showed antibody levels induced by PEG-IFN were lower than those stimulated by CpG (Fig. 2). This decreased antibody production may be dose-dependent since previous reports have shown that the Ig subclass was highly increased at a high dose of IFN-α adjuvant administration (Tovey et al., 2006); or possibly the IFN-α2b subtype could not fully activate the DC or B cell, since IFN-α1 is the main subtype produced by pDC after virus infection.

CFA has been used for decades in animal models to generate high levels of antibody against antigens. It is known that MDP, the NOD2 ligand, is the minimal essential component in CFA (Ellouz et al., 1974). Unlike previous findings (Kufer, 2008), the results of our microarray profiling in ferrets showed that CFA-mediated vaccination did not stimulate a proinflammatory milieu but instead only showed the elevation of the MAPK pathway gene ERK. Weak proinflammatory cytokine responses, e.g. absence of IFN-γ gene expression, may be the result of a low dose of CFA treatment (100 μl, one injection) given to the ferrets. Even though a robust inflammatory response was not observed, the transcription enhanceosome RFX5 was upregulated upon CFA-adjuvanted vaccination along with MHC gene induction, which is associated with DC maturation (Mellman & Steinman, 2001). Previous reports have shown strong synergism between TLR ligands and MDP when administered together (Tada et al., 2005). Therefore, it is possible that there may be another ligand in CFA that contributes to the adjuvant activity. In particular, the TLR4 ligand can increase the maturation potential of MDP on human DC by
inducing co-stimulatory molecules and the MHC class II gene (Fritz et al., 2005). Furthermore, NOD2/TLR mediated MHC upregulation may be regulated by the induced SOCS3, which is involved in the negative regulation of STAT3 and associated with Th2-type signaling in DC (Kubo et al., 2003). This is supported by our finding that the Th1-directing cytokine IL12A (Table 2.2) was significantly down-regulated in CFA-adjuvanted vaccination.

Mature DCs efficiently present antigens on the cell surface and directly promote expression of B cell high-affinity receptors. This results in strong BCR signaling and subsequent interaction with T helper cells to initiate antibody secretion cells and memory B cells (Dustin & Dustin, 2001). In this study, we found that several important genes involved in B cell activation were induced in CFA mediated vaccination, in particular, the molecules involved in Lyn-Syk-PI3K signaling pathway, calcineurin-NFAT pathway and Ras-MEK-ERK pathway. Furthermore, our pathway analysis suggested that B cell activation may have induced Calcineurin-NFAT, Ras-MEK-ERK and NF-κB signaling (Healy & Goodnow, 1998). These pathways in turn initiate the transcription of genes involved in B cell expansion, plasma cell differentiation and antibody production, such as Bam32 (Han et al., 2003), and Oct2 (POU2F2), which binds to the AICDA promoter and activates AID transcription (Park et al., 2009) for determining the antibody SHM and CSR (Chaudhuri et al., 2003). In addition, the molecules engaged in cell adhesion and motility, complement components, and chemoattractant were highly stimulated in CFA adjuvanted vaccination.

In contrast to type I IFN activation by CpG ODN or PEG-IFN, CFA stimulated a relatively low level of ISG expression, which is unlike previously published findings that robust type I IFN activation is a hallmark of CFA activity in mouse models (Le Bon et al., 2001). Given that CFA was administered with vaccine intramuscularly, the slow release of this emulsion from the injection site to the peripheral circulation, or the more localized activity of this adjuvant, may result in the limited ISG stimulation in peripheral blood at 24 hours post vaccination. It will be important to evaluate gene regulation in the peripheral blood at later time points following CFA adjuvanted vaccination to demonstrate the role of IFN responses in the adjuvanticity of CFA.

Altogether, our data suggests that the low dose of CFA activates the expression of MHC molecules which are associated with DC activation through NOD2 and/or TLR signaling rather than type I IFN receptor ligation. We contend that the activated DC may strongly activate BCR
signaling to initiate the B cell proliferation and plasma cell differentiation through high-Ca\textsuperscript{2+}-induced NFAT, ERK and NF-κB-regulated transcription. Given the paucity of ferret-specific reagents, we could not isolate DC in this study to investigate this potential mechanism further. Also, we were limited to one time point of gene expression profiling and cannot rule out that some of the expression differences may be affected by different gene expression kinetics or different dose of adjuvants.

In conclusion, we have identified both common and disparate signaling pathways activated downstream of \textit{in vivo} adjuvant activity during vaccination. Of note, RFX5 was a common transcript induced by both IFN-α and CFA-adjuvanted vaccinations, representing a focal point of adjuvant activity. Additionally, the identified signature molecules in our study could be specifically targeted in future vaccines, thereby facilitating the efficacy of vaccination and the development of host immunogenicity.

References


Chapter 3

Seasonal H1N1 infection induces cross-protective pandemic H1N1 immunity through a CD8 independent, B cell dependent mechanism

A version of this chapter is published in:

3.1 Summary

During the 2009 pandemic (H1N1pdm) outbreak it was found that most individuals lacked antibodies against the new H1N1pdm, and only aged people showed anti-hemagglutinin (HA) antibodies that were cross-reactive with the new strains. Different studies have demonstrated that prior contact with the virus can confer protection against strains with some degree of dissimilarity; however, this has not been sufficiently explored within the context of a H1N1pdm infection. In this study, we have found that a first infection with A/Brisbane/59/2007 confers heterologous protection in ferrets and mice against a subsequent H1N1pdm 2009 (A/Mexico/4108/2009) infection through a cross-reactive but non-neutralizing antibody mechanism. Heterologous immunity is abrogated in B cell-deficient mice but maintained in CD8- and perforin-1- mice. We have identified cross-reactive antibodies from A/Brisbane/59/2007 sera that recognize non-HA epitopes in H1N1pdm 2009. Passive serum transfer showed that cross-reactive sH1N1-induced antibodies conferred protection in naïve recipient mice during H1N1pdm challenge. The presence or absence of anti-HA antibodies, therefore, is not the sole indicator of the effectiveness of protective cross-reactive antibody immunity. Measurement of additional antibody repertoires targeting the non-HA antigens of influenza virus need be taken into consideration in assessing protection and immunization strategies. We propose that pre-existing cross protective non-HA antibody immunity may have had an overall protective effect during the 2009 H1N1pdm, thereby reducing disease severity in human infections.

3.2 Introduction

The novel swine-origin influenza A H1N1 virus was identified as the cause of human respiratory disease in Mexico and USA in April of 2009 (Anonymous2009d; Anonymous2009b). This virus was later designated as pandemic H1N1 2009 virus (H1N1pdm). The emerging virus spread throughout the world and prompted the World Health Organization (WHO) to declare the pandemic alert to level 6 on June 11, 2009 (Anonymous2009a). It infected millions of people and at least 14,711 deaths were reported worldwide by January 29, 2010 (Anonymous2010).

Vaccination is a critical intervention intended to diminish the spread of influenza virus and reduce the symptom severity in the infected individuals. Given that H1N1pdm virus is antigenically and genetically different from previously circulating seasonal H1N1 (sH1N1) influenza virus (Garten et al., 2009), vaccines that are based on sH1N1 antigens are unlikely to
provide cross reactivity to the H1N1pdm virus (Anonymous2009c). Thus, monovalent H1N1pdm vaccines have been produced since the emergence of the new influenza strains and they are able to achieve seroprotection rates of about 85% (Clark et al., 2009).

Serological analyses performed in pre-pandemic human serum samples showed that cross-neutralizing antibodies against H1N1pdm virus were present in the elderly population, but not in children and young adults (Hancock et al., 2009; Itoh et al., 2009; Miller et al., 2010). Those antibodies are possibly a consequence of previous exposure to older viruses that were antigenically related to H1N1pdm virus (Maines et al., 2009; Skountzou et al., 2010), and their presence may explain the overall low symptom severity that was observed among the elderly during the 2009 pandemic (Chen et al., 2010; Miller et al., 2010). Furthermore, several studies in animal models have demonstrated that a prior infection with sH1N1 is able to provide substantial protection against H1N1pdm infection (Ellebedy et al., 2010; Ellebedy et al., 2011; Kash et al., 2010; Laurie et al., 2010); cross-reactive CD8 and CD4 T cell responses against H1N1pdm viruses were detected, indicating that a substantial fraction of the T cell epitopes is conserved between sH1N1 and H1N1pdm (Sun et al., 2011; Tu et al., 2010). Also, B cell responses can provide extensive cross-protection against drifted influenza strains (Waffarn & Baumgarth, 2011).

In this study, we have found that a first infection with sH1N1 A/Brisbane/59/2007 confers heterologous protection in ferrets and mice against a subsequent challenge with H1N1pdm A/Mexico/4108/2009 through a cross-reactive but non-neutralizing antibody mechanism. Heterologous immunity is heavily diminished in B cell-deficient mice but maintained in CD8− and perforin-1− (Prf1−) mice. We have identified cross-reactive antibodies from A/Brisbane/59/2007 sera that recognize non-HA epitopes from the H1N1pdm virus. Moreover, passive transfer of cross-reactive antibodies induced by sH1N1 infection provided substantial protection against H1N1pdm challenge in naïve recipient mice. Our study indicates that sH1N1 primary infection induced pre-existing non-HA antibodies and/or memory B cells, and they are essential for providing cross-protective immunity against a subsequent H1N1pdm challenge in animal models. Assuming that human immune responses will show an analogous behavior during a heterologous reinfection, we propose that previous encounters with sH1N1 exerted an overall protective effect in the human population during the 2009 pandemic.
3.3 Materials and Methods

3.3.1 Animals and viruses

Male ferrets 4-6 months old were purchased from Marshall Bioresources (New York, NY, USA) and they were proven seronegative against different influenza strains. Pathogen free C57BL/6, CD8 T cell deficient strain Cd8atm1Mak/J (CD8-/-) mice, immunoglobulin µ heavy chain mutant strain Ighmtm1Cgn/J (µMT) mice and perforin-1 deficient strain Prf1tm1sdz/J (Prf1-/-) mice (8-10 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME). Experiments with ferrets and mice were conducted at the Animal Resources Centre of University Health Network (UHN, Toronto, Canada) under BSL-2+ conditions and in accordance with the Canadian Council of Animal Care (CCAC) guidelines. The animal use protocols were approved by the Animal Care Committee (ACC) of UHN. All viruses were obtained from US Centre of Disease control (CDC, Atlanta) and grown in the allantoic cavity of 10-day old embryonated chiken eggs. The viral titer expressed as EID₅₀/ml was calculated by serially titrating the virus on chicken eggs as per Reed-Muench method. The A/California/07/2009 virion was split by Triton X-100 and inactivated by formaldehyde at 4°C overnight. Then, the split viral components were collected by high-speed centrifugation and diluted in PBS. The resulting split virus contains all the structural proteins such as HA, NA, NP and M. Specifically, we determined the presence of HA and NP by ELISA.

3.3.2 sH1N1 primary infection, vaccine immunization and H1N1pdm secondary challenge

To evaluate the cross-protection provided by human seasonal H1N1 primary infection, naïve ferrets, C57BL/J mice and gene deleted mice were infected by 10⁶ EID₅₀ of A/Brisbane/59/2007 intranasally (i.n.). The clinical signs were monitored daily post infection. Ferrets and mice were rechallenged 4-5 weeks after the first infection with sH1N1; similar period of time between the first and the second infection has been used in other studies (Guo et al., 2010; Lamere et al., 2011a; Laurie et al., 2010; O'Neill et al., 2000).

In the vaccination study, three groups of eight ferrets were immunized respectively by 500µl of Swine influenza vaccine (SwVaccine), 2008-2009 seasonal human flu vaccine Vaxigrip containing A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B/Florida/4/2006, and
PBS (Unvaccinated). All the animals were vaccinated through intramuscular (i.m.) injection. At day 14 following primary immunization, HI titers against pandemic H1N1 virus of each ferret group was evaluated. In the meantime, Swvaccine and Vaxigrip group animals were boosted correspondently by the same amount of vaccine; unvaccinated control group was boosted with PBS. At day 14 post boost, HI test for examining the antibody titer against pandemic virus was performed on the animal serum samples.

Ferrets were challenged by H1N1pdm virus at week 4 post vaccination or primary infection. Animals were moved at least 4 days prior to infection to the BSL-2 animal holding area, where they were housed in cages contained in bioclean portable laminar flow clean room enclosures (Lab Products, Seaford, Del.). Prior to infection, baseline temperatures and weights were measured once daily for at least 3 days. Ferrets were anesthetized with 5% isoflurane anesthesia and infected intranasally (i.n.) with a total of 1 ml of $10^6$ EID$_{50}$ of A/Mexico/4108/2009 or A/California/07/2009 per ml in PBS delivered to the nostrils. Temperatures were measured daily using a subcutaneous implantable temperature transponder (BioMedic Data Systems, Inc., Seaford, Del.). Clinical signs in terms of temperature change, weight loss, nasal discharge and inactivity were recorded daily post infection as described previously (Fang et al., 2010).

In our mouse study, seasonal virus infected wild type (WT) C57BL/6 mice, gene deleted mice and uninfected wild type mice were challenged with $10^5$ EID$_{50}$ of A/Mexico/4108/2009 virus at week 5 post primary infection. Body weights were monitored once daily and mice were humanly euthanized when losing 20% or more of their original weights.

### 3.3.3 Viral load assay in nasal wash samples and lung tissue

Ferret nasal wash samples and mouse lung tissue were collected post H1N1pdm infection (p.i.). Dissected lung tissue was homogenized in DMEM by 1:10 (W/V) and then centrifuged at 3000 rpm for 15 minutes to collect the supernatant. Viral load in nasal wash and lung homogenate samples was determined by using a 6-day MDCK cell-culture based assay as described in our previous study (Fang et al., 2010). The virus titer expressed as TCID$_{50}$/ml from each nasal wash sample was calculated by Reed-Muench method.
3.3.4 Histopathology

Lung tissue collected from the infected animals was perfused and fixed in 10% formalin and then paraffin embedded. Tissue section was placed on positive charged slide and stained with hematoxylin and eosin (H&E) for histopathologic examination.

3.3.5 Haemagglutination inhibition (HI) test

Serum samples were treated with receptor destroying enzyme (RDE) at 37°C overnight. Fresh turkey red blood cells (TRBC) were washed and diluted in PBS to a concentration of 0.5% (v/v). The sera were serially diluted in PBS in 96-well V bottom cell culture plates. The serial diluted sera were incubated with 25 µl (8HA units/50 µl) virus for 15 minutes. Then, 50 µl of 0.5% TRBC was added and the plates were incubated at room temperature for 30 minutes. The HI titer was the reciprocal of the highest serum dilution to completely prevent agglutination.

3.3.6 Microneutralization assay

The serum neutralizing antibodies were determined using the H1N1pdm virus A/Mexico/4108/2009 (H1N1) by microneutralization (MN) assay described previously (Fang et al., 2010). Briefly, the 50% tissue culture infectious dose (TCID50) of each virus was determined by titration in MDCK cells under bio-safety level 2 conditions. The serially 2-fold diluted RDE treated serum at a starting dilution of 1:10 was tested for neutralizing the 100 TCID50/50 µl of each virus in MDCK cell monolayer. The cytopathic effect was read after incubation for 20 hours.

3.3.7 ELISA assay

Serum and lung homogenate samples were assessed for antibody level by ELISA assay. Briefly, ELISA plates were coated with inactivated split pandemic H1N1 A/California/07/2009 viron (5 µg/ml of HA), recombinant H1N1 (seasonal H1N1 A/Bribane/59/2007, pandemic H1N1 A/California/07/2009; Sino Biological Inc., China) HA (5 µg/ml) or recombinant H1N1 (A/Puerto Rico/8/34) NP (5 µg/ml) overnight at 4°C. Plates were washed with PBS containing 0.05% Tween 20 (T-PBS) and blocked with 1% bovine serum albumin (BSA) for one hour at 37°C. Antigen-coated plates were washed with T-PBS and incubated with 1:1,000 diluted samples overnight at 4°C. After washing with T-PBS, plates were incubated with goat anti-mouse immunoglobulin (IgG, IgG1, IgG2a and IgA) HRP conjugates (Santa Cruz) in a 1:2000
dilution for one hour at 37°C. The reaction was developed by o-phenylenediamine and the optical density was read by excitation and emission wavelengths of 490nm and 570nm, respectively. The IgG1 and IgG2a standard curves generated by plotting the density readout and the serially diluted amount of protein standard were used for calculating the concentration of samples.

3.3.8 Western blot

The boiled recombinant sH1N1 HA, H1N1pdm HA, conserved H1N1 NP and H1N1pdm M1 (A/California/04/2009; Immune Technology Corp) protein was equally loaded (0.5µg/lane) and run through 12% SDS-PAGE (polyacrylamide gel electrophoresis). The gels were subsequently transferred onto nitrocellulose membrane for 1 hour at 100V. The membrane was blocked with 5% milk-TPBS for 45 minutes at room temperature and then incubated with 1:1000 diluted serum samples (developed by Kelvin lab) overnight at 4°C followed by HRP conjugated goat anti-mouse secondary antibody incubation. Bands were developed with BM chemiluminescence western blotting substrate kit (Roche Diagnostics, Laval, Quebec) and visualized using Kodak film developer.

3.3.9 Enrichment of lymphocytes from lung and spleen

Methods have been described in previously published studies (Lin et al., 2007; Lin et al., 2010). In brief, after lung tissue was collected, it was cut into small pieces and then mechanically homogenized into RPMI 1640 medium. The suspension was passed through 40µm nylon mesh filter to remove major tissue fragments and fibroblasts. The filtered clear suspension was placed onto a mouse Lympholyte (Cedarlane) layer and centrifuged at 1600 rpm for 20 minutes. The lymphocyte band was collected and washed three times with PBS before using.

Lymphocytes from spleen were also enriched in a mechanical way. The filtered cell suspension was treated with red blood cell lysis buffer and then washed three times with PBS to generate a single cell suspension.

3.3.10 Flow cytometry analysis

Lung lymphocytes and splenocytes isolated post H1N1pdm infection were analyzed for T cell and B cell activation and/or memory phenotype by staining with mouse anti-CD3, anti-CD8, anti-CD44, anti-CD19, anti-CD69 and anti-CD27 antibodies (eBioscience). After staining, the
samples were run through a BD FACS Calibur (BD Biosciences) and the data was analyzed by FlowJo software (TreeStar).

3.3.11 Lymphocyte in-vitro stimulation and intracellular staining

For IFN-γ intracellular staining, isolated lung lymphocyte and splenocyte at the concentration of 1 x 10^6 cells/ml were cultured with live virus A/Mexico/4108/2009 or A/Brisbane/59/2007 in complete RPMI 1640 medium at 37°C and 5% CO2 for 10 hours. To minimize the indirect manner of IFN-γ stimulation by live virus, the low MOI number (MOI = 0.1) of viruses was used for the ex vivo cell co-culture. After stimulation, cells were washed by 1 x PBS and stained with mouse anti-CD3 and anti-CD8 antibody. After surface staining, cells were fixed and permeabilized before staining with IFN-gamma antibody. Samples were run on a BD FACS Calibur (BD Biosciences) and the data was analyzed by FlowJo software (TreeStar).

3.3.12 Analysis and comparison of B cell epitope sequences

Predicted B cell linear epitopes toward haemagglutine (HA), neuraminidase (NA), nucleoprotein (NP) and matrix protein 1 (M1) of A/Briabane/59/2007 and A/Mexico/4108/2009 were generated by using Kolaskar and Tongaonkar antigenicity scale (Kolaskar & Tongaonkar, 1990) from the IEDB analysis resources (http://tools.immuneepitope.org/main/). Similarity of predicted B cell epitope sequences of influenza antigens from two influenza viruses were evaluated by IEBD epitope conservancy analysis tool.

3.3.13 Serum passive transfer

Ferret serum was collected 14 days after infection with A/Brisbane/59/2007 or A/Mexico/4108/2009; the presence of moderate to high HI titers against the same virus strain was confirmed to assure the validity of the infections. Also, serum collected from naïve ferrets was used as negative control. Naïve mice received 200 μl of serum by intraperitoneal injection daily from day -3 to day 0 before infection with 10^5 EID_{50} of A/Mexico/4108/2009. After infection, mice were monitored daily for weight loss and lethality.
3.3.14 Statistics

Student’s t-test and one-way ANOVA were used for statistical analysis of the results. Log-rank (Mantel-Cox) test was used to analyze the difference among survival curves. A P value of ≤ 0.05 was considered as significant.

3.4 RESULTS

3.4.1 Live infection with sH1N1 protected from subsequent H1N1pdm challenge in mice and ferrets

In order to investigate whether a prior infection with sH1N1 is able to induce protection against a secondary challenge with H1N1pdm, ferrets were infected with $10^6$ EID$_{50}$ of sH1N1 A/Brisbane/59/2007 (Brisbane/59) and subsequently challenged with $10^6$ EID$_{50}$ of H1N1pdm A/Mexico/4108 (Mexico/4108). Ferrets infected with sH1N1 only developed mild clinical symptoms. Ferrets showed increased temperature only at day 2 post infection and a maximum weight loss of 3.9%; the clinical findings were very similar to those shown in a previous work published by our group, which included a more extensive pathological evaluation of ferrets infected with A/Brisbane/59/2007 (Rowe et al., 2010). In this study, serological assays demonstrated that Brisbane/59 virus primary infected animals had neither cross-reactive HI titers nor cross-neutralizing antibodies against H1N1pdm virus before challenge (Fig. 3.1 A, B). Nonetheless, we found that the primary infection with sH1N1 in ferrets conferred robust protection against a secondary infection with either H1N1pdm Mexico/4108 or A/California/07/2009 (Fig. 3.1 C, D; see supplementary material in Appendix 3.1). The immunity induced by Brisbane/59 infection dramatically reduced the disease severity in ferrets, in terms of weight loss, temperature change, viral load and lung pathology (Fig. 3.1 C-E; see supplementary material in Appendix 3.2). Furthermore, we demonstrated the same protective effect in C57BL/6 mice. Mice infected with $10^6$ EID$_{50}$ of Brisbane/59 showed slight weight loss of maximal 3.1% without lethality, and during a second challenge with $10^5$ EID$_{50}$ of Mexico/4108 a significant reduction in the disease severity was observed. sH1N1 primary infected mice showed a peak of 12% weight loss on day 4 post infection with no mortality (Fig. 3.2 A, B), while the H1N1pdm-only infection group showed 95% mortality, significantly higher viral titers in lungs and also extensive lung pathology featured by acute alveolitis and bronchiolitis (Fig. 2 A-C; Appendix 3.2). Taken together, these results demonstrate that prior
infection with sH1N1 confers protection against H1N1pdm. On the other hand, we found that immunization with a seasonal vaccine was of little value in providing cross protection against H1N1pdm (see supplementary material in Appendix 3.3), confirming previous results (Ellebedy et al., 2011).

Figure 3.1 (Legend is on next page)
Figure 3.1 Evaluation of antibody response, clinical signs and viral titer for seasonal H1N1 primary infected ferrets following secondary infection with A/Mexico/4108/2009.

Ferrets were primary infected with $10^6$ EID$_{50}$ of A/Brisbane/59/2007 or left uninfected as control. Purified serum was treated by RDE at 37°C overnight before HI and microneutralization assays. (A) Antibody titers against H1N1pdm virus in A/Brisbane/59/2007 infected ferret (Bris/59+Mex/4108) serum as well as uninfected ferret serum samples (n=4) collected at day 0 and day 14 post H1N1pdm secondary infection were measured by HI assay. (B) Neutralizing antibody titers for blocking H1N1pdm virus were measured by microneutralization assay in the same serum samples investigated by HI test. (C) Weight loss and (D) Body temperature change were determined daily for the respective groups (n=8/group) after the secondary infection with $10^6$ EID$_{50}$ doses of A/Mexico/4108/2009. Data points represent the mean value and error bars demonstrate standard error of the mean. The number of HI titers and neutralizing antibody titers shown in the figure was log2 transformed from the original data. Student t test was utilized for statistical analysis. Viral load in (E) ferret nasal wash samples (n=4) collected at day 3 post H1N1pdm infection was determined. Horizontal bars demonstrate mean value. Student t test was performed for statistical analysis. *: $p \leq 0.05$, ***: $p \leq 0.001$; LOD: limit of detection.
Figure 3.2 Assessment of infection outcome and viral titer for seasonal H1N1 primary infected mice following secondary infection with A/Mexico/4108/2009.

C57BL/6 mice were primary infected with $10^6$ EID$_{50}$ of A/Brisbane/59/2007 or left uninfected as control. Five weeks later, mice were rechallenged with $10^5$ EID$_{50}$ of A/Mexico/4108/2009. (A) Weight loss and (B) lethality of A/Brisbane/59/2007 infected C57BL/6 mice and uninfected C57BL/6 mice (n=20/group) were monitored post H1N1pdm secondary infection. Comparison of survival curves was analyzed by Log-rank (Mantel-Cox) test. Only one mouse (n=1) survived in primary uninfected group mice post H1N1pdm infection. Viral load in (C) mice lung tissue samples (n=3) collected at day 4 post H1N1pdm challenge was determined. Horizontal bars demonstrate mean value. Student t test was performed for statistical analysis. ***: $p \leq 0.001$; LOD: limit of detection in viral load assay.
3.4.2 Cross-reactive but non-HA antibodies against H1N1pdm virus were induced by sH1N1 infection

We next evaluated the antibody responses that were generated after influenza infection in mice. First, HI titers showed that antibodies induced by Brisbane/59 infection were not cross-reactive with Mexico/4108 (Fig. 3.3 A). The western-blot analysis also showed that anti-HA IgG1 and IgG2a antibodies are influenza strain-specific; Brisbane/59-infected animals showed only anti-Brisbane HA antibodies, while Mexico/4108-infected animals showed only anti-H1N1pdm HA antibodies (Fig. 3.3 B). Although anti-Brisbane HA antibodies were highly increased in Brisbane/59 primary infected mice at day 4 after heterologous virus challenge (Fig. 3B), no antibodies against H1N1pdm HA were seen at this time point (Fig. 3.3 A, B). These results confirm that neutralizing antibodies against the external influenza antigens HA are highly specific against closely-related influenza strains (Dormitzer et al., 2011).
Figure 3.3 Evaluation of cross reactive activity of antibodies induced by human seasonal H1N1 virus in mice.

Mouse anti-H1N1pdm sera (collected at day 14 post a sublethal infection with A/Mexico/4108/2009), anti-Brisbane/59 sera, sH1N1 primary infected mice sera collected at day 4 post H1N1pdm challenge (Bris/59+Mex/4108 day 4 P.R.) and primary uninfected mice sera collected at day 4 post H1N1pdm challenge (naive+Mex/4108 day 4 P.R.) were assessed for antibody responses. (A) HI test was performed for assessing Brisbane/59 or H1N1pdm specific HI titers. The number of HI titers shown in the figure was log2 transformed from the original data. (B) Mice sera were evaluated for cross-reactive IgG1 and IgG2a antibodies against recombinant sH1N1 HA and H1N1pdm HA by western. Cross-reactive IgG1 and IgG2a antibodies against (C) split H1N1pdm and (D) recombinant H1N1 NP (A/Puerto Rico/8/34) were analyzed by ELISA assay. (E) Isotype IgG antibodies against H1N1 NP in mice sera were shown by western blot. (F) Total IgG antibodies against recombinant H1N1pdm (A/California/04/2009) M1 (p-M1) protein in mice sera were evaluated by western blot. The average relative absorbance density from three individual samples was read by excitation and emission wavelengths of 490nm and 570nm, respectively. The concentration of IgG isotype antibodies were then calculated based on the linear standard curve. The result of a representative sample was shown in
the western blot. Student t test was performed for statistical analysis. ND: not detected; NS: not significant *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001; LOD: limit of detection in HI assay.

Antibodies targeting the internal viral proteins also represent a major component of the antibody repertoires (Greenbaum et al., 2009). Given that different putative B cell epitopes of the internal proteins are highly conserved between Brisbane/59 and Mexico/4108 (see supplementary material in Appendix 3.4), we analyzed the presence of cross-reactive antibodies against split H1N1pdm virus by ELISA. We found that cross-reactive IgG1 and IgG2a antibodies against split H1N1pdm virus were elevated in Brisbane/59-infected mice (Fig. 3.3 C), and it was after homologous infection when higher antibody titers were observed (Fig. 3.3 C). Moreover, IgG1 antibodies against split H1N1pdm virus in Brisbane/59 primary infected mice were significantly increased at day 4 post H1N1pdm secondary infection (Fig. 3.3 C). On the other hand, the viral nucleoprotein (NP) also showed high antigenic similarity between sH1N1 and H1N1pdm (Appendix 3.4); detection of anti-NP antibodies in serum was carried out by using recombinant NP from PR8 virus, which shows 91% similarity with NP from H1N1pdm. ELISA and western blot analyses showed that anti-NP IgG1 and IgG2a antibodies were induced during the infection with either Brisbane/59 or Mexico/4108 virus (Fig. 3.3 D, E), and they were preferentially stimulated towards the IgG1 isotype (Fig. 3.3 D, E). Next, we examined the presence of antibodies targeted against the M1 antigen by using recombinant M1 protein from H1N1pdm A/California/04/2009. Serum from Brisbane/59 infected mice showed less than one half of the band intensity as compared with serum from H1N1pdm infected mice, suggesting that anti-M1 IgG antibodies are only partially cross-reactive between sH1N1 and H1N1pdm (Fig. 3F). Taken together, the presence of antibodies reactive against split H1N1pdm virus, viral NP and also viral M1 to a lesser extent, prove that the primary infection with Brisbane/59 induce the production of cross-reactive antibodies against the conserved viral antigens; these cross-reactive antibodies may partially account for the in vivo protection that is induced by a previous exposure to sH1N1 (Fig. 3.2). Although HI titers and levels of neutralizing antibodies are still useful indicators of specific immunity against influenza, the measurement of the cross-reactive antibodies against the internal viral antigens may provide a global picture of the overall cross-protective immunity during a heterologous virus infection.

The levels of specific antibodies in the infected tissues correlate well with the level of protection against secondary infections (Ito et al., 2003). Upon a heterologous infection, we were
able to detect the presence of cross-reactive antibodies in the lungs, showing an expression pattern similar to the one found in the sera (see supplementary material in Appendix 3.5), except for the higher levels of IgA antibodies that were detected at the site of infection. These results are consistent with a role that local antibody responses play during the resolution of influenza infections (Ito et al., 2003).

3.4.3 Cross-responsive CD8+ T cells and B cells were induced by sH1N1 infection

Apart from inducing high titers of cross-reactive antibodies, prior infection with influenza virus Brisbane/59 also influenced the cellular response kinetics during the subsequent heterologous infection with Mexico/4108. Cross-reactive memory/effector CD8⁺CD44^high T cells as well as CD8⁺IFN-γ⁺ T cells were significantly increased in enriched lung lymphocytes (Fig. 3.4 A, B) and splenocytes (data not shown) of Brisbane/59 primary infected mice during H1N1pdm rechallenge. We also observed significantly increased percentages of activated B cells (CD19⁺CD69⁺) and memory B cells (CD19⁺CD27⁺) in the lungs of primary infected mice which showed limited viral shedding at day 4 post H1N1pdm infection (Fig. 3.4 C, D; Fig. 3.2 C), nevertheless, it is possible that the higher levels of B cells observed during the reinfection may be due partially to remaining cells that were induced during the primary infection. Taken collectively, these results indicate that sH1N1 primary infection induces cross-reactive memory T cells and B cells, resulting in enhanced local immune responses during the second infection.
Figure 3.4 Evaluation of cross responsive CD8 T cells and B cells in lungs of seasonal H1N1 primary infected mice following H1N1pdm rechallenge.

Lymphocytes were enriched from seasonal virus primary infected and uninfected mice lung tissue (n=3/group) at day 4 and day 7 post H1N1pdm secondary infection. (A) Analyses of memory/effector phenotype CD8 T cell (CD8^+CD44^high) response in mouse lung lymphocytes by flow cytometry. (B) Antigen specific CD8^+IFN-γ^+ T cell in mouse lung was determined by restimulating the enriched lung mononuclear cells (day 7) with the sH1N1 or H1N1pdm virus at MOI = 0.1, and the percentage of the CD8^+IFN-γ^+ T cell was analyzed by flow cytometry (dot plot figures of a representative sample are shown in upper panel); the total number of antigen specific CD8^+IFN-γ^+ T cell in mouse lung lymphocytes is shown in the lower panel. (C) Percentages of activated B cells (CD19^+CD69^+) and (D) memory phenotype B cells.
(CD19^+CD27^+) in lungs collected from primary infected and uninfected mice (n=3/group) at day 4 post H1N1pdm secondary infection were measured by Flow cytometry analyses. Student t test was utilized for statistical analysis. *: p ≤ 0.05, **: p ≤ 0.01.

3.4.4 B cells but not CD8^+ T cells were required in sH1N1 infection-induced cross-protection

In order to further evaluate the importance of B cell and cytotoxic T cell responses in heterotypic immunity, B cell deficient mice (µMT), CD8 deficient and Prf1 deficient mice were infected first with Brisbane/59 and five weeks later they were challenged with Mexico/4108. As mentioned above, primary infection with Brisbane/59 conferred immunological protection in wild-type mice during heterologous reinfection with Mexico/4108 (Fig. 3.2). However, B cell deficient mice showed 90% lethality, as well as a high level of lung viral titers similar to those found in the group of naïve mice infected with Mexico/4108 (Fig. 3.5 A). This result indicates that the protection induced by Brisbane/59 primary infection significantly relies on the presence of B lymphocytes. Interestingly, CD8 deficient and wild-type mice infected with sH1N1 showed a similar pattern of weight loss during Mexico/4108 reinfection (data not shown) and 100% of the CD8 deficient mice survived from H1N1pdm rechallenge (Fig. 3.5 B). Therefore, the lack of CD8 T cells is not detrimental for the induction of cross-protective immunity (Fig. 3.5 D). Moreover, we found that cross-protective immunity was not affected by the impairment of granzyme/perforin-mediated cytotoxic response. During secondary infection with Mexico/4108, both Prf1-deficient and wild-type mice primary infected with Brisbane/59 showed 100% survival and had similar levels of viral shedding in lungs (Fig. 3.5 C, D).

3.4.5 Adoptive transfer of cross-reactive but non-neutralizing antibodies promoted survival in naïve mice infected with H1N1pdm infection

In order to assess the capacity of pre-existing antibodies to confer protection during a heterologous rechallenge, serum from naïve ferrets or previously infected with sH1N1 or H1N1pdm, was passively transferred to naïve mice from day -3 to day 0 before H1N1pdm infection. As expected, the serum from naïve ferrets was unable to alter the course of the infection in mice (Table 3.1). Ferret anti-Mexico/4108 serum was able to confer protection in naïve mice against H1N1pdm infection without showing any significant weight loss (Table 3.1). Interestingly, the ferret anti-Brisbane/59 serum, which showed no cross-reactive HI titers against H1N1pdm virus, was capable of significantly reducing weight loss and resulted in the rescue of
Figure 3.5 Mortality and viral titer assessment of primary infected gene deleted mice post H1N1pdm rechallenge.

Mice were primary infected with $10^6$ EID$_{50}$ of A/Brisbane/50/2007 virus and five weeks later challenged with $10^5$ EID$_{50}$ of A/Mexico/4108 virus. Lethality of (A) primary infected B cell deficient (µ MT) mic, (B) primary infected CD8 T cell deficient (CD8$^-$) mice and (C) primary infected perforin-1 deficient (Prf1$^{-/-}$) mice (n=20/group) were monitored post H1N1pdm secondary infection. Primary infected wild-type (WT) C57BL/6 mice and primary uninfected WT C57BL/6 mice (n=20/group) were used as positive and negative controls in the study, respectively. Comparison of survival curves was analyzed by Log-rank (Mantel-Cox) test. (D) Viral titer in lungs (n=3) collected at day 4 post H1N1pdm secondary infection was determined.
The virus titer was calculated by Reed-Muench method and expressed as TCID$_{50}$/ml. Horizontal bars demonstrate mean value. One-way ANOVA was performed for statistical analysis. **: $p \leq 0.01$, ***: $p \leq 0.001$; LOD: limit of detection in viral load assay.

90% of the naïve recipient mice from H1N1pdm infection (Table 3.1). These results confirm that high levels of neutralizing antibodies are required to achieve the maximum level of protection, but at the same time, the cross-reactive non-HA, and possibly non-neutralizing antibodies can also play an important protective role in the absence of HI titers (Lamere et al., 2011a).

<table>
<thead>
<tr>
<th>Donor Serum</th>
<th>HI titer against sH1N1 in donor serum</th>
<th>HI titer against pdmH1N1 in donor serum</th>
<th>% Weight loss (day) of treated mice</th>
<th>Lethality (%) in treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferret anti-Mex/4108</td>
<td>&lt;10</td>
<td>1280</td>
<td>0.6 (D6)</td>
<td>0***</td>
</tr>
<tr>
<td>Ferret anti-Bris/59</td>
<td>1280</td>
<td>&lt;10</td>
<td>8.2 (D7)</td>
<td>10***</td>
</tr>
<tr>
<td>Ferret naïve</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>22.9 (D9)</td>
<td>100</td>
</tr>
</tbody>
</table>

$a$ Ferret anti-influenza serum was intraperitoneally injected into naïve mice (10 mice/group) daily from day -3 to day 0 before infection with 10$^5$ EID$_{50}$ of A/Mexico/4108/2009.

*b Day post challenge of peak weight loss from original weight

***Log-Rank sum test respect the group treated with serum from naïve ferret, $p < 0.001$

### 3.5 Discussion

Although many studies have tried to establish the relative contribution of antibodies and cytotoxic responses in the immunity against influenza infection, the answer is still not clear-cut and the experimental variables of each study need to be carefully considered. In the context of heterologous immunity, our results showed that efficient antibody production was required to achieve protection in mice, this protection was not ameliorated in CD8 deficient or perforin deficient mice and passive transfer serum was able to confer heterologous protection. An analogous scenario was observed by Nguyen et al. in the context of heterosubtypic immunity (first H3N1 and later H1N1), where protection was ameliorated in antibody deficient mice but not in CD8 deficient mice (Nguyen et al., 2001). On the other hand, Guo et al. found that heterosubtypic immunity was strongly dependent on the presence of CD8 cells in their experimental model in which passive serum transfer was unable to confer protection (Guo et al., 2010). While keeping in mind the contribution of influenza-specific cytotoxic CD8 cells during influenza re-challenge (Hillaire et al., 2011), we hypothesize that cytotoxic CD8 cells may be dispensable in those scenarios where the antibody response meets certain levels of strength and
specificity and it is able to limit the infection on its own. It is reasonable to think that vaccines induce a less efficient antibody response as compared with a proper influenza infection, and this would explain why vaccine-induced immunity relies more heavily on the cytotoxic response (Ellebedy et al., 2010; Sambhara et al., 2001).

Influenza infection induces the production of antibodies against most of the 10 viral proteins (Sealy et al., 2003), however, it is the subset of neutralizing anti-HA antibodies that exerts the largest contribution to block the virus entry to the cells. Due to the significant antigenic dissimilarity between the HA protein from seasonal and pandemic H1N1, the neutralizing antibodies induced by the seasonal vaccination or prior infection are unable to accomplish their mission against H1N1pdm (Laurie et al., 2010; Janjua et al., 2010). The HA2 region of the HA protein possesses a relatively high degree of similarity among different influenza strains, and this fact has caused an increasing interest in HA2-based peptides for vaccine development (Wang et al., 2010). However, anti-HA2 antibodies represent a relatively small fraction of the neutralizing antibodies induced by seasonal vaccines (Corti et al., 2010). Moreover, a critical antigenic determinant on position 89 of the HA2 region from sH1N1, which had been present in the circulating influenza strains for decades, is no longer present in pandemic H1N1 and this has been suggested to be one of the causes of the antigenic escape of the new variants (Wang et al., 2011). Considering the results from these studies, it seems that the biological relevance of HA2-targeted antibodies in the context of a heterologous infection or vaccination is still unclear and it requires further study. Other approaches towards the design of “universal influenza vaccines”, effective against different influenza subtypes, are based on antigens derived from NP or M2e (Carragher et al., 2008; El Bakkouri et al., 2011; Lamere et al., 2011b). Unlike anti-HA neutralizing antibodies that act through direct blockage of the virus, the anti-NP and anti-M2e antibody mediated heterosubtypic immunity requires FcRs which are involved in phagocytosis and/or antibody-dependent cell-mediated cytotoxicity (El Bakkouri et al., 2011; Lamere et al., 2011a); therefore, it is likely that those antibodies relying on a receptor-dependent pathway also play a relevant role in the heterologous protection found in our experimental model.

Here, we have provided solid experimental evidence of the protective immunity that sH1N1 induces against a later challenge with H1N1pdm in both ferrets and mice. If humans behave immunologically in a similar fashion then prior infection with seasonal H1N1 likely
resulted in attenuated disease severity. This may partially explain the modest number of severe cases reported during the pandemic phase of the H1N1pdm outbreak. Our results favor an immunological model where B cells generate cross reactive non-HA and non-neutralizing antibodies that confer protection against H1N1pdm. We also explored the role of seasonal vaccines in providing cross protection and found, like others, that vaccination was of little value.

References


Chapter 4

Adjuvant induced attenuation of excessive inflammation in mice infected with pandemic H1N1 (H1N1pdm) virus

A version of this chapter is submitted to Journal of Virology as Adjuvant induced attenuation of excessive inflammation in mice infected with pandemic H1N1 (H1N1pdm) virus by Fang et al.
4.1 Summary

The reassorted human 2009 pandemic H1N1 (H1N1pdm) virus infected millions of people and caused thousands of deaths with associated immunopathology. Traditional immunosuppressants have limited capabilities in controlling inflammatory responses associated with severe influenza infections. A new therapeutic strategy, therefore, must be developed. We evaluated several adjuvants including toll-like receptor (TLR) agonists, TLR independent adjuvants and Complete Freund’s adjuvant (CFA) for limiting severe H1N1pdm infection outcomes in a mouse model. In contrast to the TLR agonists and TLR independent adjuvants, CFA which contains multiple pattern recognition receptor (PRR) agonists significantly restrained pro-inflammatory responses and promoted survival in mice from lethal H1N1pdm infection. CFA reduced expression of the plamacytoid dendritic cell (pDC) markers and interferon alpha (IFN-α) after infection, whereas it elevated the T regulatory (Treg) cell suppressive molecules galectin-1 and CTLA-4 expression. Consequently, Th1 cell differentiation and CD8+ effector T cell responses were diminished via downregulated myeloid DC (mDC) costimulation. Furthermore, CTLA-4 expressing Treg cells were dramatically increased when the CFA primed splenocytes were restimulated ex vivo with the adjuvant containing stimuli-killed mycobacterium tuberculosis (M. TB). The elevated CTLA-4 on Treg cells led to reduced CD86 expressing pDCs/mDCs and less CD4+ effector T cells. Overall, our study highlights that the stimuli of innate immunity potentially controls overactive host immune responses in certain situations and the uncovered mechanism(s) sheds light on the development of anti-influenza therapies.

4.2 Introduction

Influenza infection is a prominent threat to human health around the world and can cause severe morbidity and mortality in susceptible individuals due to acute respiratory disease. The factors associated with severe influenza symptoms include excessive cytokine expression, aberrant immune response and tissue damage (Osterholm, 2005). Failure of traditional methods for preventing influenza infection is result of highly frequent mutation of influenza virus. In particular, conventional vaccination has little value of protection in immunized population during influenza pandemic which is caused by the virus with antigenic reassortment (Anonymous, 2009a). Furthermore, the anti-viral drug neuraminidase inhibitor causes selection pressure on viral progeny which mutate to be drug resistant strains (Anonymous, 2009b; Garten et al., 2009).
Thus, it is necessary to explore an antigen independent treatment to regulate nonspecific immune response for limiting influenza caused disease.

Classical immunomodulators such as corticosteroids and Cox-2 inhibitor have been tested for preventing influenza caused immunopathology, because they inhibit inflammation and are less susceptible to virus mutation. Limited efficacy, however, has been shown by the treatment (Carter, 2007; Falagas et al., 2010). Recently, a published study has demonstrated that sphingnosine analog AAL-R, a chiral sphingosine analog of clinical drug FTY720 (flinglimod), provided significant protection against pandemic 2009 H1N1 (H1N1pdm) infection in mouse model (Walsh et al., 2011). AAL-R inhibited cytokine and chemokine expression to limit immunopathology and poor disease outcome. Furthermore, Norton et al. has found that prophylactic treatment with bacterially derived immunomodulator cholera toxin (CT) protected mice from lethal influenza challenge (Norton et al., 2010). Although CT induced an initial inflammatory process and augmented immune responses against influenza infection, it coincidently prevented the pulmonary chemokine expression which is usually involved in infection induced cytokine storm and exacerbates disease outcome (La Gruta et al., 2007). On the other hand, T regulatory (Treg) cells are well characterized to play a vital role in immune suppression that controls effector T cell responses and cytokine/chemokine production (Shevach, 2009). In a murine model of respiratory syncytial virus (RSV) infection, depletion of Treg cells caused severe infection outcomes with increased weight loss/mortality and exacerbated inflammation due to altered CD8 T cell kinetics (Fulton et al., 2010). Additionally, a recent study has shown that Treg cells are capable of reducing effector T cell responses and cytokine expression during in a non-lethal influenza mouse model (Betts et al., 2011b). Therefore, Treg cell mediated suppression plays a role in modulating immune responses in acute respiratory virus infection. A therapeutic strategy that regulates Treg cell responses for decreasing immunopathology may benefit the patients against severe influenza infection.

Pattern recognition receptors (PRRs) such as TLRs provide a crucial link between innate and adaptive immunity. TLR signaling induces interferon/cytokine expression by the innate immune cells and promotes maturation of antigen-presenting cells (APC) which subsequently enhance effector T cell responses (Iwasaki & Medzhitov, 2004). Some studies have shown that the agonists of TLR2, 3, 6 and 9 play a direct role in control of influenza infection in mice (Tuvim et al., 2012; Wong et al., 2009), however, the mechanism of this TLR mediated
protection is still unclear. Recently, increasing evidence has shown that stimulation with PRR ligands also directly regulates T cell responses in addition to their canonical role on APC (Caramalho et al., 2003; Gelman et al., 2004; Komai-Koma et al., 2004). It has been reported that a CD4 T cell subpopulation CD4+CD25+ T regulatory (Treg) cells express TLR1, 2, 4, 5, 7 and 8 (Sutmuller et al., 2007). Lipopolysaccharide (LPS) which binds to TLR4 enhanced suppressive capacity of Treg cells in certain circumstances (Caramalho et al., 2003). In addition, stimulation with TLR5 ligand flagellin increased Foxp3 expression in Treg cells and promotes immune suppression on effector T cells in vitro (Crellin et al., 2005). Thus, PPR ligands may be able to regulate adaptive immunity in controlling aberrant activation of inflammatory responses during pathogenic influenza infection. Furthermore, PPR agonists are potential to provide broad protection against different influenza strains because they are not susceptible to virus mutation.

In this study, we have assessed the protective roles of TLR agonists, TLR independent adjuvants and Complete Freund’s adjuvant (CFA) in the context of severe H1N1pdm infection in mice. Our results demonstrated that treatment with poly i:c (TLR3), LPS (TLR4), flagellin (TLR5) or CpG ODN (TLR9) was limited in controlling the lethal H1N1pdm infection. Also, we observed that TLR independent adjuvant MF59 (squalene), aluminum and Incomplete Freund’s adjuvant (IFA) were unable to enhance survival in infected mice. Interestingly, CFA which contains IFA and killed mycobacterium tuberculosis (M. TB) provided substantial protection against H1N1pdm virus in mice. It was determined that CFA treatment or killed M. TB stimulation induced the expression of Treg cell suppressive molecules, blunted dendritic cell (DC) maturation, and downregulated effector T cell responses for producing pro-inflammatory cytokines. Given that CFA or killed M. TB is enriched with PRR agonists including NOD2 ligand muramyl dipeptide (MDP) and possible TLR2/4 agonists, our findings suggest that PRR ligands have the potential to regulate host immunity during severe influenza infection.

4.3 Materials and Methods

4.3.1 Animals and virus

Pandemic virus A/Mexico/4108/2009 was obtained from the US Centers for Disease control (CDC, Atlanta) and grown in the allantoic cavity of 10-day old embryonated chiken eggs. The viral titer expressed as EID<sub>50</sub>/ml was calculated by serially titrating the virus on chicken eggs per the Reed-Muench method. Pathogen free C57BL/6 mice (8-10 weeks of age) were
purchased from Jackson Laboratory (Bar Harbor, ME). Experiments with mice were conducted at the Animal Resources Centre of University Health Network (UHN, Toronto, Canada) under ABSL-2+ conditions and in accordance with the Canadian Council of Animal Care (CCAC) guidelines. To determine the 50% Mouse lethal dose (MLD$_{50}$), 4 groups of mice (n = 5/group) were inoculated intranasally with serial dilutions that contain $10^3$ to $10^6$ EID$_{50}$ A/Mexico/4108/2009 virus in 50 µl. Body weights were monitored once daily and mice were humanely euthanized when losing 20% or more of their original weights. The MLD$_{50}$ was calculated by using the Reed-Muench method. Mice were infected with $10^4$ EID$_{50}$ (3.2 MLD$_{50}$) virus with treatment of different TLR agonists and adjuvants. The tissue analysis of CFA treated mice was performed in context of $10^4$ EID$_{50}$ infection. Also, the CFA treated mice were inoculated with $10^5$ EID$_{50}$ (32 MLD$_{50}$) virus to validate its protective effect in a more lethal model. Our mouse model has been described in the previously published studies which included a more extensive pathological evaluation of mice infected with A/Mexico/2009/4108 (Fang et al., 2012; Paquette et al., 2012; Rowe et al., 2010).

4.3.2 PRR ligand/adjuvant treatment and pandemic H1N1 infection

In brief, 1mg/kg of CpG ODN (Operon), lipopolysaccharide (LPS, Sigma), flagellin (Enzo) and poly (i:c) (Sigma); 50 µl of MF59 (squalene, Sigma), aluminum hydroxide (alum, Sigma), Incomplete Freund’s adjuvant (IFA, Sigma) and Complete Freund’s adjuvant (CFA, Sigma) were mixed with PBS for a total volume of 100 µl, respectively. Mice were administered intramuscularly (i.m.) in each quadriiceps with 50 µl. Two days after first administration, mice were infected by $10^4$ EID$_{50}$ of A/Mexico/4108/2009. At day 2 post infection, another administration was performed. Body weights were monitored once daily and mice were humanely euthanized when they lost 20% or more of their original weights.

4.3.3 Viral load assay in nasal wash samples and lung tissue

Mouse lung tissue samples were collected post pdmH1N1 infection (p.i.) and homogenized in DMEM by 1:10 (W/V) and then centrifuged at 3000 rpm for 15 minutes to collect the supernatant. Viral load in the lung homogenate samples was determined by using a 6-day MDCK cell-culture based assay as described in our previous study (Fang et al., 2010). The virus titer expressed as TCID$_{50}$/ml from each sample was calculated by Reed-Muench method.
4.3.4 Histopathology

Lung tissue collected from the infected animals was perfused and fixed in 10% formalin and then paraffin embedded. Tissue sections were placed on positive charged slide and stained with hematoxylin and eosin (H&E) for histopathologic examination.

4.3.5 Haemagglutination inhibition (HI) test

Serum samples were treated with receptor destroying enzyme (RDE) at 37°C overnight. Fresh turkey red blood cells (TRBC) were washed and diluted in PBS to a concentration of 0.5% (v/v). The sera were serially diluted in PBS in 96-well V bottom cell culture plates. The serial diluted sera were incubated with 25 μl (8HA units/50 μl) virus for 15 minutes. Then, 50 μl of 0.5% TRBC was added and the plates were incubated at room temperature for 30 minutes. The HI titer was the reciprocal of the highest serum dilution to completely prevent agglutination.

4.3.6 RNA extraction and microarray analysis

Mouse lung tissue was homogenized in Trizol and RNA was extracted by using isopropanol and chloroform following the manufacture’s instruction (Invitrogen). The isolated RNA was then analyzed by microarrays. Briefly, cRNA was prepared from 500 ng mouse lung total RNA by two-cycle cRNA synthesis according to the manufacturer’s protocol (Illumina). cRNA samples (20 μg) were labeled and hybridized to Illumina mouse WG-6v 2.0 expression chip. The arrays were scanned using an Illumina BeadStation 500GX system according to standard Illumina protocols. Raw data collection was performed using Illumina GenomeStudio V2010.3 software. The datasets of each individual target on the Illumina chips were pre-processed with variance stabilization, log2-transformation and were normalized against the PBS (uninfected) control group with quantile normalization. T-test was performed with Benjamin-Hochberg false discovery rate (FDR) correction. Genes with a statistical difference ($p \leq 0.05$) between CFA and vehicle groups were selected for agglomerative hierarchical clustering with Pearson distance metrics and average linkage distance measurements between clusters using MultiExperimental Viewer 4.1. To annotate the significantly distinguished genes ($p \leq 0.05$; fold change $\geq 1.5$ or $\leq -1.5$) identified in the CFA group when compared to the vehicle control, DAVID Bioinformatics Resources (NIAID) were utilized to subgroup the genes associated with multiple biological functions by the enrichment score. Additionally, Ingenuity Pathway Analysis (IPA) 5.0 software (Ingenuity Systems Inc.) was used to annotate and organize the gene
expression data into networks and pathways. The microarray data has been submitted to the NCBI GEO dataset. The accession number is GSE41088.

4.3.7 Enrichment of mononuclear cells from lung and spleen

Methods have been described in previously published studies (Lin et al., 2007; Lin et al., 2010). In brief, after lung tissue was collected, it was cut into small pieces and then gently homogenized into RPMI 1640 medium. The suspension was passed through 40µm nylon mesh filter to remove major tissue fragments and fibroblasts. The filtered clear suspension was placed onto a mouse Lympholyte (Cedarlane) layer and centrifuged at 1600 rpm for 20 minutes. The lymphocyte band was collected and washed three times with PBS before using.

Lymphocytes from spleen were also enriched in a mechanical way. The filtered cell suspension was treated with red blood cell lysis buffer and then washed three times with PBS to generate a single cell suspension.

4.3.8 Ex vivo stimulation

In the ex vivo restimulation experiment, 1 x 10^6 cells/ml of splenocytes isolated from CFA treated mice at day 4 post administration were restimulated with or without killed M. TB at 5µg/ml in complete RPMI 1640 medium at 37°C and 5% CO₂ for 18 hours. Then, cultured splenocytes were collected and washed for the following FACS analysis.

4.3.9 Flow cytometry analysis

Lung lymphocytes and splenocytes isolated post pdmH1N1 infection or ex vivo stimulated splenocytes were analyzed for T cell response and DC costimulation respectively by staining with mouse anti-CD3 (Percp-Cy5.5), anti-CD8 (Pacific blue), anti-CD4 (APC-Cy7), anti-CD25 (PE-Cy7), anti-CTLA4 (APC) and anti-CD44 (FITC); anti-Gr1 (Pacific blue), anti-F4/80 (PE-Cy7), anti-CD11b (APC-Cy7), anti-CD11c (FITC), anti-CD86 (APC) and anti-CD69 (Percp-Cy5.5) antibodies (eBioscience). For Foxp3 (PE) intracellular staining, cells were fixed and permeabilized before staining with anti-Foxp3 antibody. All samples were run through a BD FACS CANTOII Flow Cytometer (BD Biosciences) and the data was analyzed by FlowJo software (TreeStar).
4.3.10 Statistics

The student’s *t*-test was used for statistical analysis of the results which has been specifically described in each figure legend. Log-rank (Mantel-Cox) test was used to analyze the difference among survival curves. A *P* value of ≤ 0.05 was considered significant.

4.4 Results

4.4.1 Treatment with CFA improves infection outcome caused by H1N1pdm

In order to investigate the direct function of TLR agonists and adjuvants for preventing H1N1pdm infection, several TLR ligands, adjuvants and vehicle (PBS) were administered intramuscularly (i.m.) into C57B6/J mice at day -2 and day 2 after 10⁴ EID50 of A/Mexico/4108/2009 infection (p.i.). The doses of TLR agonists were chosen based on the previously published studies which have shown treatment with TLR agonist successfully prevented non-H1N1pdm influenza virus or other viral infections in mouse models (Li *et al*., 2011; Norton *et al*., 2010; Shinya *et al*., 2012; Vijay-Kumar *et al*., 2008). We found that the TLR agonists poly (i:c), LPS, flagellin, and CpG ODN were unable to limit H1N1pdm infection caused weight loss and lethality in mice when compared to the vehicle control group. Also, TLR independent adjuvant MF59, alum and IFA failed to promote survival in mice from the H1N1pdm infection (see supplementary material in Appendix 4.1). Interestingly, we observed that gold standard adjuvant CFA (IFA + M.TB) significantly restricted weight loss of the treated mice between day 4 and day 8 post infection when compared to the vehicle treated mice (Fig. 4.1 A). The weight change paradigm of CFA treated mice was distinguished from that of TLR agonists or TLR independent adjuvant treated mice which failed to survive after infection (please see the representative results of TLR agonists and adjuvants that are unsuccessful to delay weight loss of H1N1pdm infected mice in Appendix 4.2). Additionally, treatment with CFA dramatically increased survival in mice from the H1N1pdm infection than the vehicle control (Fig. 4.1 B). To verify that CFA is capable of protecting mice against H1N1pmd infection in a more lethal mode, we infected mice with 10⁵ EID₅₀ (32 MLD₅₀) virus. It was demonstrated that CFA significantly delayed weight loss and promoted survival in mice when compared to the vehicle control (please see in Appendix 4.3). These results indicate that CFA treatment is protective against a lethal influenza infection.
To assess whether CFA hampered the virus replication at site of infection, we evaluated viral titers in the lung tissues collected at day 3, day 5 and day 7 p.i. Interestingly, no significant differences of viral titer in the lung were observed between the CFA and vehicle treated mice (Fig. 4.1 C). In addition, we did not observe CFA treatment altered HI antibody responses in mice after infection (Fig. 4.1 D). Nonetheless, we found decreased number of infiltrated inflammatory cells and mild pathology in the lungs of CFA treated mice when compared to the vehicle control at day 5 p.i. (see supplementary material in Appendix 4.4). Taken together, our results suggest that CFA treatment may reduce severity of the outcome by limiting inflammatory responses during the course of infection.

**Figure 4.1 (Legend is on next page)**
Figure 4.1 Assessment of infection outcome, viral titer and HI titers for CFA treated mice following infection with A/Mexico/4108/2009.

C57BL/6 mice were treated with CFA and vehicle (PBS) at day -2 and day 2 post A/Mexico/4108/2009. (A) Weight loss and (B) lethality of CFA or vehicle treated C57BL/6 mice (n=15/group) were monitored post H1N1pdm infection. Weight changes between CFA and vehicle group of mice from day 4 to day 8 p.i. were compared by student t test. Comparison of survival curves was analyzed by Log-rank (Mantel-Cox) test. Two mice survived in vehicle group of mice post H1N1pdm infection. Viral load in (C) mouse lung tissue samples collected at day 3, day 5 and day 7 (n=3/group) after H1N1pdm challenge was determined; horizontal bars demonstrate mean value. (D) Mouse sera collected at day 5, day 7 and day 14 were analyzed for HI titers against A/Mexico/4108/2009 virus. The number of HI titers shown in the figure was log2 transformed from the original data. *: p ≤ 0.05, **: p ≤ 0.01; LOD: limit of detection in viral load assay.

4.4.2 CFA treatment limits expression of the genes involved in host inflammatory responses during H1N1pdm infection

We have shown here that treatment with CFA restrained lung pathology and promoted survival from H1N1pdm infection. In our following study, microarray analysis was performed to demonstrate the gene expression profiling associated with the CFA treatment for uncovering the mechanism(s) leading to the protection against H1N1pdm infection. Lung tissue samples collected from CFA and vehicle groups of mice at day 3 and day 5 p.i. were processed for RNA extraction and then used for expression array analysis. Our microarray data demonstrated that a total number of 4994 genes with a statistical difference (p ≤ 0.05) were regulated by CFA in the lung when compared to the vehicle control at day 3 p.i. At a later time point (day 5 p.i.), 2375 genes with a difference of p ≤ 0.05 in expression were identified between CFA and vehicle groups (Fig. 4.2 A). To further uncover the genes with a significantly different expression level, we filtered the total distinguished genes between CFA and vehicle groups with an expression dissimilarity of fold change ≥ 1.5 or ≤ -1.5 and p ≤ 0.05 at day 3 and day 5 p.i. When compared to the vehicle control, 394 upregulated and 71 downregulated genes were found in the lung of CFA treated mice at day 3 p.i.; furthermore, we uncovered 367 upregulated and 245 downregulated genes in the lung at day 5 p.i. (Fig. 4.2 B). By utilizing functional annotation tool of the DAVID Bioinformatics Resources, we annotated the genes significantly regulated by CFA
with the enrichment analysis ($p \leq 0.05$). It was shown that the upregulated molecules in CFA treated mice were extensively involved in regulation of transcription and translation at day 3 p.i. (Fig. 4.2 C), whereas the genes with increased expressions at day 5 p.i. were dramatically enriched in the signaling molecules, phosphorylation and protein kinas activity (Fig. 4.2 D). Interestingly, we found the genes downregulated by CFA at day 3 and day 5 p.i. were significantly associated with the functions of immune effector process, antigen presentation, T cell differentiation, cytokine/chemokine activity and inflammatory responses (Fig. 4.2 C, D). Furthermore, the fold changes of some surrogate genes involved in the multiple arms of host immune responses have shown that CFA treatment significantly altered host gene expression induced by the H1N1pdm infection; most strikingly, CFA downregulated expression of the genes including chemokines and proinflammatory cytokines CCL2, CCL3, CCL5 and TNF-α that contribute largely to immunopathology caused by the pathogenic influenza (Table 4.1). Altogether, our results suggest that CFA specifically regulated mediators may play a role in suppression of overactive host immune responses to promote survival outcome in mice during H1N1pdm infection.
Figure 4.2 (Legend is on next page)
Figure 4.2 CFA treatment downregulated the genes involved in host inflammatory responses post infection with A/Mexico/4108/2009.

Microarray analysis was performed on the lung RNA samples collected from CFA and vehicle treated group of mice at day 3 and day 5 p.i. (A) Heat maps were generated from the expression profiles from CFA and vehicle groups by selecting the genes with a statistical difference (p ≤ 0.05) at day 3 and day 5 p.i. (B) Significantly regulated genes (p ≤ 0.05 and fold change ≥ 1.5 or ≤ -1.5) were filtered from the total genes with dissimilar expressions in CFA group at day 3 and day 5 p.i. (C) and (D) Functional annotation tool of the DAVID Bioinformatics Resources was used to annotate the CFA significantly regulated genes with enrichment analysis.

| Table 4.1. In vivo differential microarray gene expression analysis on lung tissue of CFA and vehicle treated mice infected with A/Mexico/4108/2009 virus (n=3) versus uninfected controls (n=3) at day 3 and day 5 post vaccination. | Mean of Fold Change$^b$
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$^a$ The gene symbol is compatible to the human ortholog

$^b$ The mean fold change is normalized to the uninfected control group. Boldface indicates the gene expression is significantly different between CFA and vehicle groups by at least 1.5 fold change (p ≤ 0.05) versus controls, while others still showed statistical differences of p ≤ 0.05.
4.4.3 CFA treatment augments Treg suppressive molecule expression after H1N1pdm infection

Due to the finding that CFA treatment suppressed inflammatory responses after H1N1pdm infection, we next looked into the possible suppression mechanism(s) mediated by CFA. To exclude the possibility that CFA may induce granuloma which attracts pro-inflammatory cells at site of injection and limits their migration to the circulation, we analyzed pro-inflammatory cell populations in the spleen post infection. Although CFA transiently enhanced activated macrophages/monocytes at day 3 p.i, it did not significantly altered the populations of effector CD4 and CD8 T cells in the spleen after infection when compared to the vehicle control (Appendix 4.5). Therefore CFA may utilize other mechanisms that lead to immune suppression during severe influenza infection. Given that CFA contains PRR ligands which are capable of enhancing Treg cell suppressive capacity, we asked whether CFA augmented Treg cell activities that mediated immune suppression during H1N1pdm infection. Thus, expressions of the genes associated with Treg cell functions have been investigated in the CFA and vehicle groups at day 3 and day 5 p.i. It was shown that galectin-1 (Lgaasl1) which is overexpressed in Treg cells was upregulated by CFA treatment at day 3 p.i (Fig. 4.3 A). Galectin-1 potentially plays a role in Treg cell-DC and Treg cell-T cell interactions and inhibits responder cells by promoting cell growth arrest (Garin et al., 2007). Interestingly, we found a group of genes Mcm, Cdt1, Dbf4, Orc6 and Cdk which are involved in control of chromosome replication were significantly reduced in CFA treated mice (Fig. 4.3 A). Furthermore, we observed that Treg cell marker CTLA-4, which plays a major role in downregulation of DC costimulation, was significantly upregulated by CFA in contrast to the vehicle control at day 5 p.i. (Fig. 4.3 B). Interestingly, the mRNA level of anti-apoptotic molecule BCL2 which prevented apoptosis in activated Treg cells was significantly increased in CFA group of mice (Strauss et al., 2009), implying Treg cell population may survive better with CFA treatment during infection. To validate this result, we assessed the level of Treg cells in the lung by FACS at day 3 and day 5 p.i. It was shown that the numbers of Treg cells were increased from day 3 to day 5 p.i. in both CFA and control groups. This suggest that Treg cells are also induced by the influenza infection (Betts et al., 2011a). Interestingly, the CFA treated mice showed 1.4 fold more Treg cells in the lung when compared to the control ($p < 0.05$) at day 5 p.i. (Fig. 4.3 C). Altogether, our results indicate that CFA treatment enhances expressions of Treg cell
suppressive genes and promotes accumulation of Treg cells in the lungs following H1N1pdm infection.

Figure 4.3 CFA treatment induced Treg cell associated genes and increased accumulated Treg cells in mouse lung following infection with A/Mexico/4108/2009.

Lung tissues collected at day 3 and day 5 p.i. from CFA or vehicle treated group of mice were processed for expression microarray and FACS analyses. (A) and (B) The heat map generated from the gene expression profile of the CFA and PBS groups (n = 3/group) shows the galectin-1 (Lgals1), DNA replication, Treg cell and effector T cell cytokine/chemokine related genes (red: upregulation; blue: downregulation). (C) Percentages of Treg cells identified as Foxp3+CD25+.
CD4⁺CD25⁺Foxp3⁺ in CD4⁺ T cells in mouse lung at day 3 and day 5 p.i. (left panel). CD25⁺Foxp3⁺ cells were gated on CD3⁺CD4⁺ cells. Absolute number of CD4⁺CD25⁺Foxp3⁺ cells in mouse lung was shown as bar graph in right panel. Student t test was performed for statistical analysis. The result of a representative sample (n = 4) was shown in FACS analysis. *: p ≤ 0.05.

4.4.4 CFA treatment blunts DC maturation and reduces effector T cell responses post H1N1pdm infection

Having shown that CFA treatment induced Treg cell suppressive molecules, we went on to investigate the DC activity and effector T cell responses in the lung post infection. Our microarray data demonstrated that CFA treatment significantly reduced the mRNA level of IL3R, TLR7 and TLR9 which are preferentially expressed by the pDCs, when compared to vehicle control at day 3 p.i. (Fig. 4.4 A). Given that engagement of TLR7 and TLR9 is needed for optimal production of IFN-α (Hemmi et al., 2003; Lund et al., 2004), we further analyzed the expression level of IFN-α in the lung. Interestingly, the decreased mRNA levels of TLR7/9 were associated with downregulated expression of IFN-α1 and IFN-α5 that are specifically expressed by the pDCs (Fig. 4.4 A). These results suggest that CFA treatment may inhibit activity of the pDCs which are the primary sources for producing IFN-α at the infection site.

Given that CFA significantly induced CTLA-4, which downregulates CD80/CD86 on the mDCs (Misra et al., 2004), we next evaluated the mRNA level of mDC functional genes in CFA treated mice. Interestingly, CFA treatment not only significantly reduced the level of mDC costimulation molecules CD80 and CD86, but also the CD40 and MHCI/II genes which are the indicators of mDC maturation in the lung at day 3 p.i. (Fig. 4.4 B). Since mDC maturation is also regulated by the pDCs (Kadowaki et al., 2000), our results suggest that the limited pDC activity may lead to further downregulation of mDC maturation in the CFA treated mice. FACS analyses confirmed that the numbers of CD86⁺ mDCs were significantly lower in the lung at both day 3 and day 5 p.i in the CFA treated mice (Fig. 4.4 D). To better understand the correlation between DC maturation and T cell responses, we used IPA analysis to annotate the microarray data. It was demonstrated that CFA treated mice had decreased levels of signaling molecules involved in the Th1 cell differentiation due to reduced APC maturation and costimulation, when compared to the vehicle control at day 3 p.i. (Fig. 4.4 C). Taken collectively, our results suggest that CFA treatment downregulates mDC maturation and Th1 cell
differentiation in the lung and consequently diminish hyper-activation of the pro-inflammatory T cells during H1N1pdm infection.

**Figure 4.4 (Legend is on next page)**
Figure 4.4 CFA treatment downregulated DC maturation and costimulation in mouse lung following infection with A/Mexico/4108/2009.

(A) pDC associated genes (B) mDC maturation/costimulation markers were determined by microarray analysis from the lung RNA of CFA and vehicle treated groups (n = 3/group) of mice at Day 3 p.i. (red, increase; blue, decrease) and plotted by heat map. (C) Histograms are CD86 staining for mDC cells in mouse lung at day 3 and day 5 p.i. (left panel). The arrow pointing percent is proportion in Gr1^CD11b^CD11\text{high} cells. Absolute number of CD11b^CD11\text{high}^CD86^ cells in mouse lung was shown as bar graph in right panel. Student t test was utilized for statistical analysis. The result of a representative sample (n = 4) was shown in FACS analysis. (D) IPA canonical pathways of T helper cell differentiation were created by using the microarray data of CFA and vehicle treated mice at day 3 p.i. (red, upregulation; blue, downregulation). *: p ≤ 0.05.

We next continued to assess the accumulation of effector T cells in the lung at day 3 and day 5 p.i. CFA treatment statistically reduced the number of Foxp3^CD44^ effector CD4 T cells in the lung at day 3 p.i. (Fig. 4.5 A). The significant differences of effector CD4 T cells, however, were not seen between CFA and vehicle groups at a later time point after infection (Fig. 4.5 A). In contrast, we observed that CD44^ effector CD8 T cells were decreased in the CFA treated mice at day 5 p.i. and CFA-induced inhibition of T cell responses was more marked on effector CD8 T cells (Fig. 4.5 B). Altogether, our results suggest that CFA treatment restrains pro-inflammatory T cell responses associated with controlling levels of cytokines/chemokines to improve infection outcome (Table 4.1).
Figure 4.5 (Legend is on next page)
Figure 4.5 CFA treatment reduced effector T cell responses in mouse lung post infection with A/Mexico/4108/2009.

(A) CD44+ staining for Foxp3- CD4+ T cells in mouse lung was analyzed by flow cytometry at day 3 and day 5 p.i. (upper panel). Percent is proportion of Foxp3-CD4+ T cells. Absolute number of Foxp3-CD4+CD44+ T cells in mouse lung was shown as bar graph in lower panel. (B) FACS analysis identification of effector CD8 T cells in mouse lung at day 3 and day 5 p.i. (left panel) Gate is plotted on CD3+CD8+ cells. Percent is proportion in CD8+ T cells. Absolute number of CD8+CD44+ T cells in mouse lung was shown as bar graph in right panel. Student t test was performed for statistical analysis. The result of a representative sample (n = 4) was shown in FACS analysis. *: p ≤ 0.05.

4.4.5 Restimulation with killed M. TB enhances CTLA-4 expressing Treg cells and downregulates pDC and mDC maturation ex vivo

In contrast to the treatment of IFA, CFA which contains IFA and killed M.TB significantly enhanced survival in mice from H1N1pdm infection. In addition, our results showed that CFA treatment elevated Treg cell suppressive molecules associated with reduced host immune responses after infection (Fig. 4.2-4.3). Therefore, we next investigated whether CFA containing stimuli----killed M. TB was capable of directly activating Treg cell mediated immune suppression ex vivo. Since CFA was administered twice for inducing Treg responses in vivo, we resimulated the splenocytes isolated from CFA primed mice (day 4 post treatment) with or without 5µg/ml killed M. TB for 18 hours at 37°C. It was observed that killed M. TB restimulation did not increase Foxp3 expressing Treg cell population, however, the percentage of CTLA-4 expressing Treg cells was significantly increased (Fig. 4.6 A). Furthermore, we analyzed the level of cotimulation molecules on both pDCs and mDCs to assess whether increased CTLA-4 on Treg cells restricted their expression. Our results showed that killed M. TB restimulation highly reduced CD86 expression on mouse pDCs which were identified as Gr1+B220+CD11c+ cells (Fig. 4.6 B; upper panel) (Ferrero et al., 2002). Also, CD86 expressing F4/80+CD11b+CD11c+ mDCs were significantly diminished by the ex vivo stimulation (Fig. 4.6 B; lower panel). Interestingly, we observed that reduced DC costimulation resulted in significantly lower percentage of Foxp3- effector CD4 T cells, whereas no significant change of effector CD8 T cell population was seen after killed M. TB restimulation (Fig. 4.6 D). Altogether, our results indicate that killed M. TB directly enhanced CTLA-4 expressing Treg cells that play a role in inhibition of DC costimulation and T cell responses.
Figure 4.6 (Legend is on next page)
Figure 4.6 *Ex vivo* restimulation with killed M.TB increased CTLA4 expressing Treg cells and downregulated pDC/mDC maturation in splenocytes isolated from CFA primed mice.

Splenocytes isolated from mice at day 4 after CFA treatment were restimulated with or without killed M. TB for 18 hours at 37°C. (A) Histograms are CTLA4 staining for individual gate of CD4^+CD25^+Foxp3^+ T cells. Percentage shown within gate is proportion in CD4^+ T cells. Arrow pointing percent is proportion in CD4^+CD25^+Foxp3^+ T cells. (B) Histograms are staining for CD86 on pDC (upper) and mDC (lower) identified as Gr1^+B220^-CD11c^+ cells and F4/80^-CD11b^-cd11c^+ cells, respectively. Arrow pointing percent is in pDC or mDC cells. (C) FACS identification of Foxp3^-CD44^-CD4^+ T cells and CD44^-CD8^-T cells. Percent is in Foxp3^-CD44^-CD4^+ T cells or CD8^-T cells. Total percentage of CD44^-Foxp3^-CD4^-T cells and CD44^-CD8^-T cells are shown as bar graph in right panel. The result of a representative sample (n = 4) was shown in FACS analysis. Student *t* test was utilized for statistical analysis. *: *p* ≤ 0.05; NS: not significant.

4.5 Discussion

Immunopathology caused by influenza is associated with cytokine storm (Osterholm, 2005). Synergistic responses of IFN-α and IFN-γ activate pro-inflammatory cells and induce multiple cytokines and chemokines (Galligan *et al.*, 2006). TNF-α secretion is correlated with pulmonary tissue damage, and CCL2, CCL3 and CCL5 production is directly associated with disease severity during pathogenic influenza infection (de Jong *et al.*, 2006; La Gruta *et al.*, 2007; Lin *et al.*, 2008). It is well known that Treg cell suppression limits effector CD4^+ and CD8^+ T cell responses efficiently (Grossman *et al.*, 2004); therefore, this cell population is considered as a therapeutic candidate that may directly diminish overactive host immune responses during severe infection. Partial deficiency of Treg cells potentiated disease severity and resulted in excessive cytokine expressions by CD8^+ T cells during RSV infection (Fulton *et al.*, 2010). Also, conditional depletion of Treg cells developed lethal infection outcome in mice when infected with WNV (Lanteri *et al.*, 2009). These findings suggest that Treg cells may play crucial roles in control of host immune responses during serious virus infections. Although a recent study has reported that partial depletion of Treg cells did not alter the infection outcome in a lethal influenza infection (Betts *et al.*, 2011a), this study did not rule out the possibility that Treg cell suppression may have been inhibited by severe infection due to the induced hyper-inflammatory environment in the host. Therefore, increase of Treg cell suppression has the potential to assist the host in maintaining a balanced immune environment during pathogenic infection.
Previously published studies have shown that innate immune receptor PRRs play crucial roles in regulation of Treg cell suppression in vitro (Caramalho et al., 2003; Crellin et al., 2005; Komai-Koma et al., 2004; Rahman et al., 2010), which suggests PRR agonists are likely to modulate host immune responses in certain circumstances. This proposition led us to evaluate the function of several TLR agonists and adjuvants for regulating host immunity in context of severe H1N1pdm infection in mouse model. The treatment with single TLR (3, 4, 5 or 9) agonist or TLR independent adjuvant (MF59, alum or IFA) are unable to significantly rescue the mice from H1N1pdm infection, whereas treatment with CFA which contains multiple PRR agonists highly reduced severity of the infection. Furthermore, the protective role of CFA is validated in a more lethal model wherein the CFA promoted survival in mice infected with 32 times of MLD_{50}. These results may suggest that activation of multiple PRR signaling pathways is more efficacious in controlling H1N1pdm infection. Interestingly, this improved outcome was closely related to restrained inflammation in the lung and, independent of limited viral burden and/or robust antibody responses. Our microarray analyses and annotation suggest that the mechanism of CFA treatment is restraining multiple activities of host immunity including antigen presentation, T cell differentiation and cytokine/chemokine activity following infection, indicating that attenuated host immune responses to H1N1pdm infection confers better outcome. It has been verified that this pro-inflammatory resolution is not correlated with CFA-induced granuloma which may limit pro-inflammatory cell migration to the circulation. Interestingly, the microarray data uncovered that CFA treatment is strongly associated with Treg cell responses. It was found that galectin-1 preferentially expressed by the Treg cells was highly induced by CFA. Galectin-1 can bind to several glycoproteins such as CD43, CD45 and CD7 expressed on the responder cells (Pace et al., 1999). It has been found that Treg cell secreted galectin-1 is able to directly reduce effector T cell responses by activating cell cycle arrest (Shevach, 2009). In addition, galectin-1 expressed on the mesenchymal stromal cells (MSCs) inhibited DC differentiation and function by preventing entry into cell cycle (Ramasamy et al., 2007). Consistent with these findings, our results have shown that several genes involved in the chromosome replication were significantly downregulated in the lung of CFA treated mice. It is suggested that binding of galectin-1 may activate the signaling pathways that inhibit expression of the genes involved in the cell division. In this scenario, proliferation of the galactin-1 bound T cells or DCs is restrained to reduce pro-inflammatory cytokine production during H1N1pdm infection.
Interaction between CTLA-4 on Treg cells and costimulatory molecules CD80/CD86 on DCs is considered as a major pathway that is used by Treg cells for mediating suppressive function (Shevach, 2009). The binding between CTLA-4 and its ligand CD80/CD86 blocks the increase or even downregulates the expression of CD80 and CD86 on DCs, which in turn to reduce effector T cell responses (Misra et al., 2004). Our results showed that CTLA-4 was highly upregulated at transcription level in the lung by CFA post infection. This finding was further validated by our ex vivo restimulation assay which showed CTLA-4 expression was significantly increased on the Treg cells. In contrast, our in vivo study demonstrated that the CFA treated mice had about 1.4 fold more Foxp3 expressing Treg cells accumulated in the lungs after infection, possibly due to the reduction of their apoptosis. Furthermore, no significant augmentation of Foxp3 positive Treg cells was seen by the killed M. TB restimulation. Therefore, the increased mRNA level of CTLA-4 more likely resulted from the enhanced expression of this molecule on the Treg cells following engagement of the CFA containing stimuli and possibly the PRR agonists. It is suggested that the innate immune signaling such as PRR ligation may play a role in regulation of CTLA-4 expression in Treg cells. Secretion of suppressive cytokines is another major mechanism by which the Treg cells directly inhibit responding T cells and DCs (Shevach, 2009). Specifically, cytokine IL10 and TGF-β suppress the host immune responses during chronic infection and play crucial roles in restriction of inflammatory bowel disease (Guyot-Revol et al., 2006). In our study, however, suppressive cytokines are unlikely involved in the CFA-induced Treg cell suppression since the IL10 and TGF-β signaling pathways were not dramatically activated. This observation implies that the CFA-induced Treg cells may utilize alternative mechanisms for suppressing immune responses at the acute stage of infection. Similar to our results, a previous study has also shown that CTLA-4 expression was vigorously increased on the Treg cells for controlling RSV infection in the mouse lung (Fulton et al., 2010). Therefore, CTLA-4 mediated inhibition of mDC costimulation may represent a main strategy utilized by CFA-induced Treg cell suppression in severe influenza infection.

pDCs are a subpopulation of DCs and capable of inducing T helper cell differentiation for generating effective immunity (Mckenna et al., 2005). In particular, the pDC expressed IFN-α contribute largely to mDC maturation and lead to a Th1-like phenotype by stimulating IFN-γ expression (McKenna et al., 2005). Controversially, pDCs have been found to develop Treg cell
mediated suppression. These tolerance-inducing pDCs activate Treg cells which inhibited effector T cell responses via IL10 dependent manner in the human gut (Bilsborough & Viney, 2002). To date, it is still unknown what factors induce tolerogenic pDCs and how to distinguish tolerance-inducing pDCs from stimulatory pDCs. Interestingly, our study revealed that stimulatory pDCs may be also targeted by the Treg cells to suppress host immune responses. With CFA treatment, the pDC specifically expressed IFN-α1 and IFN-α5 were highly reduced after infection with associated downregulation of TLR7 and TLR9, which are preferentially expressed by the pDCs to induce type I IFN expression (Hemmi et al., 2003). This occurrence may partially result in the diminished Th1 cell differentiation and restricted number of inflammatory CD8 T cells in the lung. Furthermore, the CFA-induced Treg cells are capable of reducing CD86 expression on the stimulatory pDCs after restimulation with the killed M. TB. Since the killed M. TB restimulation increased CTLA-4 expression on Treg cells, we suggest that CTLA-4 expressing Treg cells may also bind to the costimulation molecule on the pDCs to downregulate their activities. In this scenario, mDC costimulation and effector T cell responses may be further diminished in the presence of Treg cell suppression. Future studies will aim at identifying the effector molecules or signaling pathways that regulate CTLA-4 expression and function in context of pDC maturation.

It is acknowledged that effector T cells are vital for controlling influenza infection; however, hyper-response causes immunopathology (Osterholm, 2005). The shift from immunopathogenicity to normal immunogenicity may be achieved by limiting host immune responses. In this study, we have shown CFA is capable of suppressing overactive effector T cell responses during H1N1pdm lethal infection via downregulation of DC costimulation/maturation. Of note, this suppression is mainly mediated by the Treg cell preferentially expressed CTLA-4, which is induced by the CFA or the killed M. TB. CFA or killed M. TB contains NOD2 and possible TLR2/4 ligands which are reported to enhance Treg cells survival and suppressive capacity in vitro (Caramalho et al., 2003; Komai-Koma et al., 2004); (Rahman et al., 2010), thus, PRR engagement has the potential to modulate host immune responses in certain conditions. Here, we have uncovered a scenario in which the PRR ligation may trigger a costimulatory signaling in the influenza infection-induced Treg cells to augment the suppressive molecules which mediate a more balanced immune-environment in the host and consequently limit severe disease symptoms.
References


Chapter 5

General Discussion and Future Directions
5.1 General discussion

Influenza virus is an infectious pathogen that causes respiratory illness in human populations worldwide. Antigenic variation of the influenza viruses may result in yearly epidemic outbreaks and occasional pandemic events by transmitting among humans. Currently, vaccines are primarily used to prevent influenza infections. Successful immunogenicity in the vaccine recipients largely reduces frequency of the infection and severity of the symptoms caused by the viruses which display similar antigenicity to the vaccine strains. Influenza (A) virus, however, mutates quickly and undergoes antigenic drift or shift in its evolution. In particular, influenza A virus with antigenic shift has distinct surface antigens when compared to the recently circulating seasonal virus. Usually, the viral strain with antigenic shift is considered as a pandemic influenza virus which is less likely to be prevented by the seasonal influenza vaccines.

To achieve the optimal vaccine immunogenicity against influenza virus, adjuvant has been extensively studied in the context of vaccination in both humans and animals (El Sahly, 2010; Krieg et al., 1995). On the other hand, prior exposure to influenza virus infection has been reported to provide heterosubtypic immunity against different subtype strains. Specifically, several studies have shown that infection with seasonal influenza virus prevents 2009 H1N1pdm challenge in the past two years (Ellebedy et al., 2010; Hillaire et al., 2011; Laurie et al., 2010). Therefore, live seasonal virus infection is thought to provide a broader immunity against different strains in certain situations. Additionally, a therapeutic strategy is needed to control severe symptoms during a pathogenic influenza infection when pre-existing immunity fails to provide protection. It has been known that an overactive host immune response is considered a major hazard that causes pulmonary damage and acute respiratory failure during pathogenic influenza infection. Thus therapies that directly limit excessive host immune responses must be developed. In this thesis, the adjuvanticity during influenza vaccination, prior infection-induced cross-protection, and potential immune therapeutic strategy have been well characterized. The mechanistic view of this thesis has provided new insights in the prevention and control of influenza caused illness.

5.1.1 Molecular signatures associated with adjuvanticity
To date, it has been shown that several TLR dependent and independent adjuvants induce strong immunogenicity when coadministered with influenza vaccines (Cooper et al., 2004; Jelinek et al., 2011; Puig & Gonzalez, 2007). The published results suggest that the vaccine adjuvant is able to increase maturation of the DCs that mediate antigen presentation to the adaptive immune cells (Iwasaki & Medzhitov, 2004). Analysis of human use TLR independent adjuvants MF59 and alum indicates that they facilitate differentiation of monocytes into DCs and that MF59 further enhances migration of DCs to the tissue draining lymph nodes to activate adaptive immunity (Seubert et al., 2008).

Activities of TLR dependent adjuvants such as poly i:c (TLR3), LPS (TLR4), flagellin (TLR5) and CpG ODN (TLR9) have been well characterized in influenza vaccination to understand their TLR signaling pathways that contribute to the augmentation of adaptive immune responses (Iwasaki & Medzhitov, 2004). In this thesis, TLR9 agonist CpG (ODN), type I IFN (PEG-IFN) and gold standard adjuvant CFA were coadministered with seasonal influenza vaccination in ferrets. The gold standard CFA showed the highest efficacy of inducing neutralizing antibodies among the three adjuvants and that CpG is more efficacious than PEG-IFN. To correlate the immunization outcome and gene expression signatures, microarray analysis has been performed to uncover the common and differing mediators that are involved in the adjuvanted vaccinations. Analysis of expression profiling demonstrated that DC maturation was significantly enhanced by PEG-IFN, CFA and CpG to a lesser extent. PEG-IFN and CFA induced the expressions of several genes including MHC class I/II and antigen processing molecules that are involved in DC maturation and APC function, whereas CpG increased only one or two genes associated with DC antigen presentation. Interestingly, MHC transcription enhancesome RFX5 has been discovered as a common mediator in PEG-IFN and CFA adjuvanted vaccination. This suggests that RFX5 can be considered as a common indicator for successful immunization showing enhanced DC maturation. CpG was found to be a weak adjuvant inducing DC maturation; therefore, it may function differently from the canonical adjuvants. The results suggest that CpG is likely to anchor TLR9 directly in B cells to initiate MyD88-mediated NF-κB and/or AP-1 activation to induce expressions of IRF4, which has been identified as a regulator of antibody class switching and plasma cell differentiation (Fillatreau & Radbruch, 2006). Further analysis revealed that PEG-IFN and CFA were also capable of directly activating B cell responses, although different signaling pathways were utilized by each
adjuvant. CFA fully induced B cell activation through Lyn-Syk-PI3K, calcineurin-NFAT and Ras-MEK-ERK pathways. The synergism of these signaling pathways subsequently activated expression of the transcription factors Bam32 and/or Oct2 to initiate expression of AID, which is vital for antibody SHM and CSR (Chaudhuri et al., 2003). In contrast, PEG-IFN may bind to the type I IFN receptor expressed by the B cells to activate the Ras-MEK-ERK pathway to generate high affinity antibodies. Given that innate receptors are also expressed by the adaptive immune cells, these findings suggest that adjuvant can also affect adaptive immune responses directly in addition to their function of mediating DC maturation.

TLR dependent adjuvants have not yet been licensed for use in humans because of safety concerns. However, it is suggested that the mediators or signaling pathways that are involved in the TLR adjuvanted vaccination are considered the indicators leading to successful immunization. It is noteworthy that RFX5 is a common indicator of adjuvant vaccine mediated DC maturation and that an efficacious vaccine is capable of activating the pathways involved in antibody SHM and CSR. Therefore, the crucial mediators and pathways identified in the successful vaccinations are the potential targets which need be considered in the design of the future influenza vaccines.

5.1.2 Broad protection induced by prior exposure to influenza virus

Influenza A viruses can generate novel reassorted viruses when coinfection occurs in the same host cell. The newly generated influenza virus has different surface antigens from the parental strains; therefore, it is capable of escaping the pre-existing host immunity which is commonly acquired via vaccination. Vaccine development is aiming to generate the ideal vaccines that induce successful seroconversion as well as CTL immunity against conserved influenza antigens to provide broad cross-protection in the vaccine recipients. To date, however, solid evidence showing cross-reactive vaccination against different strains is very limited (Anonymous, 2009). The results shown in this thesis concur with those of other studies which have shown that the currently used seasonal influenza vaccines are unable to generate cross-protective immunity against the novel reassorted pandemic strain. On the other hand, during the past 2009 pandemic, increasing evidence demonstrates that prior infection with seasonal
influenza virus plays crucial roles in cross-protection and control of disease severity (Ellebedy et al., 2010; Hillaire et al., 2011; Laurie et al., 2010). Some controversial findings, however, indicate that prior infection may inhibit forming protective humoral immunity against the secondary infection or even exacerbate the symptoms of disease in some situations (Kim et al., 2009). It is suggested that this “original antigenic sin” occurs when the first viral antigen has the intermediate similarity to the second virus which causes the following infection (Pan, 2011). Although evaluation of the benefit provided by the prior infection must be carefully addressed, understanding of the mechanism of cross-protection induced by the previous exposure is valuable for developing efficacious vaccines.

Cross-reactive CTL responses have been considered the main mechanism involved in the heterosubtypic immunity. The CD8+ CTLs targeting the conserved influenza antigens are cross-responsive to different viral strains (Tu et al., 2010). It has been found that CD8+ T cell deficiency significantly diminished cross-protection induced by the prior infection in some publications (Guo et al., 2010; Hillaire et al., 2011). These findings support the view that it is beneficial to develop pre-existing immunity toward CTL responses in the host. This thesis also shows that cross-responsive CTLs targeting the 2009 H1N1pdm virus were detected in sH1N1 infected mice, which indicates that CTLs may be important for providing heterologous protection. By using gene deleted mice, it has been found that prior infection with sH1N1 Brisbane/59 still conferred cross-protection against H1N1pdm in CD8 and perforin deficient mice. In contrast, cross-protection was significantly abrogated in the B cell knockout mice. It is suggested that cross-reactive antibodies but not CTLs are essentially required for preventing heterologous virus infections in some situations.

It has been known that neutralizing and non-neutralizing antibodies were induced by natural influenza infection (Sealy et al., 2003). Typically, cross-neutralizing antibodies are considered as effective for protecting against the secondary infection. This cross-protection, however, is only limited to prevent infection with the virus showing similar surface antigens. Due to distinct antigenicity of the HA, cross-neutralizing antibodies are not commonly induced following heterologous viral infections. The work described in this thesis confirms that anti-HA IgG1 and IgG2a antibodies are very specific to the subtype strain. Recently, nonetheless, several studies have shown that antibodies against the conserved region HA2 are capable of cross-neutralizing the group 1 and 2 influenza A viruses (Corti et al., 2011). It is suggested that anti-
HA2 antibodies can be used in therapeutic treatment against serious influenza infection in humans. Also, immunization with the vaccines containing the conserved HA2 antigen may have universal protection against different influenza strains. On the other hand, anti-HA2 antibodies are induced to a very restricted extent by influenza infection (Corti et al., 2010). This raises the question whether the natural infection-induced anti-HA2 antibodies are sufficient to prevent heterologous reinfection. Moreover, it has been reported that the H1N1 strain Brisbane/59 studied in this thesis has a conserved mutation on HA2 which abrogates cross-neutralization against the 2009 H1N1pdm (Wang et al., 2011). This finding suggests that neither Brisbane/59 prior infection nor vaccination is capable of inducing the anti-HA2 cross-neutralizing antibodies against H1N1pdm. Therefore, it is very likely that the cross-reactive but non-neutralizing antibodies potentially play crucial roles in heterosubtypic immunity.

Non-neutralizing antibodies targeting the internal antigens are induced following influenza infection. Among them, anti-NP and anti-M2e antibodies have been extensively studied to approach the development of “universal influenza vaccines” because NP and M2e are highly conserved. Several studies have shown that anti-NP and anti-M2e antibodies are capable of preventing infections with different viral strains (Carragher et al., 2008; El Bakkouri et al., 2011). This leads to a proposition that prior infection utilizes cross-reactive but non-neutralizing antibodies to restrain the heterologous viral infection. The results presented in this thesis confirm that sH1N1 Brisbane/59 infection induces antibodies cross-reactive with split H1N1pdm virus, H1N1pdm NP and H1N1pdm M1 to a less extent. The cross-reactive antibodies were dramatically increased in circulation and in the respiratory tract after H1N1pdm reinfection. Moreover, the elevated level of antibodies in the lung is closely associated with the increased percentage of activated and/or memory B cells detected at the early stage after secondary infection. This indicates that the sH1N1 infection-induced B cells, which secrete the antibodies against the conserved internal antigens, are immediately recalled upon reinfection.

To validate if the non-neutralizing antibodies are responsible for controlling the infection outcome, anti-sH1N1 hyper-immune sera were adoptively transferred into the naïve mice from day -3 to day 0 post H1N1pdm infection. The results confirmed that the non-neutralizing antibodies significantly promoted survival in mice from H1N1pdm infection. Unlike anti-HA antibodies which directly block the virus entry into host cells, non-neutralizing antibodies need FcRs to mediate phagocytosis and ADCC to lyse the infected cells (El Bakkouri et al., 2011;
Lamere et al., 2011). It is suggested that early treatment of high titer non-neutralizing antibodies are capable of achieving optimal protection against heterologous infection. Thus the antibody responses dependent on the FcR pathways are likely to play a crucial role in cross-protection when neutralizing antibodies and cross-responsive CTLs are absent. This study also supports the implication that prior infection with circulating seasonal virus is responsible for controlling severity of the disease caused by the 2009 H1N1pdm, which has been confirmed by a recently published human study indicating that patients previously infected with the Brisbane/59 virus displayed mild symptoms during the 2009 pandemic year (Couch et al., 2012).

5.1.3 Immune therapy for the pathogenic influenza infection

Prevention of infection is the main strategy used in control of influenza disease. In most circumstances, pre-existing host immunity acquired from the vaccination or prior infection is capable of inhibiting the potential infection and limiting severity of the disease. However, serious outcome occurs in the individuals who fail to generate successful anti-influenza immunity or recall the memory immune responses when infected with pathogenic influenza viruses (Rowe et al., 2010). In particular, severe human cases caused by the H5N1 or 2009 H1N1pdm infections demonstrate pulmonary damage and acute respiratory syndrome which correlate with excessive inflammatory responses in the host (Itoh et al., 2009; Taubenberger & Morens, 2009); hence therapeutic treatment is needed to control severe influenza infection. As discussed above, anti-HA2 antibodies are effective to treat the infections caused by variant influenza viruses. By using animal models, it has been validated that anti-HA2 treatment can provide full protection against H5N1 infection in mice and ferrets (Corti et al., 2011). This suggests that anti-HA2 antibodies have applicable value for use as therapeutic antibodies in human influenza infections. However, the high cost of therapeutic antibodies may prohibit the broad use of HA2 antibodies in influenza patients. Currently, antiviral drugs such as oseltamivir (NA inhibitor) have been commonly used for serious influenza infection to reduce viral burden and subsequently limit hyper-inflammation in the patients. Antiviral drugs, nonetheless, have two major limitations in treatment. The first is that antiviral drugs raise the selective pressure on the viruses that reproduce the mutated offspring virus which is resistant to the treatment; second, antiviral drugs are unable to directly regulate the excessive host inflammatory responses to
control pulmonary damage. Therefore, a new therapeutic strategy that is less susceptible to virus selection and capable of directly regulating host immune responses must be developed.

Recently, a chiral analog compound AAL-R of the human immunomodulator FTY720 was investigated in influenza infection (Walsh et al., 2011). AAL-R has shown promising value for limiting excessive pro-inflammatory cytokine expression and promoting survival in mice with 2009 H1N1pdm infection by reducing macrophage activation and effector T cell responses. This finding confirms that the restriction of overactive host immune responses is in favor of severe influenza resolution. Given that effector T cells are the major source cells for producing pro-inflammatory cytokines, control of their activity may contribute to the improvement of immunopathology caused by pathogenic influenza infection. It has been well-recognized that Treg cells are capable of limiting CD4$^+$ and CD8$^+$ T cell responses as well as reducing DC maturation to downregulate host immune responses (Shevach, 2009). The suppressive functions of Treg cells suggest that it may be worthwhile to develop a potential therapeutic strategy by which Treg cells are targeted to control hyperactive immune responses during influenza infection.

Treg cells are induced following influenza infection. Proliferation of Treg cells was observed in the lungs and major lymphoid organs after non-lethal infection in mice (Betts et al., 2011b). It has been shown that the MHC class II molecule was required to activate influenza-antigen specific Treg cells and, the infection-induced Treg cells were capable of reducing effector T cell responses and limiting cytokine expression. During acute infections with RSV or WNV, similarly, the induced Treg cells are essential to control excessive immune responses and improve infection outcome (Fulton et al., 2010; Lanteri et al., 2009). These findings suggest that Treg cells may balance the host immune milieu for controlling immunopathology caused by a serious viral infection. Therefore, enhancement of Treg cells’ suppressive capacity may benefit host immune responses against pathogenic influenza infection. Previous studies have shown that PRR engagement in Treg cells enhances their suppressive capacity to reduce effector T cell responses in vitro (Caramalho et al., 2003; Crellin et al., 2005; Rahman et al., 2010), which suggests that PRR agonists have the potential to downregulate host immune responses in certain situations. The results presented in this thesis demonstrate that treatment with CFA containing NOD2 and TLR2/4 ligands significantly promoted survival in mice with H1N1pdm infection. The improved infection outcome was closely related to the downregulation of cytokine
expression and restricted immunopathology in the lungs, but independent of reduced viral burden or robust antibody responses. It is suggested that CFA treatment induces galactin-1 and CTLA-4 mediated Treg cell suppression which inhibits DC costimulation/maturation, effector T cell responses, and cytokine/chemokine activity. This hypothesis supports the view that attenuated host immunity is beneficial for the control of severity of the disease caused by pathogenic infection.

Although NOD2 and TLR2, 4, 5, 7 and 8 are expressed by Treg cells (Caramalho et al., 2003; Rahman et al., 2010; Shevach, 2009), other types of cells including innate immune cells also have these receptors. Typically, PRR ligands are efficiently consumed by the innate immune cells which represent the largest proportion of the PRR expressing cells to generate effective immunity. As shown in this thesis, the PRR agonists facilitate Treg cell suppression during acute influenza infection in vivo. In contrast, one study reported that partial loss of Treg cells did not alter the infection outcome and immunopathology in mice infected with a lethal H1N1 virus (Betts et al., 2011a). Given that severe influenza infection commonly induces excessive host immune responses, it is possible that Treg cell suppressor functions may have been inhibited by the excessively expressed proinflammatory cytokines such as TNF-α during the infection (Valencia et al., 2006). In this circumstance, PRR ligation is more likely to play a role in costimulatory signaling for enhancing influenza-induced Treg cell suppression. Furthermore, the CFA containing stimuli induced Treg cell responses ex vivo. With the concern that activated Treg cells do not need TCR signaling to mediate immune suppression (Caramalho et al., 2003; Shevach, 2009), the CFA-induced Treg cells may be capable of restricting inflammatory responses via an antigen independent manner.

5.2 Future directions

5.2.1 Elucidation of T cell responses and cross-protection induced by the adjuvanted vaccines

Apart from the adjuvanticity for augmenting antibody responses, it is of interest to discover the role that CpG, PEG-IFN and CFA play in CD8\(^+\) CTL activity. Since type I IFN is capable of leading to Th1-like responses, IFN and ISG expression were analyzed in the adjuvanted vaccinations. CpG was found to induce transient IFN-α and ISG expressions post vaccination and CFA had an even less extent of ISG expression. Limited IFN expression may
result in the absence of Th1 signaling in both CpG and CFA adjuvanted vaccinations. Furthermore, CFA preferentially stimulated Th2 responses by reducing Th1 cytokine expression. On the other hand, PEG-IFN consistently induced ISG expression and augmented MHC class I genes that present antigen to the CD8\(^+\) cells. Is PEG-IFN more capable of inducing CD8\(^+\) CTL responses than the other two adjuvants? In the future study, antibody or a CTL preferential adjuvant will be identified by analyzing the Th1 or Th2 responses in their mediated vaccinations.

It is known that CTL responses provide broader protection against different subtype strains. Additionally, cross-reactive but non-neutralizing antibodies have shown protection from different viral infections. Thus the adjuvant that induces cross-responsive immunity following vaccination can protect recipients from a heterologous infection. Because CpG, CFA or PEG-IFN can regulate T cell and antibody repertoires, is it possible these adjuvants are capable of providing broad protection when coadministered with the vaccines containing the conserved influenza antigens? Also, are the common or differing mechanisms utilized by the adjuvants for providing cross-protection? In the future studies, further analyses will be performed to answer these questions. The results will bring new insights in the adjuvanticity that play a role in cross-protection.

### 5.2.2 Evaluation of cross-reactive antibodies in long interval protection and their capacity associated with the cross-responsive CD4 T cells

In practical situations, long intervals of protection are considered for evaluating the effectiveness of vaccination or immunization. In the third chapter, it is shown that cross-reactive but non-HA antibodies are capable of providing protection against H1N1pdm in week 5 after prior infection with sH1N1. Is it possible that this protection can be achieved after a longer period? This knowledge is important to estimate the infection outcome when a new virus emerges. It has been shown that numerous conserved CD4 T cell eptiopes exist among different viral strains and that CD4 T cells play crucial roles in CD8 T cell mediated cross-protection (Greenbaum et al., 2009; Sun et al., 2011). Also, a recent study suggests that CD4 T cells favor the production of neutralizing antibodies during a second infection (Alam & Sant, 2011). In the context of cross-reactive but non-HA antibody protection, are the CD4 T cells involved in the generation of this cross-protective immunity? Are they required to assist the memory B cell recall during a heterologous infection? Further studies will clarify whether CD4 T cells are vital
for cross-protection. Also, this work will investigate whether CD4 T cells need be targeted in future vaccine design.

5.2.3 Determination of antigen specificity of Treg cell suppression in pathogenic influenza infection

Treg cells are induced following influenza infection, and a recent study has shown that influenza-specific Treg cells are capable of limiting effector T cell responses and cytokine expressions in a non-lethal infection (Betts et al., 2011b). It is suggested that antigen specific Treg cells are required to suppress inflammation during the infection. On the other hand, it has been known that activated Treg cells do not require second signaling via TCR to suppress. As discussed in the fourth chapter, CFA enhances Treg cell suppression to downregulate host immune responses during a serious H1N1pdm infection. Additionally, it was found that CFA containing PRR ligands alone can activate Treg cell suppression \textit{ex vivo}. Is it possible that influenza virus antigen is not required for CFA induced Treg cells to suppress hyper-inflammation caused by the H1N1pdm virus, or that CFA containing PRR ligands function as costimulatory molecules to fully activate the Treg cells in the presence of viral antigen? Further analyses will determine whether PRR ligand-induced Treg cell suppression is antigen dependent or independent.

5.3 Closing remark

This thesis has investigated the mechanisms of adjuvanted vaccination, prior exposure-induced cross-protection, and adjuvant mediated protection in the context of prevention and control of influenza caused illness. The study of adjuvanted vaccination has illustrated the common and disparate signaling pathways involved in the different adjuvant vaccinations and provided novel insight in the design and evaluation of potential adjuvant for human vaccination. The assessment of prior infection-induced cross-protective immunity highlighted the crucial roles that cross-reactive but non-HA antibodies play in prevention from reassorted pandemic virus infection. This finding suggests that measurement of additional antibody repertoires must be considered in the evaluation of protective immunity. Also, this work raises a possibility that the conserved influenza antigens that induce cross-reactive antibodies have the potential to be the “universal influenza vaccines”. Lastly, the third project of this thesis has uncovered the novel
and potential therapeutic strategy that CFA adjuvant augment Treg cell suppression to provide a more balanced host immune environment in pathogenic influenza infection. Altogether, this thesis has improved our current understanding in influenza vaccine design, prevention and treatment strategies, and has made a significant contribution to the knowledge of anti-influenza research.

References


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hemagglutinin subunit 2. PLoS Pathog. 7, e1002081
Appendices
### Appendix 2.1

#### Ferret Specific Gene Primer list

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Appendix 2.2

The level of ferret_DLA-64-like, ferret_FCN1-like and ferret_SOCS3-like expression as quantified by Q-PCR in adjuvant-mediated vaccination.

To confirm the gene changes from the microarray analysis, the average transcription levels of three selected genes (A) Ferret_DLA-64-like, (B) Ferret_FCN1-like and (C) Ferret_SOCS3-like in multiple vaccination groups (n=3/group) were determined by Q-PCR at day 1 following immunization. Increases in mRNA levels were relative to β-actin and then normalized to PBS control groups. Average values were obtained from three independent experiments and the error bars indicate standard deviation. Student t test was performed for statistical analysis. The asterisk showed the statistical difference found between the adjuvanted vaccine group and vaccine alone group. *: p ≤ 0.05.
Appendix 2.3

High levels of homology between canine and ferret cDNA sequences.

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Average ClustalW Score 90

$^a$ URL: www.ebi.ac.uk/Tools/clustalw2/index.html
Appendix 3.1

A

Day post A/California/07/2009 infection

B

Day post A/California/07/2009 infection

C

Viral load in nasal wash samples at day 3 post A/California/07/2009 infection

Legend is on next page
Assessment of antibody response, clinical signs and viral load in A/Brisbane/59/2007 infected ferrets after subsequent infection with pdmH1N1 virus A/California/7/2009.

Ferrets were firstly infected with $10^6$ EID$_{50}$ of A/Brisbane/59/2007 virus. Purified serum was treated by RDE at 37°C overnight before HI and microneutralization assays. (A) Antibody titers against pdmH1N1 virus in A/Brisbane/59/2007 infected ferret serum as well as uninfected ferret serum samples (n=4) collected at day 0 and day 14 post pdmH1N1 secondary infection were measured by HI assay. (B) Neutralizing antibody titers for blocking pdmH1N1 virus were measured by microneutralization assay in the same serum samples investigated by HI test. (C) Weight loss and (D) Body temperature change were determined daily for the respective groups (n=8/group) after infection with $10^6$ EID$_{50}$ of A/California/07/2009. Data points represent the mean value and error bars demonstrate standard error of the mean. Student t test was performed for statistical analysis. (E) Viral load in nasal wash samples (n=4) collected at day 3 post pandemic H1N1 influenza infection was determined. Horizontal bars demonstrate mean value. *: $p \leq 0.05$, **: $p \leq 0.01$; LOD: limit of detection in viral load assay.
Appendix 3.2

A

Prim-uninfected ferrets
(Day 6 p.i. A/Mexico/4108/2009)

A/Brisbane/59/2007 primary infected ferrets
(Day 6 p.i. A/Mexico/4108/2009)

B

Prim-uninfected mice
(Day 6 p.i. A/Mexico/4108/2009)

A/Brisbane/59/2007 primary infected mice
(Day 6 p.i. A/Mexico/4108/2009)

Legend is on next page
Pathology in lung of ferrets and mice infected with A/Mexico/4108/2009 H1N1 virus.

(A) and (B) Left panels: representative lung tissue sections stained with H&E from control uninfected ferrets and mice at day 6 post A/Mexico/4108/2009 infection, respectively. Acute alveolitis/bronchiolitis and inflammation infiltrate were observed. (A) and (B) Right panels: representative lung sections from A/Brisbane/59/2007 infected ferrets and mice at day 6 after A/Mexico/4108/2009 infection, no significant lung pathology was noted but moderate cell infiltration were seen. Magnification: x100.
Appendix 3.3

A

HI titer against pdmH1N1 (Log2)

Prime: Vaxigrip (0.5ml/dose) SwVaccine PBS (0.5ml/dose)

Boost: + + + + + +

B

% Original Weight

Day post A/Mexico/4108/2009 infection

Unvaccinated Vaxigrip SwVaccine

C

Change in temp over baseline (°C)

Day post A/Mexico/4108/2009 infection

Unvaccinated Vaxigrip SwVaccine

D

Viral load in nasal wash samples at day 3 post A/Mexico/4108/2009 infection

Legend is on next page
Assessment of protective efficacy provided by the seasonal and swine vaccines against pdmH1N1 infection.

Four groups of ferrets were immunized with either 500 µl of Swine influenza vaccine (SwVaccine, Pneumostar SIV Novartis) including killed swine H1N1 and H3N2 viruses, 2008-2009 seasonal human flu vaccine Vaxigrip (Sanofi Pasteur) contained A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B/Florida/4/2006, or phosphate buffered saline (PBS, Unvaccinated). All the animals were vaccinated through intramuscular (i.m.) injection. At day 14 following primary immunization, HI titers against pdmH1N1 virus of each ferret group was evaluated. In the meantime, SwVaccine and Vaxigrip group animals were boosted by the respective amount of vaccine; unvaccinated control group was boosted with PBS. At day 14 post boost, HI test for pdmH1N1 antibody titer quantification was performed on the animal serum samples. (A) Antibody titers against pdmH1N1 virus in ferret serum samples (n=8) collected at day 14 after primary vaccination and at day 14 post boost were measured by HI assay. Data represents the Log₂ value of the HI titer. Error bars indicate standard deviation. (B) Weight loss and (C) Body temperature change were determined daily post pdmH1N1 infection. Each point of data in (B) and (C) represents the mean value obtained from the respective group. Error bars demonstrate standard error of the mean. Student t test was performed for statistical analysis. (D) Viral load in nasal wash samples (n=4) collected at day 3 post pdmH1N1 influenza challenge was determined. The virus titer was calculated by Reed-Muench method and expressed as TCID₅₀/ml. Horizontal bars demonstrate mean value. *: p ≤ 0.05; LOD: limit of detection in viral load assay.
Appendix 3.4

sH1N1 virus A/Brisbane/59/2007 B cell epitopes conserved in H1N1pdm virus A/Mexico/4108/2009

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<td>100%</td>
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B cell epitopes of Hemagglutine (HA), Nucleoprotein (NP) and Matrix protein 1 (M1) were generated by IEDB analysis resources.

Similarity of B cell epitopes between Mex/4108 and Brisbane/59 were analyzed by IEBD epitope conservancy analysis tool.
Evaluation of cross-reactive IgG and IgA antibodies in seasonal H1N1 infected mice challenged with pdmH1N1 virus.

Purified serum was treated by RDE at 37°C overnight before HI assay. HI test was performed on RDE treated (A) primary infected and uninfected mice sera collected at day 4 and day 7 post pdmH1N1 challenge; data represents the Log₂ value of the titer. (B) Mice sera collected at day 4 were assessed for cross-reactive IgG and IgA antibodies against split pdmH1N1 virus by ELISA. The average relative absorbance density read from three individual samples were plotted graphically. Lung samples were collected in both primary infected and uninfected mice at day 4 post pdmH1N1 reinfection. (C) HI titer in mice lung homogenate samples was analyzed; data represents the Log₂ value of the titer. (D) ELISA was performed for assessing cross-reactive IgG and IgA antibodies against pdmH1N1 virus in lung homogenate samples. Student t test was performed for statistical analysis. ***: p ≤ 0.001; LOD: limit of detection in HI assay.
Appendix 4.1

Outcome of H1N1pdm infection in mice treated with TLR agonists and vaccine adjuvants

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<td>Vehicle control</td>
<td>23.5 (D8)</td>
<td>90</td>
</tr>
</tbody>
</table>

<sup>a</sup> TLR agonists and adjuvants were intramuscularly injected into naïve mice (10 mice/group) at day -2 and day 2 post infection with 10^4 EID50 of A/Mexico/4108/2009.

<sup>b</sup> Day post challenge of peak weight loss from original weight.

<sup>c</sup> Log-Rank sum test has been used for comparing the survival curve between the group treated with TLR agonist/adjuvants from the vehicle control mice.

NS: not significant
Appendix 4.2

Weight change in mice treated with TLR agonists or TLR independent adjuvant following H1N1pdm infection.

C57BL/6 mice were treated with (A) poly i:c, (B) flagellin, (C) MF59 and (D) aluminum at day -2 and day 2 post A/Mexico/4108/2009. PBS was used as vehicle control. Weight loss of treated C57BL/6 mice (n=15/group) were monitored post H1N1pdm infection. No statistical differences were observed in the group of treatment when compared to the vehicle control.
Appendix 4.3

Infection outcome of CFA treated mice following more lethal infection with H1N1pdm virus.

The (A) weight loss and (B) lethality of CFA and vehicle treated mice were monitored post 10^5 EID_{50} A/Mexico/4108/2009 infection. Statistical differences of weight change between CFA and vehicle treated groups of mice were observed from day 2 to day 5 after infection by utilizing student t test. A significant difference of survival curve between CFA and vehicle treated groups of mice was observed. Comparison of survival curves was analyzed by Log-rank (Mantel-Cox) test. **: p ≤ 0.01, *: p ≤ 0.05.
Appendix 4.4

CFA treatment diminishes pathology in the lung following H1N1pdm infection.

Representative lung tissue sections stained with H&E from (A) CFA and (B) vehicle control group of mice at day 5 post A/Mexico/4108/2009 infection were shown, respectively. Black arrow points the infiltrated inflammatory cells. Magnification: x200.
Appendix 4.5

CFA treatment does not significantly alter inflammatory cell populations in spleen.

The splenocytes were isolated from CFA and vehicle treated mice at day 3 and day 5 post infection for the FACS analyses. The percentages of (A) Foxp3-CD4+CD44+ effector T cells, (B) CD8+CD44+ effector T cells and (C) Gr1-CD11b+F4/80 activated macrophages in splenocytes were plotted as bar graph. Student t test was performed for statistical analysis. *: \( p \leq 0.05 \).