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THE IMMUNOLOGICAL MECHANISMS OF INFLUENZA VACCINATION: A COMPARISON BETWEEN A SEASONAL SUBUNIT VACCINE AND AN H5N1 SUBUNIT VACCINE WITH AND WITHOUT ALUM ADJUVANT

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by

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ABSTRACT

Highly pathogenic avian influenza (HPAI) H5N1 is an emerging infectious virus with a 60% fatality rate in humans. In the United States, a vaccine for H5N1 has been developed and stockpiled using FDA approved methods for seasonal vaccines; however, the H5N1 vaccine was shown to be less immunogenic than seasonal vaccines when evaluated in clinical trials. Adjuvants can be used to enhance the immune response to antigens. For the studies described herein, a lethal mouse model of H5N1 infection was utilized to examine the immune response to the H5N1 vaccine with and without the addition of an alum adjuvant, and these responses were compared to those induced by a seasonal influenza vaccine. Mice that received the adjuvanted vaccine displayed significantly reduced weight loss and increased survival following infection with H5N1 compared to mice that received the non-adjuvanted vaccine. Increased levels of antibodies were detected in mice that received either the adjuvanted H5N1

vaccine or the seasonal vaccine compared to mice that received the non-adjuvanted H5N1 vaccine. *In vitro*, both the seasonal and adjuvanted H5N1 vaccines more efficiently activated dendritic cells (DCs) when compared to the non-adjuvanted H5N1 vaccine, as seen by enhanced levels of cytokine production following treatment with the seasonal vaccine and an increase in co-stimulatory molecule expression following treatment with adjuvanted H5N1 vaccine. When treated with the adjuvanted H5N1 vaccine, DCs demonstrated increased antigen uptake and intracellular processing compared to cells treated with the non-adjuvanted vaccine. Pre-treatment with mannan or mannose diminished cytokine production by DCs in a dose dependent manner following seasonal, but not H5N1, vaccine treatment implicating C-type lectin receptor activation as the mechanism by which the seasonal vaccine elicits protection. These findings provide an explanation for attenuated DC function following H5N1 vaccination, and while an alum adjuvant is able to rescue H5N1 vaccine immunogenicity it does so via a different mechanism than that utilized by seasonal influenza vaccines. Furthermore, these studies provide insight into the development of more immunogenic vaccines targeting HPAI.

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CHAPTER ONE: INTRODUCTION

Background

Influenza viruses cause annual global epidemics that result in three to five million cases of severe illness and between 250,000 and 500,000 deaths worldwide with approximately 36,000 deaths occurring in the United States [1, 2]. Symptoms include fever, cough, muscle and joint pain, severe malaise, sore throat, and runny nose. Most affected people recover within a week, but as mentioned above, severe illness and death can occur [2]. Seasonal epidemics affect people of all ages, but high risk groups include children under the age of two, adults age 65 and older, and people with chronic diseases or weakened immune systems [2]. Vaccination is the most effective means of preventing influenza infection, and it is estimated that 70%-90% of vaccinated healthy adults are protected from the virus [2].

In addition to epidemics, influenza viruses can also cause occasional pandemics. The most severe pandemic in the 20th century was the 1918 "Spanish Influenza" pandemic during which it is estimated that more than 50 million people died worldwide [1]. The most recent pandemic was the 2009 novel H1N1 or "Swine Flu" pandemic. Circulation of the 2009 H1N1 virus resulted in a pandemic because it consisted of a unique combination of influenza virus genes that had never before been identified in animals or people, so pre-existing immunity to the virus did not exist [3]. It is estimated that, globally, between 151,700 and 575,400 people died as a result of the 2009 H1N1 pandemic [4].

Highly pathogenic avian influenza (HPAI) viruses are a type of influenza virus with the potential to cause a pandemic [1]. As of April 26, 2013, the World Health Organization has reported 628 confirmed cases of HPAI subtype H5N1 resulting in 374 deaths (59.5%) in 15 countries since 2003 [5]. Early symptoms of disease include fever, cough, and dyspnea, while severe cases result in fast progressing pneumonia, respiratory distress syndrome, damage to the central nervous system, and multi-organ failure with death usually occurring within 10 days of symptom onset in fatal cases [6-8]. The potential for H5N1 to reach pandemic levels arises if, as with the 2009 H1N1 pandemic, the HA or NA segments from an HPAI virus were to be present in an influenza strain circulating in humans to which there is no pre-existing immunity. Vaccines targeting HPAI are being developed but they are not readily available, and early vaccines have been shown to be ineffective unless supplemented with an adjuvant [9-11]. The development of effective vaccines for HPAI reamins a priority.

Influenza Viruses

Influenza viruses are negative sense, segmented, single-stranded, RNA viruses that belong to the family *Orthomyxoviridae*, and they are organized into three genera commonly known as Influenza A, B, and C [7, 12-14]. Influenza A viruses naturally occur in aquatic birds, and are the only influenza viruses that infect birds. Influenza A viruses also infect a wider range of species including humans, swine, and several other species of mammals [12, 14]. Influenza B viruses infect humans and seals, and are less common and less genetically

diverse than Influenza A viruses [14-16]. Influenza C viruses infect humans and pigs, and while they may cause isolated epidemics, they are less common than Influenza A and B viruses and result in a more mild form of disease [2, 14, 17-20]. Seasonal epidemics are caused by Influenza A and B viruses, while pandemics are the result of the introduction of a novel Influenza A subtype into the population for which there is limited or no pre-existing immunity [15, 21].

The influenza viral genome is organized into eight segments that encode up to 13 proteins, including surface glycoproteins, polymerase proteins, structural proteins, and non-structural proteins [7, 14]. The genome is surrounded by a lipid envelope derived from the plasma membrane of the infected host cell. The hemagglutinin (HA) and neuraminidase (NA) glycoproteins radiate outward from the lipid envelope and are the major antigenic proteins of the virus, against which, neutralizing antibodies are produced [14] (Figure 1). The HA and NA proteins are also the most variable proteins due to the lack of proof-reading by the viral polymerase which results in mistakes in genome copying and frequent mutations. This concept is referred to as antigenic drift and is the reason a new influenza vaccine is needed every year [22]. Influenza viruses are also subject to antigenic shift. Antigenic shift results when new HA and NA proteins enter the human population as a consequence of genetic reassortment between an influenza virus circulating in humans and an influenza virus circulating in another species such as pigs or birds [22]. Antigenic shift often results in a pandemic strain of influenza as the HA or NA segment of the new virus has most likely not circulated in humans before. [22].

Influenza A viruses are divided into subtypes based on the antigenic properties of the HA and NA proteins as determined by phylogeny [13, 14, 21]. As of 2012, 17 HA and 9 NA subtypes have been identified and characterized [7, 13, 23]. The subtypes H3N2 and H1N1 circulate in humans as the seasonal viruses, along with Influenza B. Influenza B viruses are not divided into subtypes, but are further classified into strains [20]. HPAI viruses are limited to the H5 and H7 subtypes, but not all H5 and H7 viruses are highly pathogenic. Some are designated as low pathogenic avian influenza (LPAI) viruses [12]. There is a standard nomenclature system for influenza viruses that consists of the antigenic type (A, B, or C), the host of origin (duck, equine, swine), geographical origin, strain number, and year of isolation. The host of origin is omitted for human isolates. For Influenza A viruses, this nomenclature is followed by the antigenic description in parentheses [20, 24]. For example, A/Vietnam/1203/2004 (H5N1) is an Influenza A virus isolated from a human in Vietnam in 2004.

Highly Pathogenic Avian Influenza

All Influenza A viruses, including HPAI viruses, occur naturally in wild aquatic birds; however, they are occasionally transmitted to other hosts, such as domestic poultry and mammals, causing transient infections and occasional deaths [7, 25, 26]. On rare occasions, when influenza viruses are transmitted from aquatic birds to other species, continuous infections are established resulting in permanent influenza lineages in those hosts such as the seasonal influenza viruses that affect humans [25]. Therefore, HPAI viruses do not

normally infect humans, but rare cases do occur. Most human infections with HPAI are the result of close contact with infected poultry. Human-to-human transmission is rare, limited, and unsustainable [26]. Reports of human-to-human transmission include isolated household clusters of infection and a case where a child transmitted the infection to her mother [27, 28].

The first H5N1 virus outbreak in humans occurred in 1997 in Hong Kong. During this time, the virus was first identified in a 3-year-old boy who died in a Hong Kong hospital from Reye's syndrome, acute influenza pneumonia, and respiratory distress syndrome. The outbreak resulted in a total of 18 confirmed cases resulting in 6 deaths [29-33]. Following the 1997 outbreak, avian influenza was not detected in humans again until 2003 [33]. The new cases of H5N1 were identified in a family of five from Hong Kong who had been visiting mainland China, and who had been in close contact with live chickens. Three of the family members became ill and two died from the disease [33]. By July 16, 2004, the HPAI epidemic had affected eight countries and resulted in at least 23 deaths [34]. Since 2003, human cases of H5N1 have persisted with 628 confirmed cases resulting in an approximate 60% fatality rate as of April 26, 2013 [5]. In 2013, a novel HPAI H7N9 virus outbreak began to affect China [35, 36]. This is the first time an influenza A virus with an N9 subtype has been documented in humans [36]. The first cases were reported in February 2013, and as of May 30, 2013, 132 cases have been confirmed resulting in 37 deaths (28%) with new cases being reported daily [37]. HPAI viruses therefore remain a current and future threat to public health.

Influenza Vaccines

Vaccination is the most effective means for the prevention and control of infectious diseases, including influenza [38, 39]. Vaccination against influenza prevents 70%-90% of influenza specific illness in healthy adults, as well as, decreases severe illness and complications in the elderly by 60% and deaths by 80% [2]. In the United States, there are two types of influenza vaccines approved for use: 1) the injectable inactivated trivalent influenza vaccine (TIV) approved for use in people aged 6 months and older, and 2) the intranasal live attenuated influenza vaccine (LAIV) approved for use in people aged 2 to 49. Neither the TIV nor the LAIV utilizes an adjuvant [40-43]. The TIV is the traditional influenza vaccine, and remains the most widely used [41]. Inactivated influenza vaccines were first shown to be effective during World War II, and since then, they have been repeatedly shown to be effective [44-48]. The TIV influenza vaccine has been recommended for use since 1960; however, its use possesses some limitations including lower immunogenicity in the very young and the elderly, and, since it is administered intramuscularly, it needs to be given by trained personnel [41, 43].

The LAIV vaccine was licensed for use in 2003 by the United States Food and Drug Administration (USFDA) [43]. It has some advantages over the TIV as it has been shown to have higher immunogenicity compared to the TIV and can be administered intranasally [40, 41]. Also, whereas the TIV only induces a systemic immune response, the LAIV induces both a systemic and mucosal immune response [41]. The LAIV contains live attenuated virus that is cold-

adapted, which means it can only replicate and grow at temperatures less than 25°C. This adaptation should prevent the virus from growing in the lungs and respiratory tract following vaccination; however, since the vaccine does contain live virus, there are safety concerns, especially in people with weakened immune systems [41, 42]. Both the TIV and the LAIV are trivalent vaccines using the same vaccine strains [40, 41]. Herein, focus will be placed on the TIV.

Influenza vaccine production is a standardized process, and the complete manufacturing process takes about five to six months [49, 50]. The World Health Organization's Global Influenza Surveillance Network, consisting of five influenza centers in London, Tokyo, Melbourne, Memphis, and Atlanta, continuously monitors circulating strains of influenza [49, 50]. Vaccine strains are chosen based on the circulating viruses and consist of three different influenza viruses, hence the trivalent nature of the vaccine. Two of the three viruses included in the annual vaccines are Influenza A viruses, an H3N2 and an H1N1, while the third virus is an Influenza B strain [49, 51].

Following selection of the vaccine strains, the viruses are grown in fertilized chicken eggs along with A/Puerto Rico/8/1934 (H1N1) (PR8), a standard laboratory virus that grows quickly and efficiently in eggs. As the viruses grow and replicate, reassortment occurs in which the eight segments from each virus recombine forming hybrid viruses that contain a mixture of segments from each strain. The target viruses contain the HA and NA segments from the viruses chosen for the vaccine and all other segments from PR8. Antibodies against the HA and NA segments from PR8 can be included during the culture process to

help select for the target viruses (Figure 2). It takes approximately three weeks to prepare the hybrid viruses. Once the hybrid viruses have been generated, they are verified at one of the influenza surveillance centers to make sure they are safe, can grow in eggs, express the correct HA and NA proteins, and to ensure that they can produce a robust immune response. The vaccine strains are then distributed to vaccine manufacturers [49, 50].

After receiving the vaccine strains from the influenza surveillance centers, the manufacturers must mass produce the viruses in order to have a large enough quantity to generate an adequate amount of vaccine. The viruses are again grown in fertilized chicken eggs, and it takes thousands of eggs to produce a sufficient amount of virus [50]. The viruses are inactivated, or “killed”, with formaldehyde, followed by purification in a linear sucrose density gradient solution using continuous flow centrifugation. The viruses are then chemically disrupted using Octylphenol Ethoxylate (Triton® X-100) producing a split virus. A split virus consists of pieces of the virus rather than an intact viral particle. The split virus is further purified so that it contains only the HA and NA proteins, thus designating it a subunit vaccine. The viral proteins are then suspended in sodium phosphate-buffered isotonic sodium chloride solution creating the final vaccine preparation [52]. Each batch, or lot, of the vaccine is tested for sterility and amount of protein, or antigen. The vaccine then undergoes clinical trials for safety evaluation, and to show that it performs as expected. Clinical trials are not required in all countries as previous studies on annual influenza vaccines were performed and the assumption is that the new vaccines will behave similarly. The

vaccine must then be approved by regulatory agencies before it can be introduced into the national immunization program [50].

An inactivated subunit vaccine targeting the HPAI strain A/Vietnam/1203/2004 (H5N1) has been developed using the methods described above, and 20 million doses of this vaccine have been stockpiled in the United States in case of a pandemic [53]. Despite the ability of seasonal influenza vaccines and the 2009 H1N1 pandemic vaccine to stimulate the production of protective antibody titers, the H5N1 vaccine was shown to have low immunogenicity [2, 11, 44-48]. Vaccine efficacy in humans is determined by the production of antibodies against HA with titers of greater than 1:40 deemed protective. When evaluated in clinical trials, 70% of Fluzone® 2009/2010 TIV vaccine recipients developed protective antibody titers, and 95%-100% of Fluzone® 2009 H1N1 pandemic TIV vaccine recipients developed protective antibody titers [47, 48]. When the H5N1 subunit vaccine was evaluated in clinical trials, only 22% of recipients developed protective antibody titers (Figure 3) [11].

Alum Adjuvants

Adjuvants are substances that can be added to vaccines in order to augment the immune response. The term adjuvant comes from the Latin word “adjuvans” which means “to help” [54, 55]. The use of adjuvants was first exploited by William Coley, who used bacterial components to treat cancer patients, and by Ramon and Glenny who used tapioca and aluminum hydroxide along with diphtheria and tetanus toxins to enhance responses in horses and

guinea pigs [54]. The adjuvant alum, which is based on aluminum salts, is the most widely used adjuvant worldwide, and until recently was the only adjuvant approved for use in the United States [54-57]. The adjuvant AS04, which is a combination of monophosphoryl lipid A (MPL), a derivative of the bacterial cell wall component lipopolysaccharide (LPS), and alum, was approved for use in 2009 for the Cervarix vaccine targeting human papilloma virus (HPV) [56, 58]. Other vaccines licensed for use in the United States that contain alum include those for diphtheria, tetanus, and pertussis (DTaP), hepatitis A and B, pneumonia, anthrax, and rabies. Approved influenza vaccines do not contain an adjuvant [59].

It is not fully understood how adjuvants function, and they have been shown to act in many different ways to enhance immunity to an antigen. The term antigen refers to a foreign substance that induces an immune response [60]. Many adjuvants seem to stimulate antigen presenting cells (APCs) such as dendritic cells (DCs) enhancing maturation, migration, antigen presentation, and the expression of co-stimulatory molecules which leads to improved responses of T and B cells [54]. Alum has been shown to work in several different ways leading to confusion and controversy surrounding its mechanism of action [54-56, 61]. In vaccines containing alum, antigens are adsorbed onto the aluminum salts and the mixture is injected intramuscularly creating a nodule at the site of injection. The original theory behind the mechanism of action of alum, which is still widely believed, is that alum acts as an “antigen depot” by slowly releasing antigen to the immune system and prolonging exposure [54-57]. One study

showed, however, that removal of the alum nodule one week following vaccination had no effect on the antibody response generated against the pathogen [62], while another study demonstrated that adsorption of the antigen to alum was not required for the ability of alum to enhance the immune response [63]. If the antigen is not adsorbed to alum, then prolonged exposure at the depot is not taking place.

Another mechanism of action demonstrated for alum is the activation of the NLRP3 (also known as NALP3) inflammasome immune complex [64]. The NLRP3 inflammasome complex is a molecular platform consisting of the proteins NLRP3, ASC, and pro-caspase-1. Activation of the inflammasome in turn leads to activation of caspase-1. Activated caspase-1 then proteolytically cleaves pro-IL1β and pro-IL18 resulting in the secretion of their biologically active forms, the pro-inflammatory cytokines IL1β and IL-18, which induce an anti-microbial response [65]. NLRP3 inflammasome activation requires two signals, and alum has been shown to provide the second signal which directly activates NLRP3. The first signal is provided by endogenous or microbial antigens that activate NF-κB and induce NLRP3 expression [64, 65]. Antigens that provide the first signal include lipopolysaccharide (LPS), muramyl dipeptide (MDP), bacterial RNA, the dsRNA analog polyI:C, and microbial lipopeptide [65]. While alum has been shown to activate the NLRP3 inflammasome pathway, other studies have demonstrated that alum can enhance the immune response in the absence of inflammasome activation [66].

A third means by which alum has been shown to function as an adjuvant is through increasing antigen uptake by DCs [67, 68]. To this end, alum was shown to interact directly with lipids on the cell surface of DCs, specifically sphingomyelin and cholesterol, promoting lipid sorting in the plasma membrane. The lipid sorting activated an endocytic response which led to increased antigen uptake. In addition to antigen uptake, the lipid sorting also triggered Syk and phosphoinositide 3-kinase (PI3K) signaling pathways inducing an immune response [67, 68]. Interestingly, the study demonstrated that while alum facilitates increased antigen uptake, the adjuvant itself does not enter the cell, rather it remains at the plasma membrane [67, 68].

Regardless of the mechanism, alum adjuvants are known to induce robust antibody responses, and have been shown to promote a T-helper 2 (T_H2) type immune response rather than a T-helper 1 (T_H1) type immune response, referring to the subset of $CD4^+$ T cells being activated [56, 57, 64]. T_H2 responses are associated with IL-4 production and the generation of IgG1 and IgE antibodies, whereas T_H1 responses are associated with the production of high levels of IFNγ, the secretion of IL-12, and the generation of IgG2 antibodies [56, 57, 64]. T_H1 responses are known to operate through Toll-like receptors (TLRs,) and it has been demonstrated that alum does not directly activate TLRs [56].

The Immune Response to Vaccination

The immune response to vaccination is initiated by the recognition of foreign antigen by innate immune cells such as dendritic cells (DCs),

macrophages, and neutrophils. DCs and macrophages function as antigen presenting cells (APCs) [61, 69], and DCs in particular, have been shown to be important for the recognition of vaccine antigens, which they detect via germline-encoded pathogen receptors known as Pattern Recognition Receptors (PRRs) [70]. PRRs recognize conserved microbial structures called Pathogen Associated Molecular Patterns (PAMPs) found on a variety of microbes, such as bacteria, viruses, yeast, fungi, protozoa, and parasites [61, 69, 71]. PAMPS include, but are not limited to, peptidoglycans, lipopolysaccharides, and viral RNA and DNA [61, 69]. Several classes of PRRs have been identified and include: Toll-like receptors (TLRs), nucleotide-oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs) which recognize a variety of pathogens; as well as retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs) which recognize viral nucleic acids [39, 72-75].

TLRs are the most widely studied PRRs [69]. Eleven TLRs have been identified in humans and 13 TLRs have been identified in mice, with TLRs 1-9 being conserved between humans and mice [69, 71]. TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface and recognize PAMPs from bacteria, fungi, and protozoa. TLRs 3, 7, 8, and 9 are located in cellular endosomal compartments and recognize bacterial and viral nucleic acids [69]. Influenza viruses are recognized by TLR3, which senses dsRNA, and TLR7/8 which senses ssRNA [76]. TLRs are type 1 transmembrane glycoproteins that consist of extracellular leucine rich repeats (LRRs) important for pathogen recognition, and a

cytoplasmic Toll/interleukin-1 receptor (TIR) domain required for signaling [69, 77].

NLRs recognize cytosolic PAMPs as well as host derived molecules associated with danger or stress referred to as Danger Associated Molecular Patterns (DAMPs) [69, 78]. DAMPs include molecular crystals, reactive oxygen species, potassium efflux, and ATP [78]. Twenty three NLRs have been identified in humans and approximately 34 have been identified in mice. NOD1 and NOD2 are among the most well studied members of the NLR family [79]. Additionally, NLRP1, NLRP3, and NLRC4 are NLR family members that form inflammasomes [74]. Structurally, NLRs consist of 3 domains: a C-terminal leucine rich repeat (LRR) domain which is important for the recognition of PAMPs and DAMPs; an N-terminal Caspase Recruitment Domain (CARD) important for signaling; and a centrally located nucleotide-binding oligomerization domain (NBD or NACHT) also required for signaling [69, 79].

CLRs are a large superfamily of proteins that contain one or more C-type lectin-like domain (CTLD), and they are divided into 17 groups based on functional and structural characteristics [72, 74]. The term C-type lectin comes from the original observation that their activities were calcium dependent; however, some CLRs have since been identified to function in a calcium independent manner [72]. Structurally, CLRs contain at least one carbohydrate recognition domain (CRD); however, not all CLRs bind carbohydrates [72, 73]. CLRs exist as both transmembrane and cytosolic receptors. Those that function as PRRs are the transmembrane receptors which recognize carbohydrates,

specifically mannose, fucose, and glucan structures. With carbohydrates as their ligands, CLRs recognize most types of human pathogens [72, 73]. There are two main ways in which CLRs induce signaling cascades. The first is through adaptor molecules that contain immunoreceptor tyrosine-based activation motifs (ITAMs), such as FcR*γ* chains. The second is through the activation of protein kinases or phosphatases that interact, either directly or indirectly, with the cytoplasmic tails of the receptors [73, 74]. CLR family members include DC-SIGN, Dectin-1, Dectin-2, Mincle, and CLEC5A [73, 74].

In general, the activation of PRRs is followed by signaling cascades which lead to the activation of transcription factors, such as NF-κB, followed by the regulation of cytokine gene expression and the secretion of pro-inflammatory cytokines such as IL-6, IL-12, IL-1β, and TNF-α [39, 72-75, 77, 79, 80]. Following recognition, antigens are internalized by DCs and degraded into small peptides. The peptides are processed and presented to T cells via major histocompatibility complex (MHC) class I or class II molecules which interact with the T cell receptor (TCR) on naïve T cells [61, 70]. Activated DCs also up-regulate the expression of co-stimulatory cell-surface molecules such as CD80/CD86 and CD40, which bind their cognate receptors CD28 and CD40L, respectively, on the surface of naïve T cells [61, 70]. Antigen-MHC interaction with the TCR, together with co-stimulatory molecule interaction with cognate receptors, provides a dual signal leading to the activation of naïve T cells.

Activated T cells produce cytokines such as IL2, IL-4, IL-7, IL-15, and IFN-γ which enhance T cell proliferation (clonal expansion) and survival, have

important anti-microbial functions, and influence B cell differentiation and antibody production [60, 61, 70]. T cells are categorized based on their function, with the most well characterized populations consisting of helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) [60]. Cytotoxic T cells eliminate infected cells, while helper T cells assist macrophages in the elimination of pathogens and also stimulate B cells responses and antibody production [60, 70].

B cells are the only type of cells that produce antibodies. Antibodies recognize microbial antigens, bind to the microbe, block the ability of the microbe to infect host cells, and target the microbe for destruction by various mechanisms [60]. B cells secrete five different types of antibodies in response to different types of pathogens, and each class of antibody has a distinct structure, function, and location within the body. The different classes of antibodies are referred to as isotypes and consist of IgG, IgE, IgM, IgA, and IgD, with the prefix “Ig” referring to immunoglobulin, another term for antibody indicative of protein structure [60, 81].

IgG is the main class of antibody found in the serum and is important for the response to viruses and bacteria, and it is the only antibody isotype that can cross the placental barrier providing passive immunity to the fetus. There are four subclasses of IgG in humans (IgG1, IgG2, IgG3, and IgG4) [60, 81]. Influenza vaccines, as with most vaccines, provide protection by inducing antibody responses, specifically IgG responses [39, 82].

Purpose of Study

Highly pathogenic avian influenza viruses are an emerging threat to public health with the potential to cause a pandemic. While a split virion subunit vaccine has been developed according to the process used for seasonal vaccines, it demonstrated low immunogenicity in clinical trials. The immunological mechanisms behind vaccination are incompletely understood. This dissertation includes the results from two original studies that examined differences in the immune response to an inactivated subunit seasonal vaccine and an inactivated subunit vaccine for H5N1 with and without the addition of an alum adjuvant. A better understanding of the mechanism by which vaccines confer protection could lead to the development of more effective vaccines.

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Figure Legends

Figure 1. Cartoon representation of an influenza virus. The influenza viral genome is comprised of eight segments of single-stranded RNA surrounded by a lipid envelope. The hemagglutinin (HA) and neuraminidase (NA) glycoproteins radiate outward from the lipid envelope.

Figure 2. Influenza subunit vaccine manufacturing process. A. The influenza virus that the vaccine will target (in this example, A/Vietnam/1203/2004 (H5N1) (VN1203)) is grown in fertilized chicken eggs along with the influenza virus A/Puerto Rico/8/1934 (H1N1) (PR8). While replicating in the eggs, genome segments from the two viruses will reassort forming hybrid viruses that contain segments from both viruses. The target virus for the vaccine will contain the HA and NA segments from VN1203 and all other segments from PR8. Antibodies against the HA and NA segments from PR8 can be included in the culture process to facilitate selection of the target virus. **B.** Once the target virus has been generated, it is again grown in fertilized chicken eggs. The virus is then harvested from the eggs and inactivated or “killed” with formaldehyde. Following purification in a linear sucrose gradient, the virus is chemically disrupted producing a split virus. The virus is then further purified so that it contains only the HA and NA proteins, which are then suspended in sodium phosphate-buffered isotonic sodium chloride solution creating the final vaccine preparation.

Figure 3. Antibody titers following influenza vaccination in human clinical trials. When tested in human clinical trials, 70% of Fluzone® 2009/2010 TIV vaccine recipients and 95%-100% of Fluzone® 2009 H1N1 pandemic vaccine recipients developed protective antibody titers greater than 1:40. Only 22% of H5N1 inactivated subunit vaccine recipients developed protective antibody titers greater than 1:40. This graph is adapted from Plennevaux, E., et al., Lancet, 2010 [47], Xie, H., et al., PLosONE, 2011 [48], and Treanor, J.J. et al., New England Journal of Medicine, 2006 [11].

Figures

Figure 1

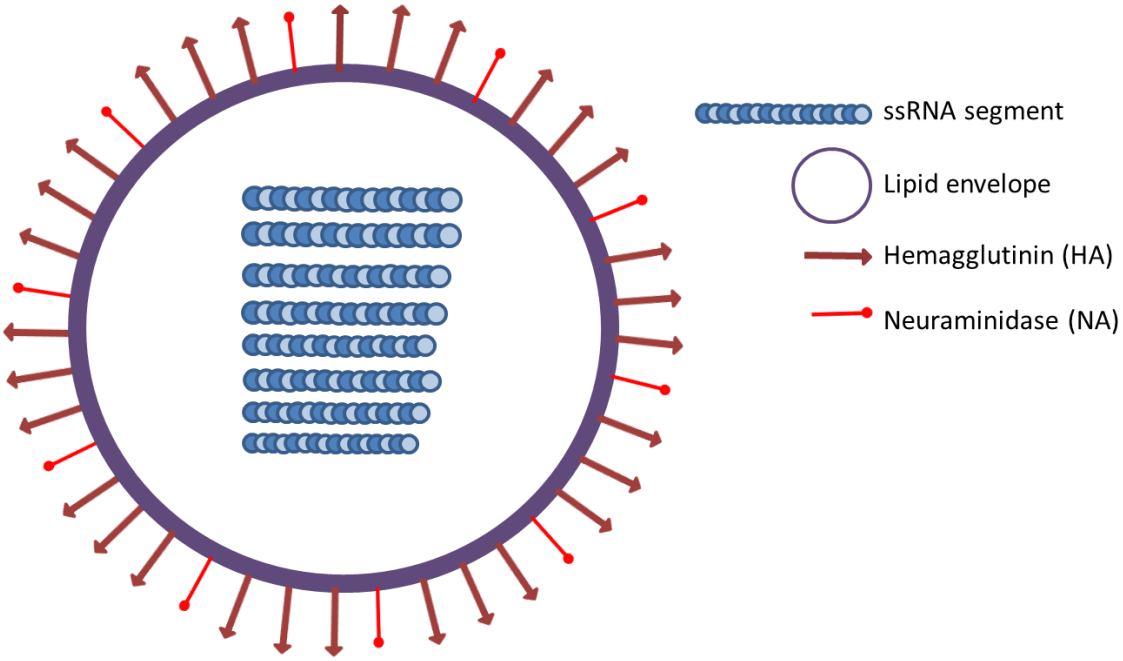


Figure 2

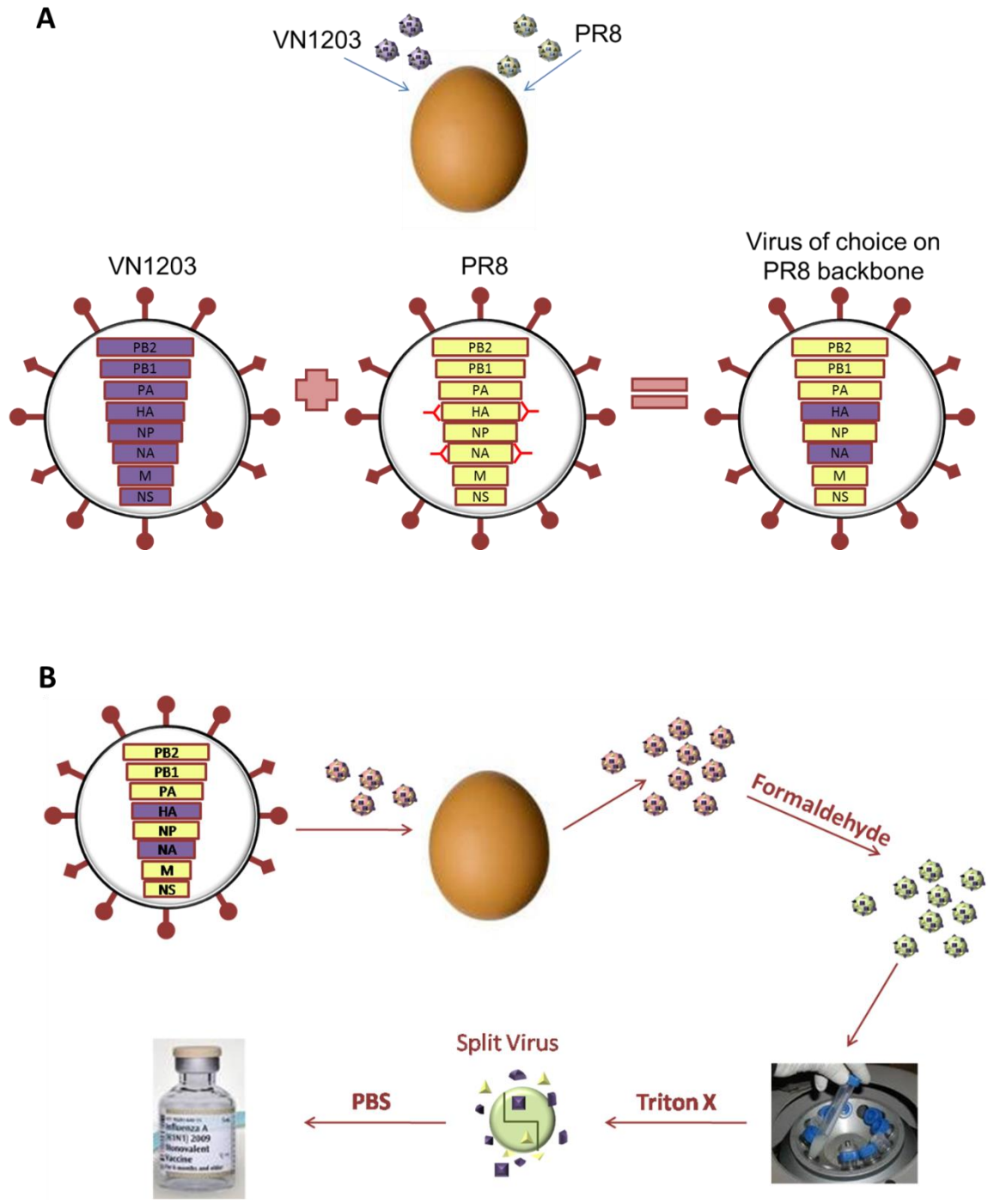
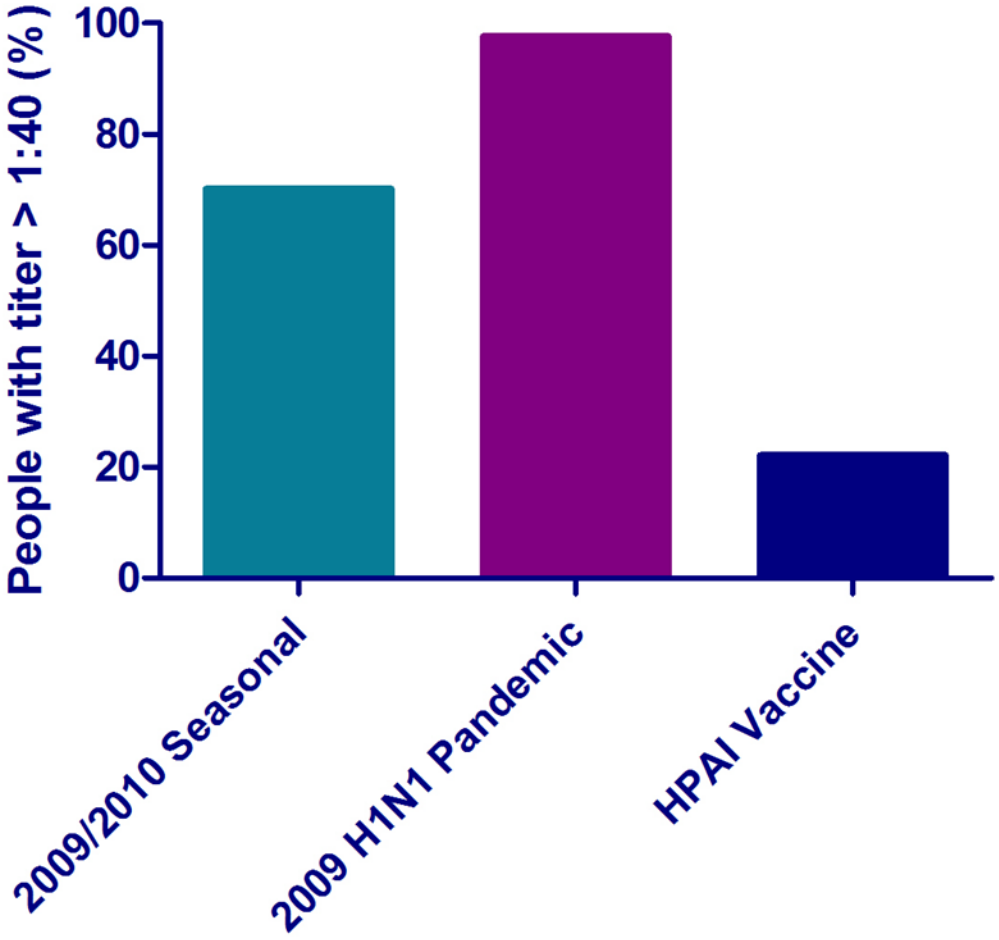

A
VN1203
PR8
VN1203
PR8
Virus of choice on PR8 backbone
PB2
PB1
PA
HA
NP
NA
M
NS
B
Formaldehyde
Split Virus
PBS
Triton X

Figure 3


People with titer > 1:40 (%)
100
80
60
40
20
0
2009/2010 Seasonal
2009 H1N1 Pandemic
HPAI Vaccine

CHAPTER TWO:

Attenuated antigen presenting cell function in BALB/c mice following HPAI vaccination results from impaired C-type lectin receptor signaling

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Abstract

Highly pathogenic avian influenza (HPAI) H5N1 has an approximate 60% fatality rate in humans. In the United States, a vaccine for H5N1 has been manufactured and stockpiled using FDA approved methods for seasonal influenza vaccines. In clinical trials, the H5N1 vaccine was shown to be less immunogenic compared to seasonal vaccines. Herein, we utilized a BALB/c mouse model to elucidate the underlying immune mechanisms by which immunization with the HPAI vaccine results in attenuated immunogenicity. Mice that received the seasonal vaccine produced a robust neutralizing antibody response whereas no neutralizing antibodies were detected following HPAI vaccination. Bone marrow derived dendritic cells (BMDCs) cultured with the seasonal vaccine produced significantly higher levels of the pro-inflammatory cytokines IL-6, IL-12, and TNF-α compared to those treated with the H5N1 vaccine, indicating increased antigen presenting cell (APC) activation. Neither vaccine stimulated Toll-like receptors nor NOD-like receptors; therefore, as C-type lectin receptors have been implicated as a class of pattern recognition receptors involved in innate immunity and immunization their involvement was examined. Pre-treatment with mannan or mannose diminished cytokine induction by DCs in a dose dependent manner following seasonal but not HPAI vaccine treatment, suggesting a role for C-type lectin receptors in DC activation by the seasonal influenza vaccine. BMDCs pre-treated with the H5N1 vaccine displayed decreased cytokine production following treatment with either the seasonal vaccine or mannan suggesting that the HPAI vaccine is binding to the receptors

but not inducing a signaling cascade. These findings provide a novel role for C-type lectin receptors in influenza vaccination, and a potential mechanism for attenuated APC function following H5N1 vaccination and may explain the decrease in immunogenicity of the currently approved HPAI vaccine.

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 is an emerging infectious virus associated with occasional illness and death in humans. Recent outbreaks have occurred in Cambodia, Bangladesh, China, and Egypt [1, 2]. So far, in 2013 documented cases have resulted in a 78% mortality rate, with the overall mortality rate being slightly lower at 60% [1]. Symptoms in affected people include fast progressing pneumonia, respiratory distress syndrome, damage to the central nervous system, and multi-organ failure, and death usually occurs within 10 days of symptom onset in fatal cases [3, 4]. The high lethality rate and occasional infection in humans make an H5N1 pandemic feasible; therefore, an effective vaccine targeting H5N1 is a high priority, especially in affected countries [5-7].

As part of the pandemic preparedness plan, the United States has stockpiled 20 million doses of an inactivated subunit vaccine targeting H5N1 [8]. The H5N1 vaccine is manufactured employing the same techniques used to generate seasonal influenza subunit vaccines [9]. In clinical trials, seasonal influenza subunit vaccines have demonstrated the ability to produce protective antibody titers, which are considered to be greater than 1:40, in the majority of recipients. One study demonstrated that 70% of recipients developed protective titers to the 2009/2010 seasonal vaccine, and another showed 95%-100% of recipients developed protective titers to the 2009 novel H1N1 influenza vaccine [10, 11]. When the H5N1 vaccine was tested in clinical trials, however, only 22% of recipients developed protective antibody titers [12].

The use of vaccines is one of the most effective means for preventing infectious diseases [13]. Recognition of foreign antigen by innate immune cells, such as dendritic cells (DCs), initiates the immune response to vaccination. DCs function as antigen presenting cells (APCs) by responding to, processing, and presenting antigen to T cells [14]. DCs detect antigen via germline-encoded pathogen receptors known as pattern recognition receptors (PRRs). PRRs recognize conserved molecular structures found on groups of pathogens referred to as pathogen-associated molecular patterns (PAMPs) [15, 16]. PRRs include Toll-like receptors (TLRs), nucleotide-oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs) which recognize a variety of pathogens, as well as retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs) which recognize viral nucleic acids [13, 15-18]. The activation of PRRs is followed by signaling cascades which lead to the activation of transcription factors and the subsequent regulation of cytokine gene expression, and this process helps shape effective adaptive immune responses [13, 15-18].

The specific mechanisms by which vaccines induce protective immunity remain to be elucidated [13]. Herein, we utilized a vaccination model in BALB/c mice to study the mechanism by which the seasonal influenza vaccine elicits a more robust antibody response than the H5N1 vaccine. We show that treatment with the seasonal vaccine results in increased total and functional antibody production in BALB/c mice. *In vitro*, BMDCs treated with the seasonal vaccine produced higher levels of pro-inflammatory cytokines compared to cells treated with the H5N1 vaccine; however, APC activation was not via TLRs or NLRs. The

seasonal vaccine stimulated CLRs to induce pro-inflammatory cytokine production, whereas the H5N1 vaccine bound CLRs but did not initiate APC activation. These findings describe a novel role for C-type lectin receptors in influenza vaccination, as well as, a potential mechanism for attenuated APC function following H5N1 vaccination providing insight into the development of more immunogenic vaccines targeting HPAI.

Materials and Methods

Mice

Male BALB/c mice, aged six to eight weeks, were purchased from the National Cancer Institute at Frederick (NCI-Frederick, Bethesda, MD), and were held for 21 days for quarantine and acclimation. Mice were housed four per cage in microisolator cages under identical husbandry conditions and fed certified commercial feed. All animal studies were approved by the Lovelace Respiratory Research Institute Internal Animal Care and Use Committee.

Vaccines

The following reagents were obtained through the National Institutes of Health (NIH) Biodefense and Emerging Infections (BEI) Research Resources Repository, NIAID, NIH: Monovalent Influenza Subvirion Vaccine, rg/A/Vietnam/1203/2004, NR-4143 and Fluzone® Influenza Virus Vaccine, 2009-2010 Formula, NR-19879 at concentrations of 30ug/ml hemagglutinin protein.

Vaccines were diluted to 0.6ug/ml HA protein within 0.05ml physiological saline immediately prior to vaccination.

Viruses

Influenza virus A/Brisbane/59/2007 subtype H1N1 was obtained from the Centers for Disease Control and Prevention (Atlanta, GA) as low-passage stock. The virus was passaged once in 10-day old embryonated chicken eggs to generate the master stock and then twice in eggs and once in Madin-Darby Canine Kidney (MDCK) cells to generate the virus for all subsequent uses. Aliquots of 0.5ml to 1ml were stored at -80°C. After storage, the virus was determined to have a concentration of 4.85×10^6 tissue culture infectious dose 50 ($TCID_{50}$).

Influenza virus A/Vietnam/1203/2004 (VN1203) subtype H5N1 was obtained from the Centers for Disease Control and Prevention (Atlanta, GA) as low-passage stock. The virus was passaged once in 10-day old embryonated chicken eggs to generate the master stock and then twice in eggs to generate the virus for all subsequent uses. Aliquots of 0.5ml to 1ml were stored at -80°C. After storage, the virus was determined to have a concentration of 1.4×10^8 plaque forming units (pfu/ml), 5.8×10^8 50% tissue culture dose ($TCID_{50}$/ml), and 1×10^8 50% egg infectious dose (EID_{50}/ml). Influenza A/Vietnam/1203/2004 is a Risk Group 3 pathogen and all experiments involving this virus were carried out in a Biosafety Level 3 (BSL 3) containment facility at the Lovelace Respiratory Research Institute and approved by the Institutional Biosafety Committee.

Vaccination and Serology

Mice (n = 6 per group, except seasonal IM where n = 18 as this group was performed separately) were given either a subcutaneous (SC) or intramuscular (IM) injection of 0.6ug seasonal influenza or HPAI vaccine in the gastrocnemius muscle. Control animals received injections of 0.05ml physiological saline either IM or SC. Blood for serum was collected in serum separator collection tubes 3 weeks following vaccination by cardiac puncture, and incubated at room temperature for 30 minutes before centrifugation at 2500rpm for 10 min. Serum was collected and kept at -20°C until analyzed. The neutralization assay was modified from previously described procedures [19]. Briefly, serum samples were treated with receptor-destroying enzyme (RDE (II)), (Enka-Seiken, Tokyo, Japan), at a ratio of 1:1 followed by heat inactivation at 56°C for 45 minutes. The serum samples were incubated with 2×10^3 $TCID_{50}$ A/Brisbane/59/2007 or A/Vietnam/1203/2004 at 37°C for one hour. After incubation, MDCK cells plated in 96 well plates were inoculated in triplicate with the samples using a 2-fold dilution and incubated at 37°C for three days. The plates were then scored for cytopathic effect (CPE), and neutralizing antibody titers were determined as the highest serum dilution at which no CPE occurred.

Total vaccine specific IgG was determined by ELISA using modified previously described techniques [20]. Briefly, 96-well plates were coated with 1ug/ml seasonal or H5N1 vaccine in coating buffer (eBioscience, San Diego, CA. Catalog # 00-0044-59) and incubated overnight at 4°C. Following incubation, plates were washed with 1x PBS containing 0.05% Tween® 20 (Sigma-Aldrich,

St. Louis, MO). Plates were blocked with 1x assay diluent (eBioscience. Catalog #00-4202) for 1 hour at room temperature before incubation with serum samples from vaccinated mice, diluted 1:10,000 in assay diluent, for 2 hours at room temperature. Following additional washes, total vaccine specific IgG was determined by incubation with horse anti-mouse IgG (H & L) conjugated to HRP (Cell Signaling Technology, Danvers, MA) at a 1:250 dilution in assay diluent for 1 hour at room temperature. Following a wash step, TMB substrate solution (eBioscience) was added to develop color, and 2N H_2SO_4 was added to stop the reaction. Plates were read at 450nm absorbance, and optical density (OD) values are reported.

Primary Bone Marrow Cell Isolation and Culture

Bone marrow cells were collected and cultured using previously described methods [21]. Briefly, bone marrow cells were isolated from the femurs and tibiae of BALB/c mice and cultured in complete RPMI media (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen), 1x penicillin/streptomycin (Invitrogen), and 25ng/ml GM-CSF (Cell Signal Technology, Danvers, MA) for 5-7 days at 37°C with 5% CO_2.

Cytokine Analysis

BMDCs in complete RPMI media were treated with either seasonal influenza vaccine or H5N1 vaccine at a concentration of 0.6ug/ml (HA protein), 100ng LPS-EB (InvivoGen, San Diego, CA), or media alone for 24 hours. Cell

culture supernatants were analyzed for IL-6, IL-12, and TNF-α production using ELISA kits purchased from eBioscience according to the manufacturer's instructions.

TLR/NLR Expression Assay

THP1-XBlue cells were purchased from InvivoGen and cultured according to the manufacturer's recommendations. The cells express TLRs 1-9 as well as NOD1/2 and are stably transfected with an NF-κB and AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Upon TLR/NLR stimulation, THP1-XBlue cells activate the transcription factors NF-κB and AP-1, and SEAP is secreted. SEAP can be detected by QUANTI-Blue (InvivoGen), which turns blue/purple in the presence of SEAP. Heat killed *Listeria monocytogenes* (HKLM) (InvivoGen) was used as a positive control for TLR2 and LPS-EB (InvivoGen) was used as a positive control for TLR4. The TLR assay was conducted according to the manufacturer's instructions. Briefly, cells were plated at 200,000 cells per well in a 96 well plate. Cells were treated with 0.6ug seasonal influenza vaccine, 0.6ug H5N1 vaccine, 10ul of 1ug/ml LPS, or 10ul of reconstituted HKLM (10^9 cells/mL) for 24 hours. Following the overnight incubation, cell supernatants were incubated with QUANTI-Blue for 2 hours and SEAP levels were determined using a spectrophotometer at 620-655 nm.

C-Type Lectin Receptor Binding Assays

BMDCs were pre-treated with increasing concentrations of either mannan (0ug/ml, 12.5ug/ml, 25ug/ml, 50ug/ml), mannose (0mg/ml, 0.5mg/ml, 5mg/ml, 10mg/ml, 20mg/ml), or HPAI vaccine (0ug/ml, 0.5ug/ml, 1ug/ml, 1.5ug/ml, 2ug/ml) for 30 min at 4°C. Cells pre-treated with mannan or mannose were treated with either seasonal influenza vaccine or HPAI vaccine at 0.6ug/ml overnight. Cells pre-treated with HPAI vaccine were treated with seasonal vaccine at 0.6ug/ml or mannose at 50ug/ml overnight. Cytokine expression was determined by ELISA as described previously.

Results

Antibody Titers in Vaccinated Mice

Human clinical trials have demonstrated that robust protective antibody responses are produced in response to seasonal influenza vaccination, but vaccination with the H5N1 vaccine leads to a much weaker antibody response [11, 12]. To examine differences in antibody production following vaccination, six to eight week old BALB/c mice were given either a subcutaneous (SC) or intramuscular (IM) injection of 0.6ug seasonal influenza or HPAI vaccine. Functional antibody titers were assessed by neutralization assay (Figure 1a) and total vaccine specific IgG was determined by ELISA (Figure 1b). Mice that received the seasonal vaccine by either route of vaccination had significantly increased levels of neutralizing antibodies compared to mice that received the HPAI vaccine, in which the amount of neutralizing antibodies was below the level

of detection. Eighty three percent of mice that received the seasonal vaccine SC, and 86% of mice that received the seasonal vaccine IM, had neutralizing antibody titers above the level considered protective (>1:40). When comparing total vaccine specific IgG, antibody titers in mice that received the seasonal vaccine mirrored the levels of neutralizing antibodies, and were significantly higher than in mice that received the HPAI vaccine. While neutralizing antibodies were not detected in mice that received an IM injection of the HPAI vaccine, low levels of total vaccine specific IgG were observed. Levels of total vaccine specific IgG in mice vaccinated IM with the HPAI vaccine were significantly higher when compared to mice that received a SC injection of the HPAI vaccine.

APC Activation Following Vaccine Treatment

It is well established that an effective adaptive immune response follows an efficient innate immune response, and the innate immune response to vaccination is initiated by recognition of foreign antigen by APCs, such as DCs. Following interaction with antigen, DCs become activated and produce pro-inflammatory cytokines such as IL-6, IL-12, and TNF-α. Pro-inflammatory cytokine secretion is crucial for the activation and differentiation of T cells and the progression of the immune response [14, 22]. Differences in DC activation were evaluated *in vitro* by assessing pro-inflammatory cytokine production following vaccine treatment. Bone marrow derived dendritic cells (BMDCs) from BALB/c mice were treated with either the seasonal or HPAI vaccine for 24 hours, and cytokine production was analyzed by ELISA. BMDCs treated with the seasonal

vaccine produced significantly higher levels of IL-6, IL-12, and TNFα compared to BMDCs treated with the HPAI vaccine, which did not induce cytokine production (Figure 2a-c). LPS was used as a positive control and resulted in the production of similar, or greater, levels of IL-6, IL12, and TNFα when compared to the seasonal vaccine.

DCs detect antigen via germline-encoded pathogen receptors known as Pattern Recognition Receptors (PRRs), specifically Toll-like receptors (TLRs) [22, 23]. TLRs are a class of PRRs that reside on the cell surface or in the endosomal compartments of innate immune cells. One of the most important consequences of TLR signaling is the transcriptional regulation of inflammatory genes, such as IL-6, IL-12, and TNF-α [24]. Influenza viruses activate DCs by signaling through TLRs, specifically the endosomal TLR3 and TLR7/8 [25-27]. To determine whether the influenza vaccine also signals through TLRs, a THP-1 reporter cell line expressing TLRs1-9 and NOD1/2 was utilized. THP-1 reporter cells were treated with either the seasonal or HPAI vaccine. Heat-killed *Listeria monocytogenes* (HKLM) and LPS were used as positive controls for TLR-2 and TLR-4 respectively. Neither vaccine treatment stimulated TLRs nor NOD1/2, showing levels of SEAP expression similar to that of cells treated with media alone (Figure 3). These data demonstrate that unlike influenza viruses, inactivated subunit vaccines targeting influenza do not signal through TLRs or NLRs.

C-Type Lectin Receptor Signaling Following Vaccine Treatment

C-type lectin receptors (CLRs) have been implicated as a class of PRRs important for antigen recognition, internalization, and presentation to T cells [18, 28-30]. The signaling cascades following pathogen binding to CLRs result in the production of pro-inflammatory cytokines [15, 18, 29]. To assess whether influenza vaccines are binding to CLRs, BMDCs were pre-treated with increasing concentrations of mannan, a CLR agonist, followed by treatment with either the seasonal or HPAI vaccine, and cytokine expression was assessed by ELISA. As expected, cells treated with the seasonal vaccine in the absence of mannan induced cytokine secretion, whereas cells treated with the HPAI vaccine in the absence of mannan did not produce cytokines. In cells treated with the seasonal vaccine, as the concentration of mannan increased, the production of the cytokines IL-6, IL-12, and TNFα decreased in a dose dependent manner (Figure 4a-c). This data suggests that mannan and the seasonal vaccine are binding to, and signaling through, the same receptors. Interestingly, in BMDCs treated with the HPAI vaccine, as the concentration of mannan increased, the expression of the cytokines IL-6, IL-12, and TNFα also increased but remained lower than cytokine levels produced by cells treated with mannan alone, suggesting that the HPAI vaccine is binding CLRs but not inducing a signaling cascade.

In order to more clearly demonstrate that the seasonal influenza vaccine is binding C-type lectin receptors, since mannan alone induces cytokine expression, mannose was used to block CLRs. CLRs recognize high-mannose structures that are able to induce receptor aggregation and consequent signaling.

The mannose monomer binds CLRs, but due to differences in valency, does not trigger receptor aggregation [15, 18, 31]. Following pre-treatment with mannose, BMDCs were treated with either the seasonal or HPAI vaccine. As with the mannan pre-treatment, in cells treated with the seasonal vaccine, as the concentration of mannose increased, the expression of the cytokines IL-6, IL-12, and TNF-α decreased in a dose dependent manner (Figure 5a-c). Again, this suggests that the seasonal vaccine is activating CLRs. As expected, treatment with the HPAI vaccine did not result in cytokine production, as this was previously demonstrated in Figure 2.

To further assess the possibility that the HPAI vaccine binds CLRs without inducing a signaling cascade, BMDCs were pre-treated with increasing concentrations of HPAI vaccine following treatment with either seasonal vaccine or mannan, and cytokine expression was assessed by ELISA. In both BMDCs treated with the seasonal vaccine or mannan, as the amount of HPAI vaccine increased, the expression of the cytokines IL-6, IL-12, and TNFα decreased in a dose dependent manner (Figure 6a-c). This data further implicates CLRs as the receptors engaged by the influenza vaccines and demonstrates that the HPAI vaccine is binding to the receptors but not initiating a signaling cascade.

Discussion

Despite utilizing similar techniques for the production of seasonal influenza subunit vaccines, the H5N1 subunit vaccine resulted in lower immunogenicity when tested in clinical trials [9-12]. Vaccination is one of the

most effective methods for preventing infectious diseases, and while the general immune response to vaccination is understood, the precise immunological mechanisms behind how vaccines work remain to be elucidated [13]. In the current study, we found that the 2009/2010 seasonal influenza subunit vaccine stimulated the immune system by binding to C-type lectin receptors and initiating signaling cascades that led to the production of pro-inflammatory cytokines, while the H5N1 vaccine bound C-type lectin receptors but did not initiate signaling.

The current study demonstrated that mice immunized with the seasonal vaccine produced higher levels of both neutralizing antibodies and total vaccine specific IgG compared to mice immunized with the H5N1 vaccine (Figure 1). The results presented herein demonstrated that 86% of mice that received an IM vaccination of the seasonal influenza vaccine produced neutralizing antibodies at a titer greater than 1:40. This is comparable to data from human clinical trials in which 70% of vaccine recipients developed protective levels of neutralizing antibodies [11]. The production of low levels of vaccine specific total IgG in mice that received an IM immunization of the H5N1 vaccine demonstrated that vaccine specific antibodies are being produced, but they are non-functional. The quantity of vaccine specific total IgG produced by mice immunized with the H5N1 vaccine is still significantly lower than in mice immunized with the seasonal influenza vaccine. As a good cellular immune response leads to a good adaptive immune response, this data suggests that the mechanism behind the attenuated function of the H5N1 vaccine occurs earlier in the immune response prior to antibody production. The ability of seasonal influenza subunit vaccines to stimulate the

production of antibodies in mice, as demonstrated herein, is in agreement with previously reported studies [32, 33]. These studies, as well as others conducted in mice, also examine cellular responses following immunization and demonstrate that our model is sufficient for investigating the immune response to influenza vaccination [32-36].

In the current study, BMDCs treated with seasonal influenza vaccine produced significantly higher levels of the pro-inflammatory cytokines IL-6, IL-12, and TNF-α compared to BMDCs treated with H5N1 vaccine, which did not induce cytokine production (Figure 2). The ability of the seasonal vaccine to induce cytokine expression in BMDCs is not surprising as previous studies, both *in vivo* and *in vitro*, have demonstrated that treatment with seasonal influenza subunit vaccines results in pro-inflammatory cytokine production [37-39]. The ability of the seasonal influenza vaccine to induce cytokine production demonstrates that the vaccine is able to stimulate APCs and initiate an immune response. Therefore, the absence of cytokine production by H5N1 vaccine treated BMDCs suggests that the HPAI vaccine is inefficient at activating APCs and results in attenuated immunogenicity.

The production of the pro-inflammatory cytokines IL-6, IL-12, and TNF-α is the result of the activation of transcription factors such as NF-κB, AP-1, and IRF3/7 downstream of PRR stimulation [13, 15-18, 25, 40]. Influenza viruses are known to activate TLR3 which recognizes dsRNA and TLR7/8 which recognizes ssRNA [25-27]. The current study demonstrated that neither the seasonal influenza vaccine nor the H5N1 vaccine activated TLRs or NLRs (Figure 3). At

first this seemed surprising, but as the manufacturing process for influenza subunit vaccines ensures that only protein and no genetic material is left in the vaccine preparation [41, 42], it was understandable that TLRs recognizing genetic material were not activated. Interestingly, vaccine components did not activate other TLRs, such as TLR2 or TLR4 which recognize various proteins found on microbes [43-45]. NOD1 and NOD2 are most associated with the recognition of bacterial pathogens [46]; however, one study showed the importance of NOD2 in the recognition of ssRNA and respiratory syncytial virus (RSV) [47], while another study demonstrated that bacteria-infected mice co-infected with murine norovirus displayed an augmentation in NOD1 and NOD2 signaling and the subsequent production of pro-inflammatory cytokines [48]. Similar to TLRs, in the case of viral pathogens, NOD1/2 appears to be recognizing viral nucleic acid which explains the absence of activation in our model.

As neither the seasonal vaccine nor the H5N1 vaccine activated TLRs or NLRs in the current study, CLRs were examined since the activation of these receptors also leads to the production of pro-inflammatory cytokines (Figure 2), and because CLRs have been identified as an important class of PRRs involved in antigen recognition and the initiation and regulation of the immune response to microbial infection [15, 18, 28-30]. Herein, pre-treatment of BMDCs with increasing concentrations of mannan (Figure 4) or mannose (Figure 5) led to a dose dependent decrease of pro-inflammatory cytokine production following

treatment with the seasonal influenza vaccine. This data implicates CLRs as the PRRs engaged by the seasonal influenza vaccine.

CLRs recognize pathogens via mannose, fucose, and glucan carbohydrate structures present on microbes, with mannose being important for the recognition of viruses [15]. CLR activation results in receptor aggregation and subsequent signaling pathways are induced by two general mechanisms. CLRs such as Mincle, Dectin 2, BDCA2, and CLEC5A induce signaling through molecules that contain immunoreceptor tyrosine-based activation motifs (ITAMs) such as F*c*R*y* or DAP12. CLRs such as Dectin 1, DC-SIGN, DCIR, and CLEC12A induce signaling through the activation of protein kinases or phosphatases, such as Src family kinases and the recruitment of the kinase Syk, which interact with the cytoplasmic domains of the receptors [15, 18]. These mechanisms can influence the design of future studies aimed to identify the specific CLRs involved in influenza vaccine recognition.

The HA, NA, and M2 surface proteins of the influenza virus are all targets of adaptive immunity [49], and the HA and NA proteins are the antigens present in the vaccine preparation [41]. The HA protein contains glycosylation sites [50] which could be recognized by CLRs. Interestingly, influenza viruses have not been shown to signal through CLRs; however, a recent study demonstrated that concurrent influenza infection and exposure to allergens resulted in an increase in CLR gene expression [51]. Our findings suggest that CLRs play an important role in immune recognition of inactivated subunit influenza vaccines.

In the current study, pre-treatment with increasing concentrations of either mannan or mannose resulted in a dose dependent decrease in cytokine production following treatment with the seasonal vaccine (Figures 4 and 5), providing a role for CLRs in vaccination against influenza. Following pre-treatment with mannan, cells that were treated with H5N1 vaccine secreted roughly the same amount of cytokines as cells treated with mannan alone (Figure 4). This data suggests that the H5N1 vaccine binds CLRs but does not initiate signaling. To substantiate this finding, BMDCs were pre-treated with increasing concentrations of H5N1 vaccine followed by treatment with either seasonal vaccine or mannan, and cytokine production again decreased in a dose dependent manner, confirming that the H5N1 vaccine is binding CLRs but not initiating a signaling cascade (Figure 6). These results provide a mechanism for the decreased immunogenicity of the H5N1 vaccine. The difference in the ability of the seasonal and H5N1 vaccines to activate CLRs suggests a variation in the glycosylation sites present on the HA proteins from the different viruses. Further studies elucidating the specific CLRs involved in influenza vaccine recognition will be important for advancing the knowledge behind the mechanisms of the immune response to influenza vaccines. Furthermore, identifying potential differences in glycosylation patterns on the HA proteins of the different viruses would contribute to the findings presented herein.

The use of vaccines is imperative for the control of infectious diseases; however, the mechanisms behind vaccine immunogenicity are incompletely understood [13]. Identifying the means by which specific vaccines confer

protection from pathogens will lead to the development of more effective vaccines. Our findings, presented herein, elucidate a mechanism by which the 2009/2010 seasonal influenza subunit vaccine gives rise to a protective antibody response whereas the H5N1 vaccine elicits an attenuated antibody response, and provide insight into the development of more immunogenic vaccines targeting HPAI.

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Figure Legends

Figure 1. Increase in vaccine specific antibody responses in BALB/c mice vaccinated with seasonal influenza vaccine but not HPAI vaccine. Male BALB/c mice were given either a subcutaneous (SC) or intramuscular (IM) injection of seasonal vaccine, HPAI vaccine, or saline. Sera were collected 3 weeks later and antibody titers determined by neutralization assay or ELISA. **A.** Neutralizing antibody titers in mice treated with seasonal vaccine SC (squares), HPAI vaccine SC (triangles), seasonal vaccine IM (circles), or HPAI vaccine IM (upside down triangles). Error bars indicate mean ± SEM (*p<.05, **p<.005, Student's t-test). **B.** Total vaccine specific IgG as determined by ELISA in mice treated with saline IM (closed circles), saline SC (squares), seasonal SC (triangles), HPAI SC (upside down triangles), seasonal IM (diamonds), or HPAI IM (open circles). Error bars indicate mean ± SEM (*p<.05, **p<.001, ***p<.0001, Student's t-test).

Figure 2. Increased cytokine production after treatment of BMDCs with seasonal vaccine but not HPAI vaccine. BMDCs were treated with seasonal vaccine (checkered bars), HPAI vaccine (white bars), LPS (striped bars), or media alone (black bars) for 24 hours. Cytokine production was determined by ELISA. **A.** IL-6 production in vaccine treated BMDCs expressed as mean ± SEM (***p<.0005, Student's t-test). **B.** IL-12 production by vaccine treated BMDCs expressed as mean ± SEM (**p<.005, Student's t-test). **C.** TNF-α production in

vaccine treated BMDCs expressed as mean ± SEM (****p<.0001, Student's t-test). Results represent three independent experiments.

Figure 3. Neither vaccine stimulates Toll-like or NOD-like receptors. THP1-XBlue reporter monocytes expressing TLRs 1-9 and NOD1/2 transfected with SEAP were treated with seasonal vaccine (checkered bars), HPAI vaccine (white bars), LPS (striped bars), HKLM (diagonal bars), or media alone (black bars) for 24 hours. Cell supernatants were then incubated in the presence of QUANTI-Blue for 2 hours and SEAP expression was determined using a spectrophotometer. LPS was used as a positive control for TLR4 and HKLM was used as a positive control for TLR2. Results represent two independent experiments.

Figure 4. Influenza vaccines bind the same receptors as mannan. BMDCs were pre-treated with increasing concentrations of mannan followed by treatment with either seasonal influenza vaccine or HPAI vaccine for 24 hours, and cytokine production was assessed by ELISA. **A.** IL-6 production by vaccine treated BMDCs pre-treated with increasing concentrations of mannan (p<.0001, Two-way ANOVA comparing all groups). **B.** IL-12 production by vaccine treated BMDCs pre-treated with increasing concentrations of mannan (p<.0001, Two-way ANOVA comparing all groups). **C.** TNF-α production by vaccine treated BMDCs pre-treated with increasing concentrations of mannan (p<.0001, Two-

way ANOVA comparing all groups). Results represent two or more independent experiments.

Figure 5. The seasonal influenza vaccine signals through C-type lectin receptors. BMDCs were pre-treated with increasing concentrations of mannose followed by treatment with either seasonal vaccine or HPAI vaccine for 24 hours, and cytokine production was assessed by ELISA. **A.** IL-6 production by vaccine treated BMDCs pre-treated with increasing concentrations of mannose ($p<.0001$, Two-way ANOVA comparing all groups). **B.** IL-12 production by vaccine treated BMDCs pre-treated with increasing concentrations of mannose ($p<.0001$, Two-way ANOVA). **C.** TNF-α production by vaccine treated BMDCs pre-treated with increasing concentrations of mannose ($p<.0001$, Two-way ANOVA). Results represent two independent experiments.

Figure 6. HPAI vaccine binds C-type lectin receptors without signaling. BMDCs were pre-treated with increasing concentrations of HPAI vaccine followed by treatment with either seasonal vaccine or mannan for 24 hours, and cytokine expression was assessed by ELISA. **A.** IL-6 production by vaccine treated BMDCs pre-treated with increasing concentrations of HPAI vaccine ($p<.05$, Two-way ANOVA comparing all groups). **B.** IL-12 production by vaccine treated BMDCs pre-treated with increasing concentrations of HPAI vaccine ($p<.0005$, Two-way ANOVA comparing all groups). **C.** TNF-α production by vaccine treated BMDCs pre-treated with increasing concentrations of HPAI

vaccine ($p<.0005$, Two-way ANOVA comparing all groups). Results were obtained from two independent experiments.

Figures

Figure 1

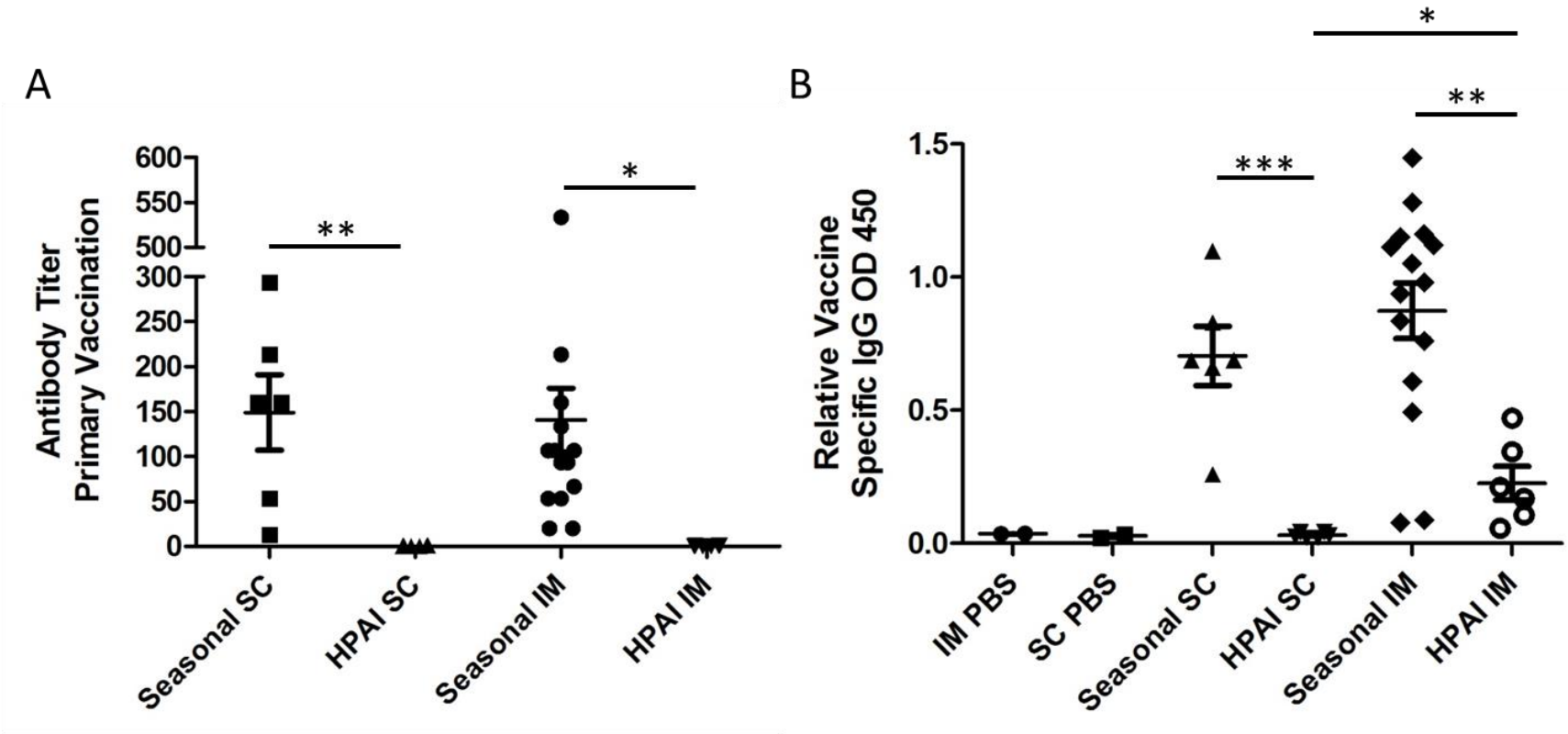

Figure 2

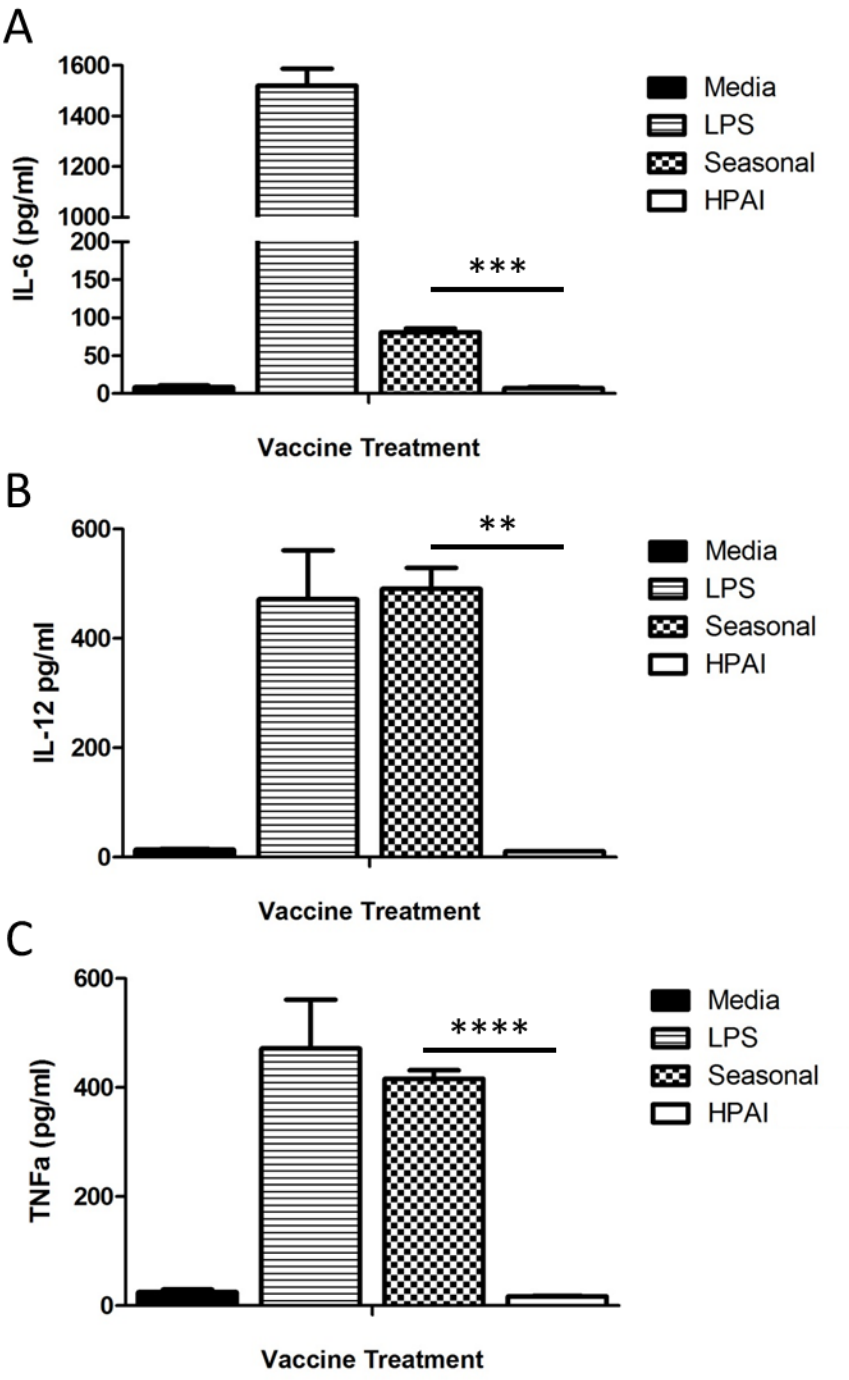
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Figure 3

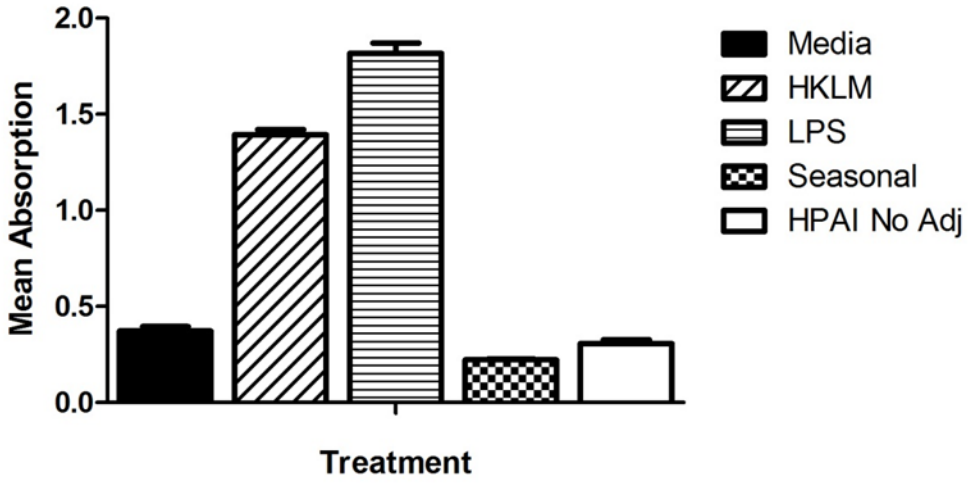
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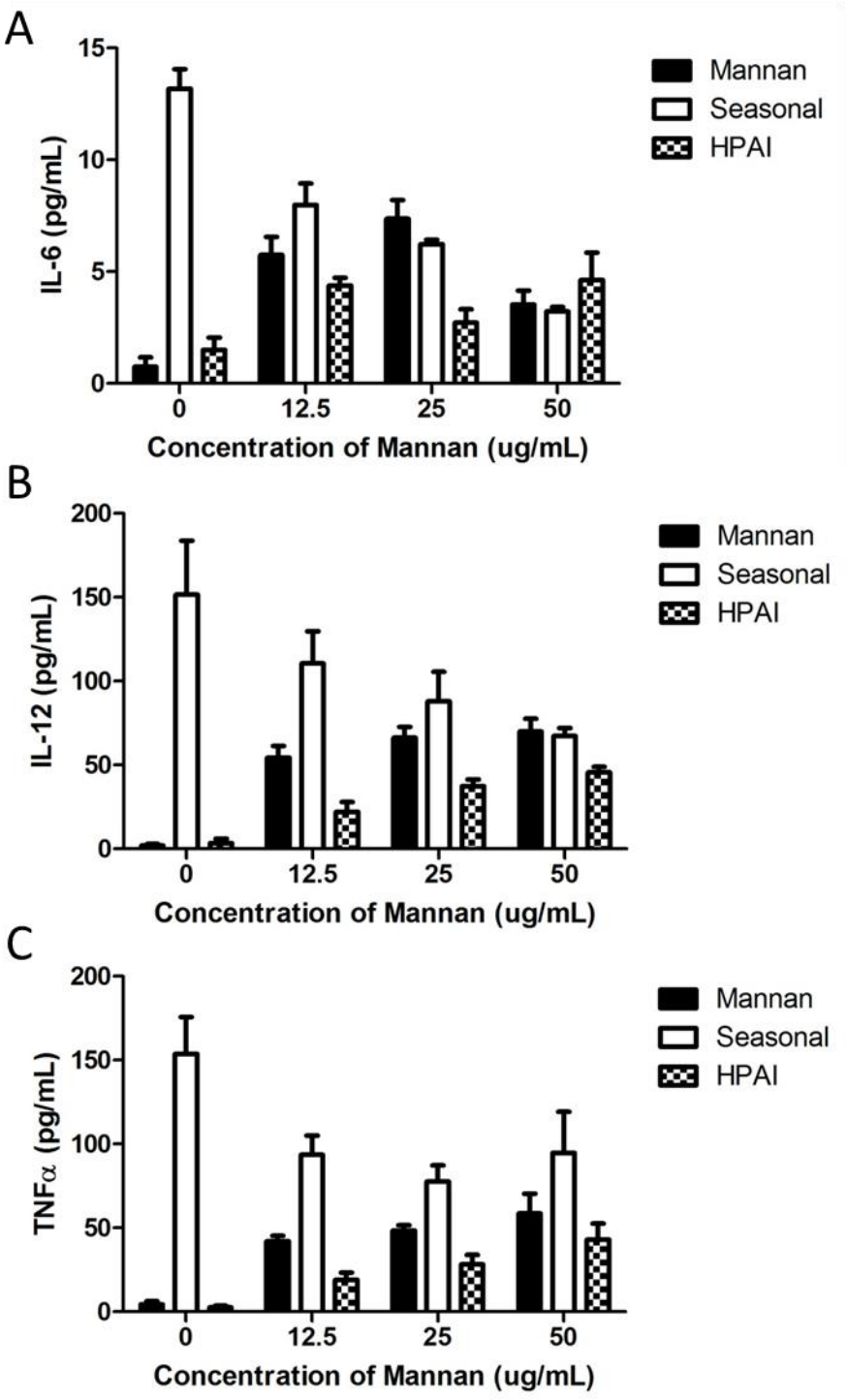
Figure 4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50
Concentration of Mannan (ug/mL)
Mannan
Seasonal
HPAI

Figure 5

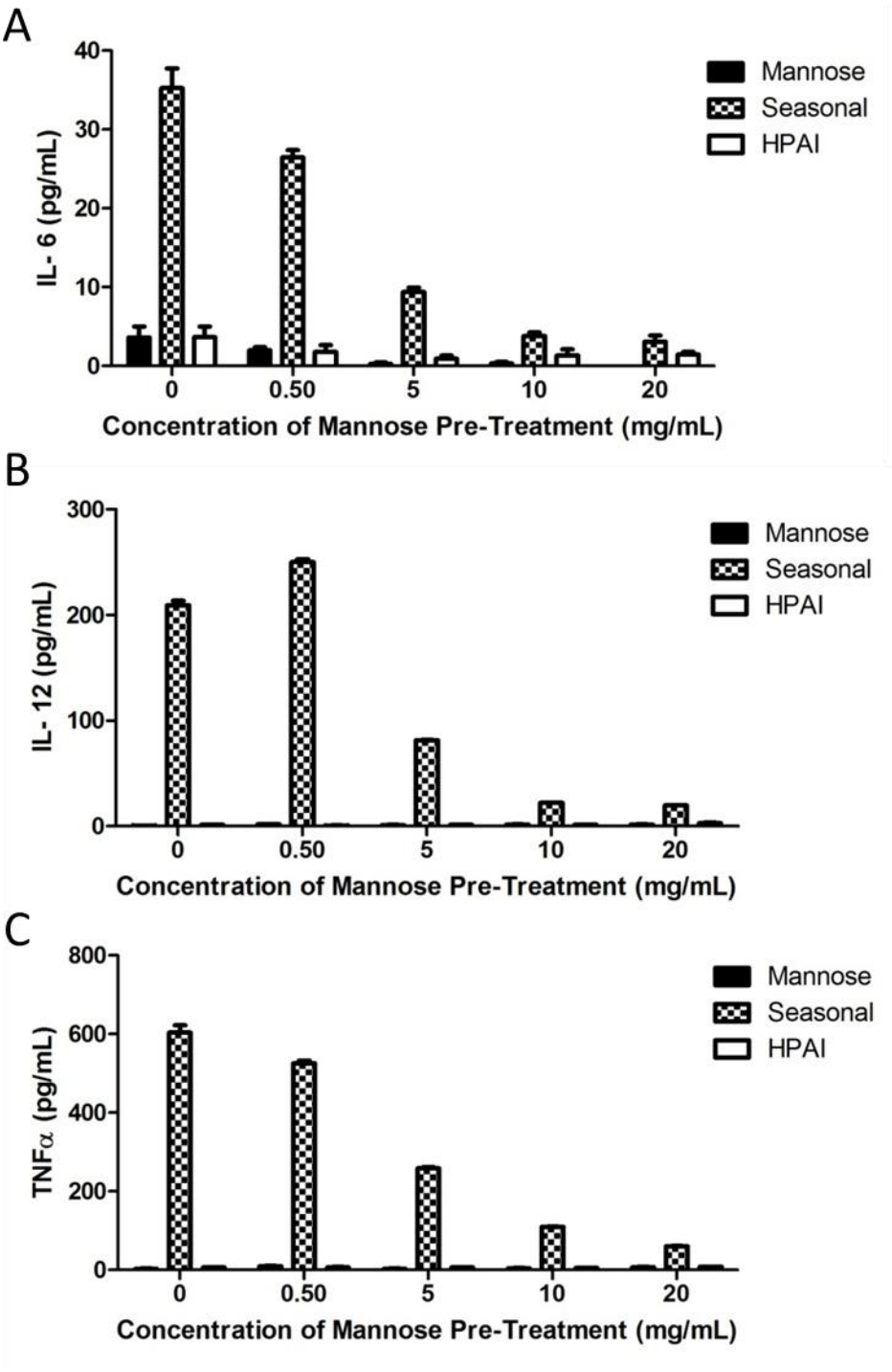
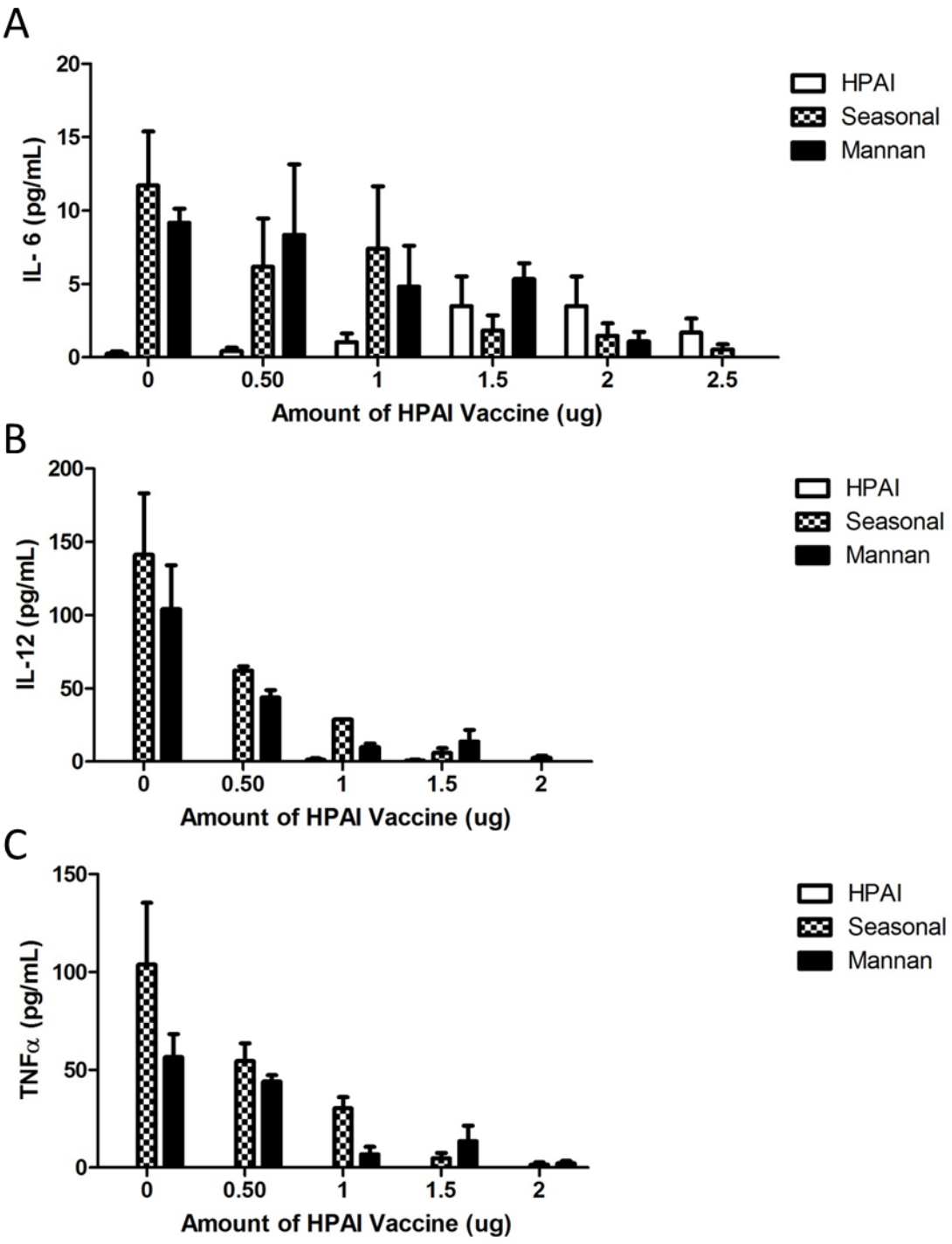

A
IL- 6 (pg/mL)
40
30
20
10
0
Mannose
Seasonal
HPAI
0
0.50
5
10
20
Concentration of Mannose Pre-Treatment (mg/mL)
B
IL- 12 (pg/mL)
300
200
100
0
Mannose
Seasonal
HPAI
0
0.50
5
10
20
Concentration of Mannose Pre-Treatment (mg/mL)
C
TNFα (pg/mL)
800
600
400
200
0
Mannose
Seasonal
HPAI
0
0.50
5
10
20
Concentration of Mannose Pre-Treatment (mg/mL)

Figure 6


A
IL- 6 (pg/mL)
20
15
10
5
0
0
0.50
1
1.5
2
2.5
Amount of HPAI Vaccine (ug)
HPAI
Seasonal
Mannan
B
IL-12 (pg/mL)
200
150
100
50
0
0
0.50
1
1.5
2
Amount of HPAI Vaccine (ug)
HPAI
Seasonal
Mannan
C
TNFα (pg/mL)
150
100
50
0
0
0.50
1
1.5
2
Amount of HPAI Vaccine (ug)
HPAI
Seasonal
Mannan

CHAPTER THREE:

Alum adjuvant rescues HPAI vaccine immunogenicity and survival in BALB/c mice by facilitating increased antigen uptake and intracellular processing

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Abstract

Highly pathogenic avian influenza (HPAI) H5N1 is an emerging infectious virus with an approximate 60% fatality rate in humans. In the United States, a vaccine for H5N1 has been developed and stockpiled in case of a pandemic; however, the vaccine demonstrated low immunogenicity in clinical trials. Adjuvants can be used to enhance the immune response to antigens. In this study, we examined differences in the immune response to the H5N1 vaccine with or without the addition of an alum adjuvant in a lethal challenge mouse model of HPAI infection. Mice treated with the adjuvanted vaccine displayed significantly reduced weight loss, increased survival, and higher neutralizing antibody titers compared to mice treated with the non-adjuvanted vaccine. Dendritic cells (DCs) cultured with the adjuvanted vaccine displayed increased expression of the activation markers CD40, CD86, and MHC II compared to DCs treated with the non-adjuvanted vaccine. DCs treated with HPAI vaccine with or without alum adjuvant did not produce pro-inflammatory cytokines nor signal through the NLRP3 inflammasome. When treated with the adjuvanted vaccine, DCs demonstrated increased vaccine uptake and phagosomal activity compared to those treated with the non-adjuvanted vaccine. Our study demonstrates that the alum adjuvant rescues vaccine immunogenicity and improves survival in the host by facilitating increased antigen uptake and intracellular processing, and provides insight into the development of more immunogenic vaccines targeting HPAI.

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 viruses typically infect avian species but are extremely lethal in humans. Symptoms include fast progressing pneumonia, respiratory distress syndrome, damage to the central nervous system, and multi-organ failure [1, 2]. These viruses have resulted in more than 600 human infections documented in 15 countries since 2003, and have had an approximate 60% fatality rate with death usually occurring 10 days after symptom onset in fatal cases [1-4]. Despite the emergence of a novel H7N9 avian influenza outbreak in humans, H5N1 cases continue and little evidence exists suggesting that H5N1 prevalence has abated. Currently, transmission between humans is sporadic; however, enhanced transmission though viral adaptation is plausible thus making an H5N1pandemic feasible [4-6].

In preparation for a pandemic, the United States government stockpiled 20 million doses of an inactivated subunit vaccine for HPAI; however, this vaccine resulted in low immunogenicity in human clinical trials with only 22% of recipients developing protective antibody titers of 1:40 or greater [7, 8]. Previous studies in our laboratory, using a lethal challenge ferret model of HPAI infection, demonstrated that the HPAI vaccine provided only limited protection with 0%-64% survival depending on the dose administered. When an alum adjuvant was added to the HPAI vaccine, survival improved significantly to 93%, and the vaccine was safe and well tolerated [9].

Adjuvants are substances that can influence and enhance the immune response to antigens [10]. Vaccines that contain purified antigen, rather than

intact pathogens, often contain adjuvants in order to amplify the immune response to the vaccine [11]. The most commonly used adjuvant in vaccines worldwide is alum, a mixture of aluminum salts [10, 12, 13]. The mechanism by which alum functions as an adjuvant remains controversial and unclear with multiple possible mechanisms described [14-17]. One proposed theory is that alum binds to vaccine components causing them to be slowly released to the immune system over time [10, 11]. Some reports have shown that alum can also work by activating the NLRP3 inflammasome immune complex, while others have shown that this is not the primary mechanism of action for alum [14, 18]. Additionally, previous studies have demonstrated that alum increases antigen internalization by dendritic cells (DCs) through binding to cell membrane lipids thereby triggering an endocytic response and signaling cascades that result in $CD4^+$ T cell activation and humoral immune responses [15-17].

Herein, immunization in a lethal challenge mouse model was utilized to elucidate the mechanism by which alum enhances HPAI vaccination. We show that, *in vivo,* the addition of alum to the HPAI vaccine increased neutralizing antibody titers and survival in BALB/c mice, while *in vitro,* treatment with the adjuvanted vaccine resulted in up-regulation of the cell surface markers CD40, CD86, and MHC II on DCs. Surprisingly, the addition of an alum adjuvant to the HPAI vaccine did not induce NLRP3 inflammasome activation nor production of pro-inflammatory cytokines. Furthermore, the addition of alum to the H5N1 vaccine increased antigen uptake and intracellular processing in DCs. Our results indicate that the H5N1 split virion vaccine is unable to induce an early

antigenic response in APCs, but this can be overcome by the addition of an alum adjuvant.

Materials and Methods

Mice

Male BALB/c mice, aged six to eight weeks, were purchased from the National Cancer Institute at Frederick (NCI-Frederick, Bethesda, MD), and held for 21 days for quarantine and acclimation. Mice were housed four per cage in microisolator cages under identical husbandry conditions and fed certified commercial feed. Animals were identified by BMDS microchips (IPTT-300 Implantable Programmable Temperature and Identification Transponder from Bio Medic Data System, Inc. (BMDS) Seaford, DE) inserted subcutaneously between the shoulder blades, and cage cards. All animal studies were approved by the Lovelace Respiratory Research Institute Institutional Animal Care and Use Committee.

Vaccines

The following reagent was obtained through the National Institutes of Health (NIH) Biodefense and Emerging Infections (BEI) Research Resources Repository, NIAID, NIH: Monovalent Influenza Subvirion Vaccine, rg/A/Vietnam/1203/2004, NR-4143 at a concentration of 30ug/ml hemagglutinin protein. Adjuvanted H5N1 Monovalent Influenza subvirion vaccine, rg/A/Vietnam/1203/2004, with a proprietary concentration of alum adjuvant was

provided under contract from DMID/NIAID/NIH at two concentrations of hemagglutinin (HA) protein: 45ug/ml (lot UD07828) and 15ug/ml (lot UD07826), or was prepared by research staff at a concentration of 1200ug alum (Invitrogen, Grand Island, NY) per 1 ml of the provided non-adjuvanted vaccine [19]. Vaccines were diluted to 0.6ug/ml HA protein with 0.05ml physiological saline immediately prior to vaccination.

Virus

Influenza virus A/Vietnam/1203/2004 (VN1203) subtype H5N1 was obtained from the Centers for Disease Control and Prevention (Atlanta, GA) as low-passage stock. The virus was passaged once in 10-day old embryonated chicken eggs to generate the master stock and then twice in eggs to generate the virus for all subsequent uses. Aliquots of 0.5ml to 1ml were stored at -80°C. After storage, the virus was determined to have a concentration of 1.4×10^8 plaque forming units (pfu/ml), 5.8×10^8 50% tissue culture dose ($TCID_{50}$/ml), and 1×10^8 50% egg infectious dose (EID_{50}/ml). Influenza A/Vietnam/1203/2004 is a Risk Group 3 pathogen and all experiments involving this virus were carried out in a Biosafety Level 3 (BSL 3) containment facility at the Lovelace Respiratory Research Institute and approved by the Institutional Biosafety Committee.

Immunization and Viral Challenge

Mice (n = 6 per group) were given a primary or a primary and secondary intramuscular injection in the gastrocnemius muscle of the H5N1 vaccine with or

without alum adjuvant. Secondary vaccinations were administered three weeks following primary vaccinations. Control animals received intramuscular injections of 0.05ml physiological saline. Seven weeks following primary vaccination, mice were challenged intranasally (IN) with a lethal dose (10 pfu) of A/Vietnam/1203/2004 (VN1203), and morbidity and mortality were assessed. Twice daily observations were conducted to determine animal health by appearance and activity. The injection site was monitored for inflammation and irritation. Body temperatures and bodyweights were recorded before vaccination, weekly prior to challenge, and daily thereafter. Animals were considered moribund if they experienced greater than 15% weight loss.

Serology

Blood for serum was collected from mice at necropsy by cardiac puncture three weeks after primary vaccination or four weeks after secondary vaccination. Blood was placed in serum separator collection tubes and incubated at room temperature for 30 minutes before centrifugation at 2500rpm for 10 min. Serum was collected and stored at -20°C until analyzed. The neutralization assay was modified from previously described procedures [20]. Briefly, serum samples were treated with receptor-destroying enzyme (Enka-Seiken, Tokyo, Japan) at a ratio of 1:1 followed by heat inactivation at 56°C for 45 minutes. The serum samples were incubated with 2×10^3 $TCID_{50}$ A/Vietnam/1203/2004 at 37°C for one hour. After incubation, Madin-Darby Canine Kidney (MDCK) cells plated in 96 well plates were inoculated in triplicate with the samples using a 2-fold dilution and

incubated at 37°C for three days. The plates were then scored for cytopathic effect (CPE), and neutralizing antibody titers were determined as the highest serum dilution at which no CPE occurred [9, 21].

Primary Bone Marrow Cell Isolation, Culture, and Treatment

Bone marrow cells were collected and cultured as previously described [22]. Briefly, bone marrow cells were isolated from the femurs and tibiae of mice and cultured in complete RPMI media (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen), 1x penicillin/streptomycin (Invitrogen), and 25ng/ml GM-CSF (Cell Signal Technology, Danvers, MA) for 5-7 days at 37°C with 5% CO_2. The following treatments were given in complete RPMI for 2 or 24 hours for *in vitro* experiments: 0.6ug/ml (HA protein) of non-adjuvanted HPAI vaccine; 0.6ug/ml (HA protein) of adjuvanted HPAI vaccine; 250ug/ml alum (Invivogen, San Diego, CA).

Treatment with Acridine Orange

Evaluation of phagosomal activity was assessed using modifications to previously described procedures [23]. BMDCs cultured as described above were pre-treated with 0.5ug/ml acridine orange (Invitrogen) for 15 min at 37°C prior to above treatments for select experiments and analyzed by flow cytometry.

Treatment with FITC Conjugated HPAI Vaccine

The adjuvanted and non-adjuvanted HPAI vaccines were conjugated to FITC (Thermo Scientific, Waltham, MA) using modifications to previously described procedures and the manufacturer's instructions [24]. Briefly, 15- to 20-fold molar excess of FITC was added to one vial of vaccine and incubated at room temperature for one hour in the dark. Excess and hydrolyzed FITC was removed by dye column removal kits (Thermo Scientific). BMDCs cultured as described above were treated with the FITC conjugated vaccine for 2 hours and analyzed by flow cytometry. For microscopy experiments, Armenian hamster anti-mouse CD11c clone N418 conjugated to PE-Cy7 (eBioscience, San Diego, CA) was used as a cell surface marker.

Flow Cytometry

In vitro experiments for cell surface marker expression, as well as those using acridine orange pre-treated cells, and cells treated with the FITC conjugated vaccines were analyzed by flow cytometry. Antibodies utilized include: Armenian hamster anti-mouse/rat CD40 clone HM40-3 conjugated to FITC (eBioscience); rat anti-mouse CD86 clone GL1 conjugated to PE (eBioscience); and rat anti-mouse MHC II I-A/I-E clone M5/114.15.2 conjugated to PerCP/Cy5.5 (BioLegend, San Diego, CA). Samples were run on a FACS Canto flow cytometer (BD Bioscience, San Jose, CA) and analyzed by FlowJo cytometry analysis software (TreeStar, Ashland, OR). Cell populations were gated on viable cells and assessed for expression in the FL1 (CD40 and FITC),

FL2 (CD86), or FL3 (MHC II and acridine orange) channels. Compensation was calculated using BD CompBeads (BD Bioscience). For each experiment, treated cells were compared to cells in culture media alone.

ELISA

BMDC culture supernatants were analyzed for IL-6, IL-12, IL-1β, and TNF-α production using ELISA kits purchased from eBioscience according to the manufacturer's instructions.

Widefield Fluorescence Microscopy

Images were acquired on an Olympus IX81 microscope using an LCAch N 40x/0.55 air UIS2m objective. Acridine orange was imaged using a 452 dichroic and a 520-550 emission filter. FITC was imaged using a 495 dichroic and a 502.5-537.5 emission filter. CD11c-PE-Cy7 was imaged using a 760 dichroic and a 765-855 emission filter. Acquisition was done using Metamorph (Molecular Devices, Sunnyvale, CA), and image processing done using ImageJ (National Institutes of Health, Bethesda, MD.).

Statistical Analysis

Bodyweights, body temperatures, and neutralizing antibody titers are expressed as mean values from each group of n = 6 mice. All statistical analysis was completed using the analysis software included in GraphPad Prism

(GraphPad Prism 5 for Windows, version 5.03; GraphPad Software, Inc., La Jolla, CA.).

Results

Morbidity and Mortality Following HPAI Challenge in Vaccinated Mice

Previous studies have shown that the subunit vaccine targeting H5N1 is not protective in a lethal challenge ferret model unless supplemented with an alum adjuvant [9]. As limited immunologic reagents are available for exploring molecular mechanisms in ferrets, we examined the immune response to H5N1 vaccination in a lethal challenge BALB/c mouse model of H5N1 infection. To assess protection conferred by the HPAI vaccine, BALB/c mice (n=6 per group) were given a primary intramuscular (IM) vaccination of the HPAI vaccine (0.6ug) with or without an alum adjuvant, and challenged intranasally (IN) with a lethal dose (10 pfu) of A/Vietnam/1203/2004 (VN1203) 7 weeks following vaccination. Non-vaccinated challenged control mice began losing weight 3 days post infection (dpi) (Figure 1a), and mortality began 5 dpi with 100% mortality occurring by 7 dpi (Figure 1b). Mice that received the non-adjuvanted vaccine began losing weight 4 dpi and weight loss peaked 7 dpi before recovering in surviving animals. Mortality in mice that received the non-adjuvanted vaccine began 6 dpi with 33% of animals surviving. Mice that received the adjuvanted vaccine had similar weight loss initially to those that received the non-adjuvanted vaccine beginning 4 dpi; however, weight loss did not progress in this group beyond 5 dpi. Animals that received the adjuvanted vaccine achieved 83%

survival with one death 8 dpi. Non-vaccinated, non-challenged control animals exhibited steady bodyweights and had 100% survival as expected. Taken together, these results demonstrate that the addition of an alum adjuvant to the HPAI vaccine provides greater protection in BALB/c mice, and these results are similar to those observed previously in a ferret model [9].

Neutralizing Antibody Titers in Vaccinated Mice

To evaluate differences in the production of functional antibody titers to HPAI in vaccine treated animals, BALB/c mice (n=6 per treatment) received a primary or a primary and secondary IM vaccination of 0.6ug of the HPAI vaccine with or without the addition of an alum adjuvant. Antibody titers were assessed by neutralization assay 21 days following primary vaccination and 28 days following secondary vaccination. Neutralizing antibody titers were observed in 50% of mice that received a primary vaccination of the adjuvanted HPAI vaccine, but were below the level considered protective (>1:40). Neutralizing antibodies were not detected in mice that received a primary vaccination of the non-adjuvanted HPAI vaccine (Figure 2a). All mice that received a primary and secondary vaccination of either the adjuvanted or non-adjuvanted HPAI vaccine developed neutralizing antibody titers. Of the mice that received the adjuvanted vaccine, 83% developed antibody titers greater than 1:40, whereas only 33% of mice that received the non-adjuvanted vaccine developed antibody titers greater than 1:40. Neutralizing antibody titers in mice that received the adjuvanted vaccine were significantly higher compared to mice that received the non-

adjuvanted vaccine (Figure 2b). These results illustrate that the addition of an alum adjuvant increased neutralizing antibody titers against VN1203 in BALB/c mice.

Dendritic Cell Activation Following Vaccine Treatment

It is recognized that an effective innate immune response contributes to a functional adaptive immune response, and dendritic cells (DCs) are well established as antigen presenting cells. The ability of DCs to process antigen and drive T cell responses makes them an important cell type in vaccination, and many vaccines are being developed to specifically target DCs [25-27]. Upon activation, DCs up-regulate the expression of cell surface markers such as CD40, CD80/86, and MHC II [28]. We examined differences in DC activation following treatment with the HPAI vaccine with or without alum adjuvant *in vitro*. Bone marrow derived dendritic cells (BMDCs) were treated with either the adjuvanted or non-adjuvanted HPAI vaccines, and stained for the cell surface activation markers CD40, CD86, and MHC II. BMDCs treated with adjuvanted HPAI vaccine showed increased expression of CD40, with approximately 60% positive (Figure 3a and d), CD86 (55% positive) (Figure 3b and e), and MHC II (80% positive) (Figure 3c and f) compared to BMDCs treated with the non-adjuvanted HPAI vaccine and relative to non-treated cells in media alone. Cell surface marker expression on BMDCs treated with the non-adjuvanted HPAI vaccine was similar to expression on non-treated cells, and treatment with alum

alone did not result in the up-regulation of cell surface marker expression (data not shown).

Antigen recognition by DCs can also result in intracellular signaling cascades that lead to the secretion of pro-inflammatory cytokines such as IL-6, IL-12, TNF-α, and IL-1β [29-31]. Alum has been previously shown to stimulate the NLRP3 inflammasome pathway resulting in pro-inflammatory and anti-microbial responses. Inflammasome activation is characterized by the secretion of the pro-inflammatory cytokines IL-1β and IL-18 [14, 32]. To assess whether alum is inducing inflammasome involvement in response to H5N1 vaccination, murine bone marrow derived dendritic cells (BMDCs) were treated with either the adjuvanted or non-adjuvanted HPAI vaccine and cell culture supernatants were collected and assessed for the expression of the cytokine IL-1β by ELISA. LPS + ATP was used as a positive control and produced a robust IL-1β response. When treated with either the adjuvanted or non-adjuvanted HPAI vaccines, BMDCs did not secrete IL-1β (Figure 4a). These results suggest that the mechanism of action of alum in this system is not NLRP3 inflammasome pathway activation.

To further assess whether vaccine stimulation resulted in pro-inflammatory responses, cell culture supernatants from vaccine-treated BMDCs were analyzed for the production of the cytokines IL-6, IL-12, and TNF-α by ELISA. LPS was used as a positive control and resulted in the secretion of high levels of all three pro-inflammatory cytokines. Surprisingly, the HPAI vaccine with or without the addition of an alum adjuvant failed to induce the production of these cytokines

(Figure 4b-d). These results demonstrate that neither vaccine stimulated signaling pathways that lead to pro-inflammatory cytokine secretion.

Vaccine Uptake and Intracellular Processing

A recent study has shown that alum can function as an adjuvant by directly promoting antigen uptake by DCs [15]. To determine if alum is functioning in this manner in this model, we next examined the ability of BMDCs to take up the HPAI vaccine with or without alum adjuvant. Briefly, BMDCs were treated with FITC-conjugated, adjuvanted or non-adjuvanted vaccine for two hours. FITC expression was measured and quantified using flow cytometry (Figure 5a and b). BMDCs treated with the adjuvanted vaccine demonstrated a clear increase in FITC expression compared to BMDCs treated with the non-adjuvanted vaccine (Figure 5a). Fluorescence intensity was quantified and increased by 10-fold in cells treated with the adjuvanted vaccine compared to cells treated with the non-adjuvanted vaccine (Figure 5b). Vaccine uptake was further assessed by fluorescent microscopy, and BMDCs treated with the FITC-conjugated, adjuvanted HPAI vaccine revealed more intense fluorescence compared to cells treated with the non-adjuvanted vaccine (Figure 5c).

To assess differences in antigen processing following vaccine uptake, we examined phagosomal activity post vaccine treatment using acridine orange quenching. The dye acridine orange has been used previously to examine activity within acidic cellular compartments [23, 33, 34]. Acridine orange becomes highly concentrated in acidic cellular compartments and is sensitive to changes in

pH; therefore, fluorescence quenching can be used to quantify phagosomal activity [23]. BMDCs were pre-treated with acridine orange followed by treatment with the HPAI vaccine with or without adjuvant for 2 hours. Cells were collected and analyzed by fluorescence microscopy and flow cytometry, and phagosomal activity was observed as a decrease in acridine orange expression. Cells treated with alum, acridine orange alone, or non-adjuvanted HPAI vaccine expressed high levels of acridine orange, suggesting little activity in phagosomal processing. Cells treated with adjuvanted HPAI vaccine had decreased acridine orange fluorescence compared to the other treatments, indicating an increase in phagosomal activity (Figure 6a-c). Taken together, these results illustrate that the addition of an alum adjuvant to the HPAI vaccine results in increased vaccine uptake by BMDCs, which in turn, results in increased intracellular processing, when compared to BMDCs treated with the non-adjuvanted HPAI vaccine.

Discussion

An inactivated subunit vaccine targeting H5N1 resulted in low immunogenicity when evaluated in human clinical trials [7]; however, studies conducted in a lethal challenge ferret model demonstrated that the addition of an alum adjuvant to the H5N1 vaccine resulted in increased survival and functional antibody production [9]. The mechanism of action of alum as an adjuvant is multifaceted and controversial [14-17]. In the current study, we found that alum enhances the immune response to the H5N1 vaccine by facilitating increased antigen uptake and intracellular processing. The addition of alum to the HPAI

vaccine resulted in decreased morbidity and mortality in a lethal challenge mouse model (Figure 1), as well as the increased production of neutralizing antibodies in vaccinated animals (Figure 2). These results coincide with previous findings in ferrets, and show that our model is sufficient for studying the immune response to HPAI vaccination [9]. The ferret model is less suited to study the immune response as limited immunological reagents are available.

In the current study, BMDCs treated with the alum-adjuvanted HPAI vaccine had increased expression of the co-stimulatory markers CD40, CD86, and MHC II compared to cells treated with the non-adjuvanted HPAI vaccine (Figure 3), which demonstrates that the addition of alum to the vaccine preparation led to the activation of APCs. These results were expected as previous studies have shown the ability of alum, when in the presence of antigen, to upregulate CD40, CD86, and MHC II on monocytes and macrophages [35-38]. The activation of APCs and the subsequent upregulation of co-stimulatory molecules on the cell surface are critical for the presentation of antigen to T cells and the progression of a productive immune response [28, 35].

While our data demonstrated that the addition of alum to the HPAI vaccine was able to activate DCs, neither treatment with adjuvanted nor non-adjuvanted vaccine resulted in the production of the pro-inflammatory cytokines IL-6, IL-12, or TNF-α (Figure 4b-d). While previous studies have shown that alum alone does not induce expression of these cytokines, as they are associated with T_H1 responses and not T_H2 responses [11, 14], it is interesting that other vaccine components did not initiate cytokine production. An *in vivo* study of influenza

vaccination in mice demonstrated that vaccination with a seasonal influenza subunit vaccine resulted in the production of the cytokines IL-6 and TNF-α in the lungs [39]. *In vitro*, a study in mice showed that following treatment with a subunit seasonal influenza vaccine, DCs produced low levels of IL-6 and TNF-α, but no detectable IL-12 [40], while another study detected low levels of TNF-α but no detectable IL-12 from DCs isolated from human PBMCs and treated with a subunit seasonal influenza vaccine [41]. Together, these studies demonstrate that cytokine production by APCs following vaccination with subunit seasonal influenza vaccines *in vivo* can be expected, while, *in vitro*, production of the cytokines IL-6 and TNF-α by vaccine treated DCs is less pronounced but still detectable. This suggests that the HPAI vaccine used in our study is less efficient than the seasonal influenza subunit vaccines in activating DCs.

In addition to IL-6, IL-12, and TNF-α, BMDCs treated with the alum-adjuvanted HPAI vaccine did not produce IL-1β (Figure 4a). This was surprising as alum has been previously shown to signal through the NLRP3 inflammasome complex, and the production of IL-1β is a result of activating this immune complex [14, 32]. The lack of inflammasome involvement in our study could be explained by the fact that inflammasome activation and the subsequent production of IL-1β require two signals. Alum is known to provide the second signal, which directly activates Nlrp3. The first signal is provided by endogenous or microbial antigens that activate NF-κB [14, 32]. It appears that, in our study, following treatment with the alum-adjuvanted HPAI vaccine, the first signal is

missing. This idea is supported by the lack of production of IL-6, IL-12, and TNF-α, which are downstream of NF-κB activation [42].

Herein, alum was able to facilitate increased antigen uptake in vaccine treated BMDCs (Figure 5). This finding is supported by a previous study that demonstrated an inflammasome independent mechanism of action for alum, in which alum acts as an adjuvant by binding to the plasma membrane of DCs and activating endocytic uptake via delayed ERK phosphorylation leading to antigen uptake [15]. The authors demonstrated that alum interacts with membrane lipids on the surface of DCs, which results in the aggregation of ITAM-containing receptors and subsequent Syk and PI3K signaling, and further demonstrated that while allowing antigen uptake by DCs, alum itself did not enter the cell. The findings from the current study suggest that this is the mechanism of action employed by alum with regard to increased H5N1 vaccine immunogenicity.

Following increased antigen uptake by vaccine treated BMDCs, an increase in antigen processing was demonstrated using acridine orange staining in BMDCs treated with the adjuvanted vaccine compared to those treated with the non-adjuvanted vaccine (Figure 6). This finding is consistent with the idea that if an increased amount of antigen is internalized by the cell that an increased amount of antigen processing will occur. This increase in antigen processing coincides with the upregulation of MHC II on the cell surface of BMDCs treated with the adjuvanted vaccine compared to those treated with the non-adjuvanted vaccine (Figure 3).

Taken together, our data suggests that the H5N1 vaccine examined in this study is unable to efficiently stimulate DCs without the addition of an adjuvant. The addition of alum to the vaccine preparation allows increased antigen uptake, possibly through an endocytic response, and subsequently, increased antigen processing. Further studies conducted *in vivo* of the immune mechanisms demonstrated by the current study would contribute to the findings presented herein. Vaccination is one of the most effective methods for preventing infectious diseases, and while the general immune response to vaccination is understood, the precise mechanisms behind the ability of vaccines to stimulate the immune system and lead to long term protection remain to be elucidated [30]. Identifying the mechanisms by which vaccines confer protection from disease will allow more effective vaccines to be developed. This is particularly important for pathogens for which current vaccines are not available or not sufficient, as is the case with highly pathogenic avian influenza. Our findings, presented herein, elucidate a mechanism by which the H5N1 vaccine offers only limited protection, and provide insight into the development of more immunogenic vaccines targeting HPAI.

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Figure Legends

Figure 1. Morbidity and mortality was decreased in BALB/c mice following vaccination with adjuvanted HPAI vaccine. BALB/c mice (n=6 per group) were vaccinated intramuscularly with adjuvanted HPAI vaccine, non-adjuvanted HPAI vaccine, or saline. Mice were challenged intranasally 7 weeks later with 10pfu A/Vietnam/1203/2004. **A.** Percent bodyweight change in surviving mice treated with the adjuvanted HPAI vaccine (triangles), non-adjuvanted HPAI vaccine (squares), or saline (diamonds). Non-challenged controls are represented by circles. Percent bodyweight change is expressed as mean ± SEM ($p<.0001$, one-way repeated measures ANOVA comparing all groups). **B.** Kaplan-Meier survival curve for mice treated with adjuvanted HPAI vaccine (dotted line), non-adjuvanted HPAI vaccine (solid line), or saline (short dashed line). Non-challenged controls are represented by the long dashed line ($p<.05$, Gehan-Breslow Wilcoxon test comparing all groups).

Figure 2. Neutralizing antibody titers were increased in BALB/c mice following vaccination with adjuvanted HPAI vaccine. BALB/c mice (n=6 per group) were given a primary, or a primary and secondary, intramuscular injection of the HPAI vaccine with or without alum adjuvant. Sera were collected 7 weeks following primary vaccination and evaluated by neutralization assay. **A.** Neutralizing antibody titers in mice following a primary vaccination of adjuvanted HPAI vaccine (squares) or non-adjuvanted HPAI vaccine (circles). Error bars indicated mean ± SEM (*$p<.05$, Student's t-test). **B.** Neutralizing antibody titers in

mice following a primary and secondary vaccination with adjuvanted HPAI vaccine (circles) or non-adjuvanted HPAI vaccine (squares). Error bars indicated mean ± SEM (*p<.05, Student's t-test).

Figure 3. Expression of co-stimulatory molecules was increased on BMDCs treated with adjuvanted HPAI vaccine. BMDCs were treated overnight at 37°C with media alone, adjuvanted HPAI vaccine, or non-adjuvanted HPAI vaccine. Following incubation, cells were collected and stained for co-stimulatory markers and analyzed by flow cytometry. **A-C.** Expression of CD40 (A), CD86 (B), and MHC II (C) on BMDCs treated with adjuvanted HPAI vaccine (dotted line), non-adjuvanted HPAI vaccine (solid line), or media alone (shaded area) depicted by histograms showing fluorescence intensity. Similar results were obtained from three independent experiments. **D-F.** Bar graphs illustrating percent positive of BMDCs treated with adjuvanted vaccine (checkered bars), non-adjuvanted vaccine (white bars), or media alone (black bars) expressing CD40 (D), CD86 (E), and MHC II (F). Error bars represent mean ±SEM (*p<.05, **p<.005, Student's t-test). Results were obtained from three independent experiments.

Figure 4. Neither vaccine induced cytokine production by BMDCs. BMDCs were treated overnight at 37°C with media alone, adjuvanted HPAI vaccine, non-adjuvanted HPAI vaccine, LPS, or LPS + ATP. Cytokine expression was assessed by ELISA. **A.** IL-1β secretion by BMDCs treated with media alone (black bars), LPS + ATP (diagonal bars), adjuvanted HPAI vaccine (checkered

bars), or non-adjuvanted vaccine (white bars). Error bars represent mean ± SEM. **B-D.** Production of IL-6 (B), IL-12 (C), and TNF-α by BMDCs treated with media alone (black bars), LPS (striped bars), adjuvanted HPAI vaccine (checkered bars), or non-adjuvanted HPAI vaccine (white bars). Error bars represent mean ± SEM. Results represent three independent experiments.

Figure 5. BMDCs treated with adjuvanted HPAI vaccine demonstrated increased antigen uptake. BMDCs were treated with FITC conjugated adjuvanted or non-adjuvanted HPAI vaccine for 2 hours at 37°C and then analyzed for FITC expression. **A.** Histogram depicting fluorescence intensity of BMDCs treated with adjuvanted HPAI vaccine conjugated to FITC (solid line) or non-adjuvanted HPAI vaccine conjugated to FITC (dotted line) analyzed by flow cytometry. Shaded area represents a non-stained control. **B.** Median fluorescence intensity (MFI), expressed as the mean ± SEM (log_{10}), in BMDCs treated with adjuvanted HPAI vaccine conjugated to FITC (checkered bar) or non-adjuvanted HPAI vaccine conjugated to FITC (white bar) as determined by flow cytometry. The black bar represents a non-stained control (*p<.05, Student's t-test). Results were obtained from three independent experiments. **C.** Fluorescence microscopy of BMDCs treated with either the adjuvanted or non-adjuvanted HPAI vaccines conjugated to FITC. CD11c conjugated to PE-Cy7 was used as a cell surface marker.

Figure 6. Intracellular processing is increased in BMDCs treated with adjuvanted HPAI vaccine. BMDCs were pre-treated with acridine orange for 15min at 37°C followed by treatment overnight with adjuvanted or non-adjuvanted HPAI vaccine. **A.** Histogram depicting fluorescence intensity of BMDCs pre-treated with acridine orange followed by treatment with adjuvanted HPAI vaccine (solid black line), non-adjuvanted HPAI vaccine (dotted line), alum (solid grey line), or acridine orange alone (dashed line) analyzed by flow cytometry. The shaded area represents a non-stained control. **B.** Median acridine orange fluorescence (MFI), expressed as the mean ± SEM (log_{10}) in BMDCs treated with media alone (black bars), acridine orange alone (crisscrossed bar), alum (dotted bar), adjuvanted HPAI vaccine (checkered bar), or non-adjuvanted HPAI vaccine (white bar) (*p<.05, Student's t-test). Results were obtained from 3 independent experiments. **C.** Fluorescence microscopy of BMDCs pre-treated with acridine orange followed by treatment with either the adjuvanted or non-adjuvanted HPAI vaccine.

Figures

Figure 1

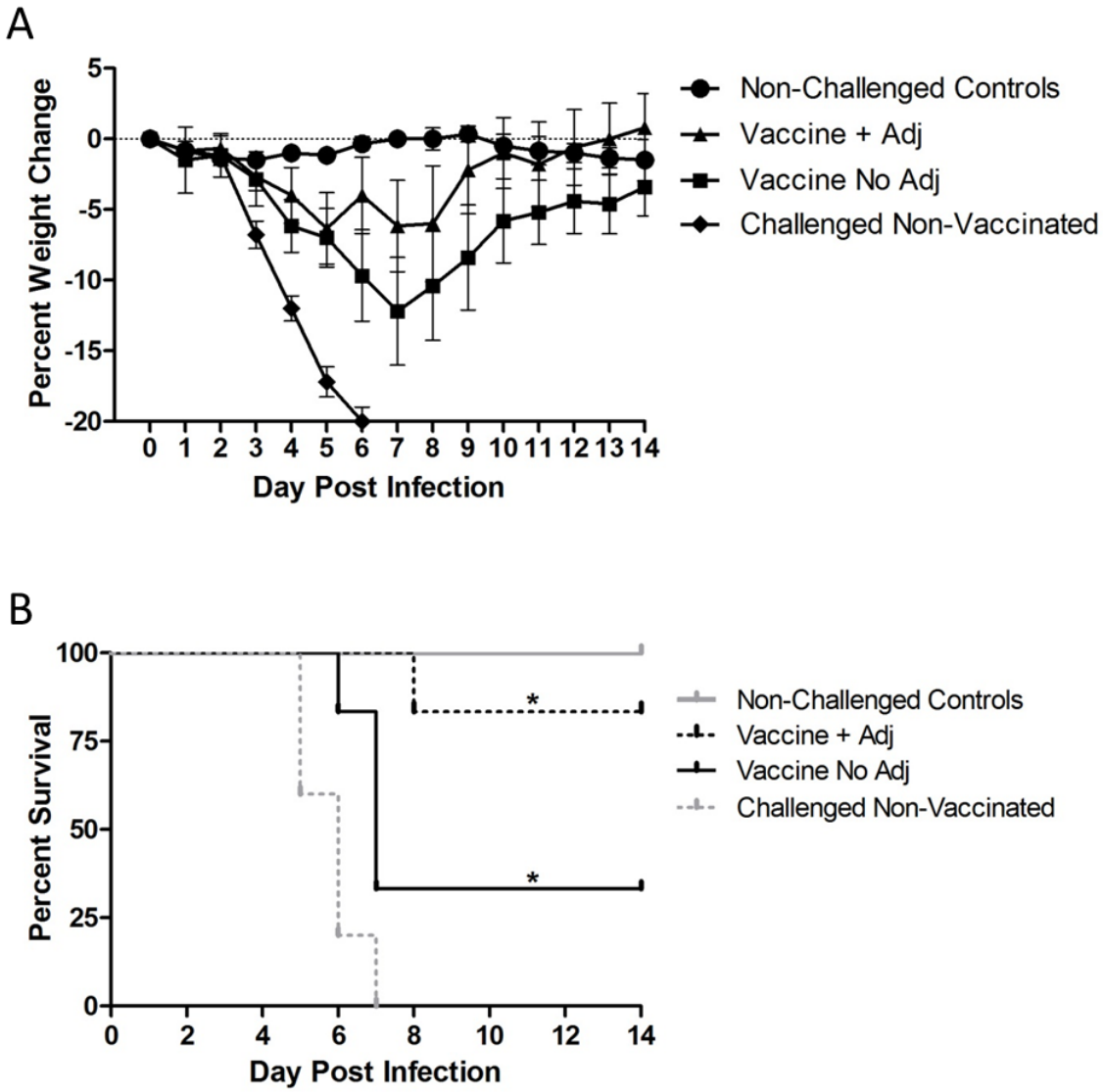

Figure 2

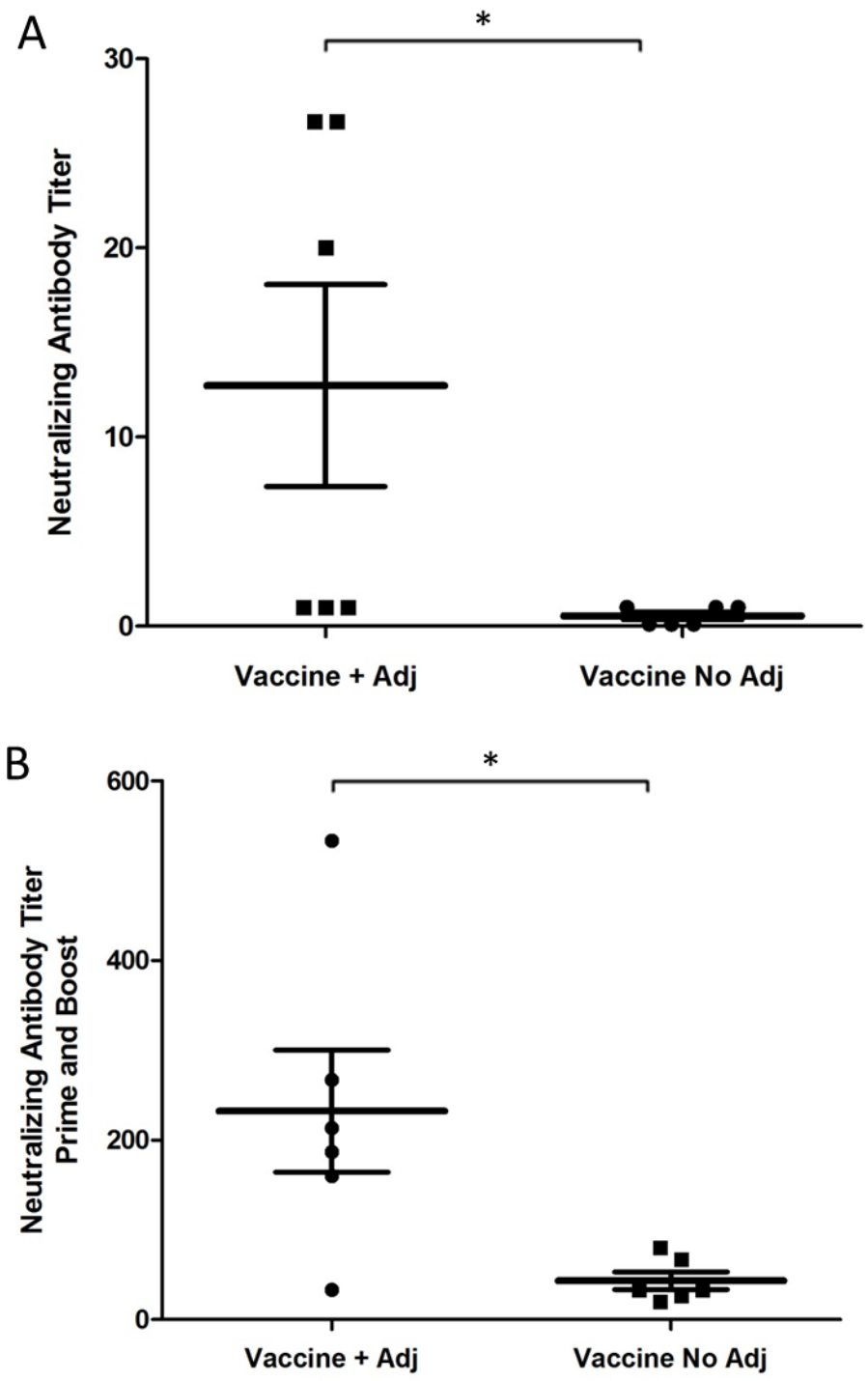
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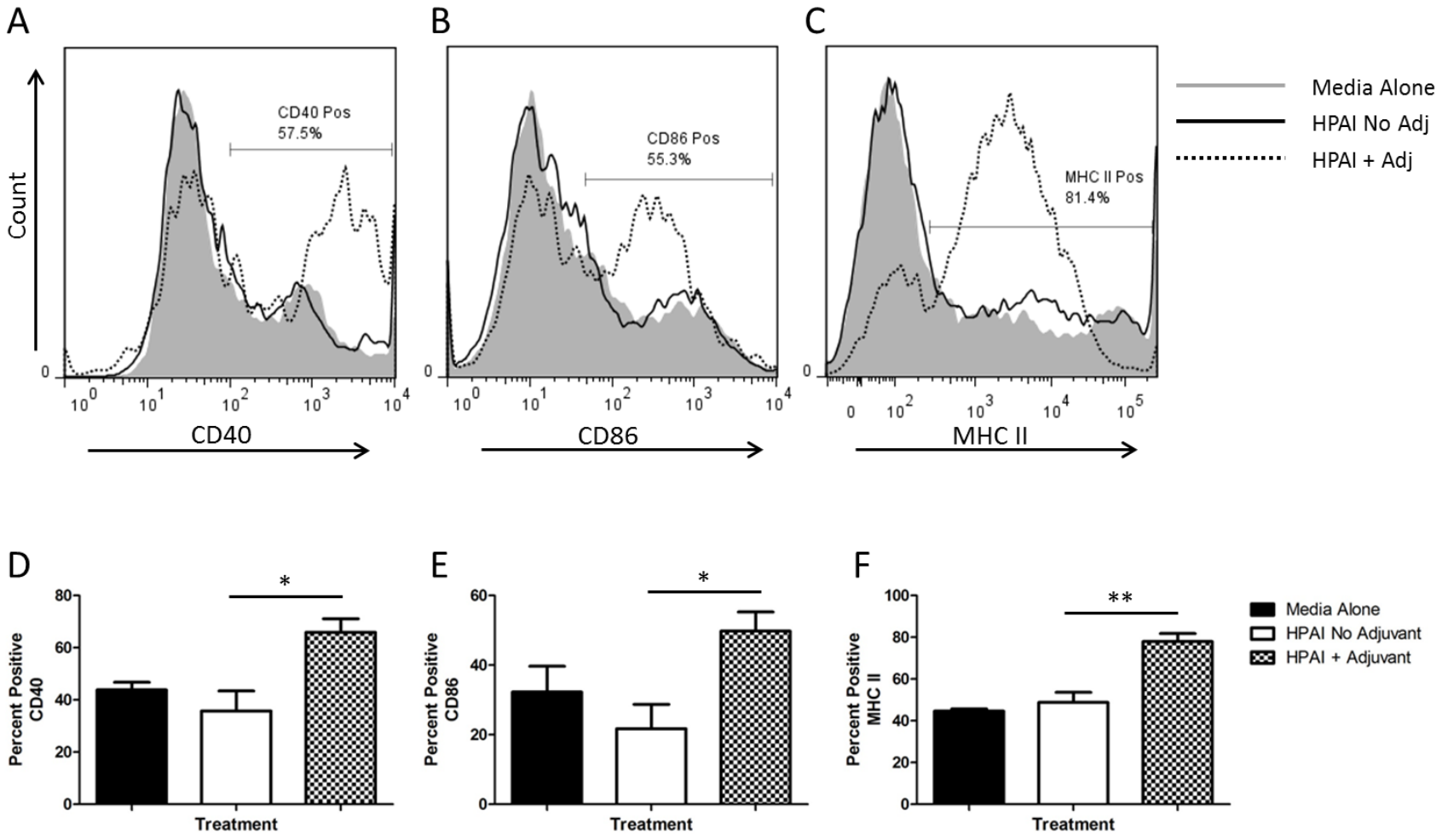
Figure 3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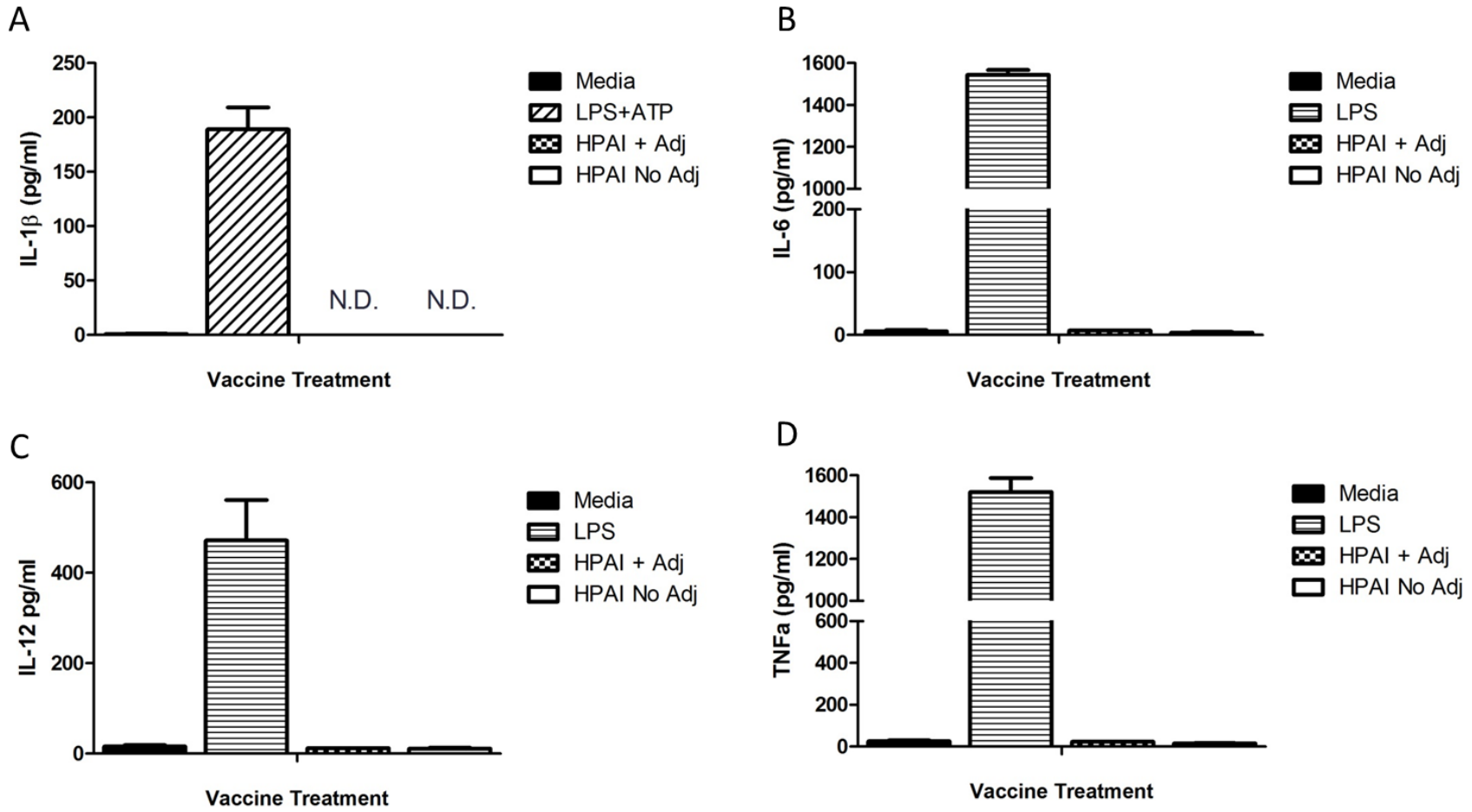
Treatment
Media Alone
HPAI No Adjuvant
HPAI + Adjuvant

Figure 4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HPAI No Adj
B
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1400
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1000
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Vaccine Treatment
Media
LPS
HPAI + Adj
HPAI No Adj
C
IL-12 pg/ml
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Vaccine Treatment
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HPAI + Adj
HPAI No Adj
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Figure 5

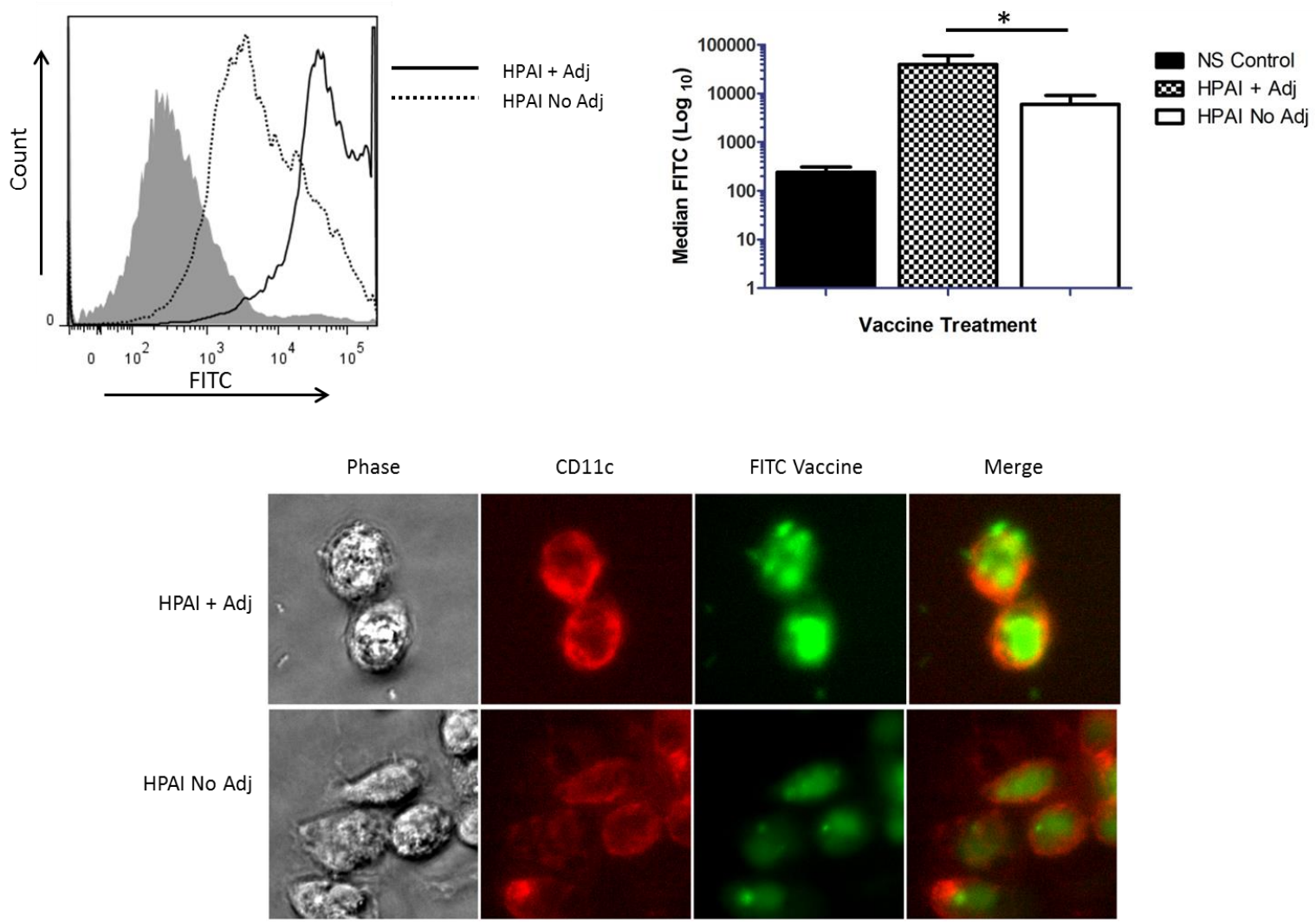
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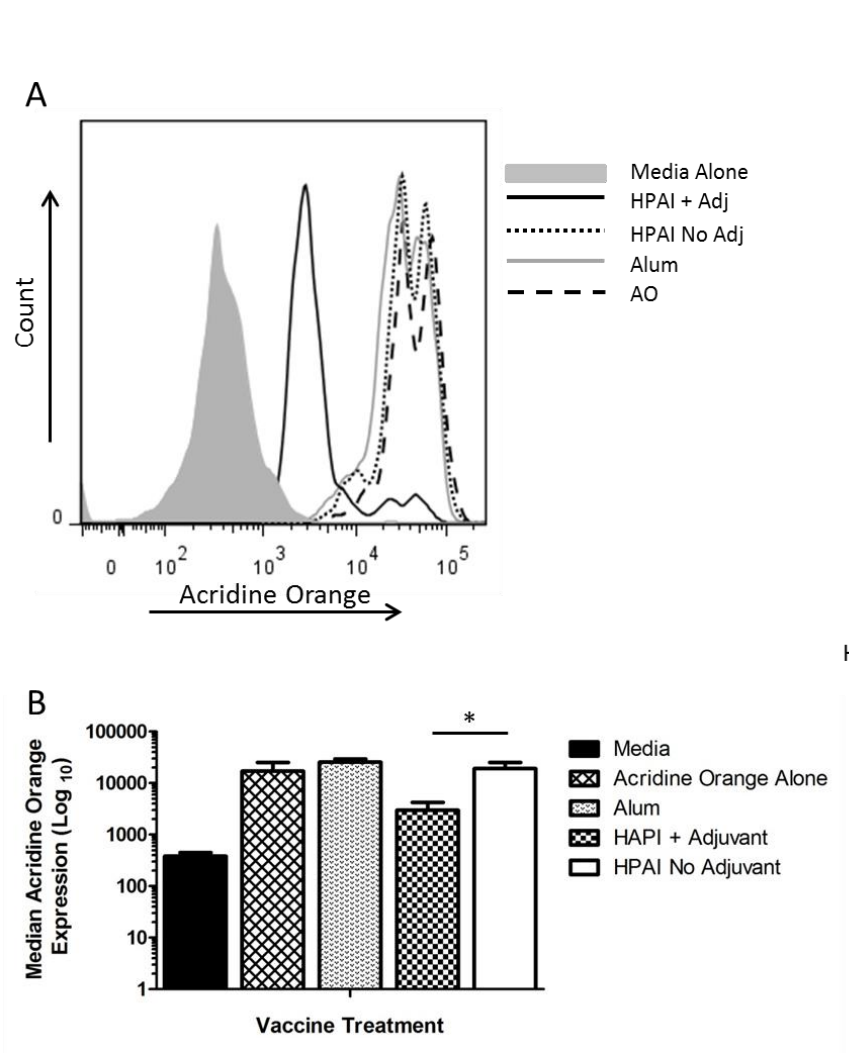
Figure 6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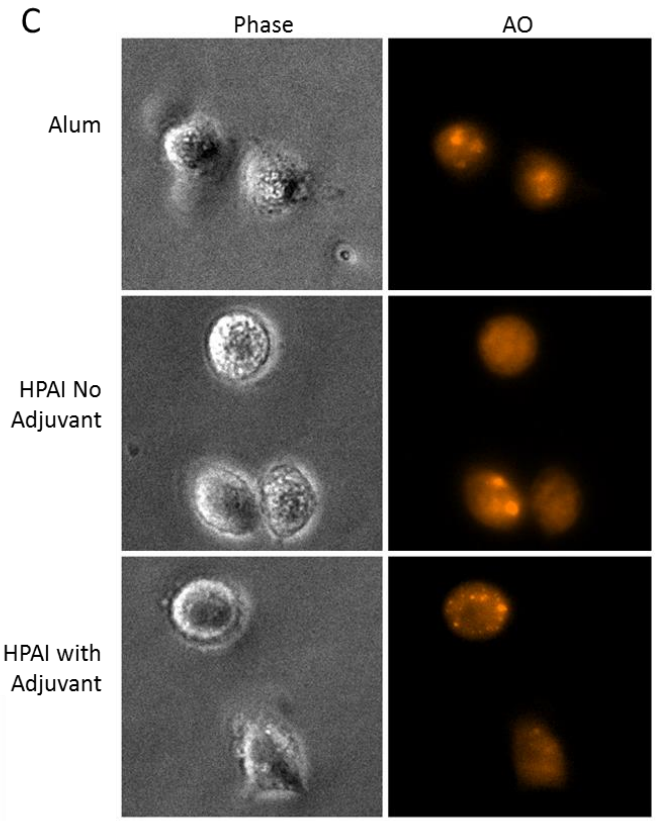
Vaccine Treatment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CHAPTER FOUR: DISCUSSION

Summary of Studies

The purpose of these studies was to further our understanding of the immunological mechanisms of vaccination, specifically with regard to inactivated split virion subunit vaccines targeting influenza viruses. More precisely, the aims of these studies were to identify mechanisms by which the seasonal and adjuvanted H5N1 vaccines elicited a protective immune response, whereas the non-adjuvanted H5N1 vaccine produced attenuated immunogenicity. Specifically, these studies investigated the ability of the vaccines to activate APCs, induce the production of neutralizing antibodies, and provide protection following lethal challenge in a BALB/c mouse model. A more complete understanding of the mechanisms of vaccination will lead to the development of more effective vaccines, and the studies presented herein provide insight into the improvement of vaccines targeting HPAI.

The immune response to subunit vaccines targeting seasonal influenza and HPAI were examined utilizing both *in vivo* and *in vitro* experimental approaches (Chapter 2). The seasonal and H5N1 vaccines were developed using the same manufacturing process, but have shown disparate efficacies in clinical trials. Regardless of the route of administration (SC or IM), the seasonal vaccine demonstrated immunogenicity, whereas H5N1 vaccination did not result in the production of detectable levels of neutralizing antibodies (Chapter 2, Figure 1). These findings validate the mouse model established herein as a suitable model for studying the immune response to influenza vaccination as vaccine

efficacy in the mouse model was comparable to that seen in humans. Studies conducted *in vitro* evaluated APC activation, and demonstrated an increase in the ability of the seasonal vaccine to activate DCs when compared to the H5N1 vaccine (Chapter 2, Figure 2). Neither vaccine stimulated TLRs or NLRs (Chapter 2, Figure 3), and this finding led to the investigation of the involvement of CLRs. For the first time, to our knowledge, this study demonstrates a role for CLRs in the immune response to vaccination against influenza (Chapter 2, Figures 4 and 5), and the ability of the H5N1 vaccine to bind CLRs but not initiate a signaling cascade provides an explanation for the attenuated response to H5N1 vaccination (Chapter 2, Figure 6).

In Chapter 3, the role of alum in the immune response to the H5N1 influenza subunit vaccine was evaluated in a lethal challenge mouse model of infection. When compared to the non-adjuvanted H5N1 vaccine, treatment with the vaccine plus alum resulted in increased immunogenicity (Chapter 3, Figure 2) and decreased morbidity and mortality (Chapter 3, Figure 1). *In vitro*, the adjuvanted H5N1 vaccine more efficiently activated APCs (Chapter 3, Figure 3); however, neither vaccine induced the production of pro-inflammatory cytokines. The absence of cytokine secretion suggests that neither the activation of PRRs, nor the NLRP3 inflammasome, is involved in the mechanism of action of alum in our model (Chapter 3, Figure 4). Herein, alum functions as an adjuvant by facilitating increased antigen uptake and intracellular processing (Chapter 3, Figures 5 and 6), and while alum rescues the efficacy of the H5N1 vaccine, it

does so through a different mechanism of action than that utilized by seasonal influenza vaccines.

Limitations of the Studies

Animal models are essential for the study of infectious diseases, as it can be difficult and unethical to study these diseases in humans. Mice are a favorable model in which to study immunology due to low cost, the broad availability of immunological reagents, and transgenic strains [1, 2]. In addition, laboratory mice are inbred and genetically identical, which facilitates reproducibility of experimental results [3]. Despite these benefits, pathogens such as influenza do not naturally infect mice, and thus limit their relevance [1, 2], and the inbred nature of laboratory mice does not reflect the genetic diversity and variability present in the human population [3]. Additionally, while studies conducted in animal models provide invaluable insight into human biological processes, differences in the correlates of protection between animals and humans may lead to results obtained in an animal model that are not a true representation of the human immune response [4, 5].

Another limitation of the current studies is that while vaccine immunogenicity was assessed *in vivo*, APC activation and the mechanisms leading to this activation were evaluated *in vitro*. As APC:T cell interactions can be influenced by the lymph node micro-environment, the addition of *in vivo* experiments evaluating APC activation and migration, antigen presentation to T cells, and T cell activation would validate the findings presented herein.

Additionally, data demonstrating APC:T cell interactions *in vitro* are not included in the current studies. Experiments were conducted to assess antigen presentation by BMDCs to T cells *in vitro,* yet these experiments were unsuccessful. Ideally, T cells from transgenic mice specific for the VN1203 HA protein would be utilized; however, such mice are not available. In an attempt to overcome this limitation, T cells were harvested from the spleens of mice vaccinated with either the seasonal or non-adjuvanted HPAI vaccines three weeks following vaccination. BMDCs pulsed with the seasonal or non-adjuvanted HPAI vaccine for 24 hours were co-cultured with T cells harvested from the vaccinated mice for three days. T cell activation was assessed by cell proliferation assays, but no T cell proliferation was detected.

An additional limitation of the studies presented herein is that the seasonal influenza vaccine evaluated in the current studies is trivalent whereas the H5N1 vaccine is monovalent. The trivalent nature of the seasonal vaccine may affect its immunogenic properties; therefore, examination of the immune response to an efficacious monovalent subunit influenza vaccine, such as the 2009 H1N1 pandemic vaccine, would expand upon and strengthen the current studies.

Future Directions

The findings presented herein provide insight into a possible mechanism for the attenuated function of the H5N1 vaccine. As mentioned in the limitations section above, *in vivo* studies examining APC activation and migration, antigen presentation to T cells, and subsequent T cell activation would expand on the

current results. Draining lymph nodes should be harvested following vaccination and assessed for cell populations and the frequencies of those populations. Types and numbers of APCs should be identified and assessed for the expression of co-stimulatory molecules such as CD40, CD80, and CD86, as well as MHC I/II. The use of fluorescently labeled vaccines may help to identify APCs that respond to antigen at the site of vaccination and then travel to the draining lymph nodes; however, an increase in the presence of APCs alone in the draining lymph nodes should indicate a response specific to vaccination. The frequency of $CD4^+$ and $CD8^+$ T cells should also be assessed as their numbers should be significantly higher in the lymph nodes of mice responding to vaccination due to T cell activation and clonal expansion. Assessment of a vaccine specific T cell response could be measured with the development and use of fluorescent tetramers that target the HA or NA proteins from the specific viruses used in the vaccines.

The current studies propose a role for C-type lectin receptors in the immune response to influenza vaccination. Future studies would expand on the involvement of CLR signaling and aim to identify the specific CLRs involved in influenza vaccine recognition. Assays determining total phosphorylation should be utilized as an indication for a general increase in signaling; however, these assays would not be specific to signaling downstream of CLRs, therefore the phosphorylation of specific CLR pathway components should be assessed. For example, ligand binding to the CLRs Dectin-1, Dectin-2, and Mincle induces receptor aggregation and initiates signaling through the immunoreceptor

tyrosine-based activation motif (ITAM) on the receptors or F*c*R*γ*. Tyrosine residues on the ITAMs are phosphorylated by Src family kinases and lead to the recruitment and activation of the kinase Syk [6]; therefore, the expression of phosphorylated Syk could be analyzed using western blot, or an inhibitor of Syk, such as piceatannol could be employed. Downstream of Syk is a protein complex consisting of CARD9, BCL10, and MALT1 (CBM), so activation of this CBM complex could also be assessed by western blot or the utilization of BCL10 knockout mice [6, 7]. Raf-1 is another signaling component downstream of Dectin-1, and its activation could also be assessed [6].

In addition to the analysis of the activation of signaling components downstream of CLRs, the involvement of specific CLRs should be assessed. This could be accomplished by using antibodies against specific CLRs that would neutralize the receptors, blocking cellular activation. The utilization of short hairpin RNAs (shRNAs) that silence CLR genes through RNA interference (RNAi) could also be used to determine the involvement of specific CLRs [8, 9].

While the current studies aimed to identify variations in the immune response to the seasonal and H5N1 vaccines, variations in the immune response ultimately result from differences in the target viruses. The ability of the seasonal influenza vaccine to activate C-type lectin receptors suggests that carbohydrate structures on the HA and NA antigens are essential for vaccine antigen recognition by these receptors. Differences in N-linked glycosylation sites on the target viruses may account for the ability of the seasonal vaccine to activate

signaling cascades whereas the H5N1 vaccine bound the receptors but did not initiate signaling.

Glycosylation sites are determined by the amino acid sequence motif N-X-T/S where X is any amino acid except proline [10, 11]. Previous studies have determined the glycosylation sites on the viruses used in the vaccines studied herein. The HA protein of A/Brisbane/10/2007, the H3N2 virus used in the 2009/2010 seasonal influenza vaccine, has potential glycosylation sites at amino acid residues 63, 122, 126, 133, 144, and 165 [11]. The possible glycosylated residues on the HA protein of A/Brisbane/59/2007, the H1N1 virus used in the 2009/2010 seasonal influenza vaccine, are at amino acid residues 15, 27, 58, 91, 129, 163, and 290 [12]. The HA protein of A/Vietnam/1203/2004 (VN1203), the virus used in the H5N1 vaccine, has potential glycosylated positions at residues 22, 34, 158, 165, 176, 204, 297, 495, and 555 [10, 13]. The glycosylation site at 158 on VN1203 has been previously shown to decrease the antigenicity and immunogenicity of a live attenuated H5N1 vaccine studied in ferrets [13]. Future studies should aim to identify whether the addition or removal of glycosylation affects the ability of the influenza vaccines to bind and activate CLRs, and would determine the viral motifs recognized by the receptors. Motif recognition could also be examined utilizing viral peptide arrays and assessing CLR activation following treatment with peptides.

Conclusions

Highly pathogenic avian influenza is an emerging infectious virus with the potential to cause a pandemic, and cases of both H5N1 and H7N9 are currently circulating in humans. The first study described in this dissertation compared the immune response to inactivated subunit vaccines targeting seasonal and H5N1 influenza viruses, as well as, defined a mechanism for the attenuated response to the H5N1 vaccine. We conclude that seasonal influenza vaccines stimulate CLRs, implicating a novel role for CLRs in influenza vaccination, whereas the H5N1 vaccine binds to CLRs but does not initiate an immune response.

The second study compared the immune response to the H5N1 vaccine with and without the addition of an alum adjuvant; studies were also performed to determine a mechanism of action for alum in our model of vaccination. We conclude that alum increases antigen uptake and intracellular processing. While the alum adjuvant is able to rescue the immunogenicity of the H5N1 vaccine, it does so though a different mechanism of activation than that employed by seasonal influenza vaccines.

The development of vaccines for use against HPAI is a high priority, especially in affected countries, and there are many challenges concerning the development of pandemic influenza vaccines targeting HPAI. For example, the amount of vaccine that can be generated globally is limited and would be insufficient for complete coverage of the world's population [14, 15]. Additionally, vaccine production capabilities are concentrated in industrialized countries, and it is estimated that deaths associated with an influenza pandemic will be greater in

developing countries than in industrialized countries due to factors such as: lack of access to adequate medical care; poor public health infrastructures; social factors such as population density and housing conditions; and host factors such as poor nutrition and co-existing medical conditions like HIV/AIDS [15]. Furthermore, it is not possible to predict which subtype of influenza will cause the next pandemic, so a vaccine stockpiled against H5N1 may not protect against an outbreak of H7N9 [16]. The production of a vaccine following the identification of a virus causing a pandemic is time consuming, and conventional methods used for growing the virus for the vaccine strain may not be sufficient as HPAI viruses are difficult to grow in eggs [14, 17]. Finally, the low immunogenicity produced by current HPAI pandemic vaccines demonstrates a necessity for multiple vaccinations, or the addition of an adjuvant, in order to provide protection [14, 18, 19]. A greater understanding behind the immunological mechanisms of influenza vaccination will lead to the development of more effective vaccines, and will help to overcome one of the challenges facing pandemic HPAI vaccine development.

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APPENDIX A

Results

Development of cross-protective neutralizing antibodies

One challenge facing the development of vaccines targeting HPAI is that a vaccine developed which is specific to the virus circulating at the time, such as the H5N1 vaccine targeting VN1203 developed in 2003/2004, may not provide protection to a different strain of HPAI that circulates in the future, such as the H7N9 HPAI virus that began circulating in February 2013, or even a different strain of H5N1. Studies were conducted to determine whether mice vaccinated with the adjuvanted H5N1 vaccine targeting VN1203 developed cross-protective neutralizing antibodies against other strains of H5N1. Sera from BALB/c mice vaccinated with the adjuvanted H5N1 vaccine, which produced neutralizing antibodies against VN1203, were tested for the ability to neutralize other H5N1 viruses, specifically: the clade 0 viruses A/Hong Kong/156/1997 (HK156), A/Hong Kong/483/1997 (HK483), and A/Hong Kong/486/1997; the clade 1 virus A/Vietnam/1194/2004 (VN1194); and the clade 2 viruses A/duck/Hunan/795/2002 (DH795) and A/common magpie/Hong Kong/645/2006 (CM645). Briefly, six to eight-week-old BALB/c mice received either a primary or a primary and secondary vaccination of the adjuvanted H5N1 vaccine with doses ranging from 0.6ug to 30ug. Secondary vaccinations occurred three weeks following primary vaccinations. Sera were collected three weeks following primary vaccination or four weeks following secondary vaccination and assessed for neutralizing antibodies using a neutralization assay. Sera from two of three

mice tested against HK156 and HK486 contained neutralizing antibody titers greater than 1:40, and sera from one of three mice tested against HK483 contained neutralizing antibody titers greater than 1:40. Sera from three of three mice tested against VN1194, DH795, and CM645 contained neutralizing antibodies greater than 1:40 (Figure A1). This data demonstrates that mice vaccinated against VN1203 produced cross-protective neutralizing antibodies against other strains of H5N1.

Antigen presenting cell migration following influenza vaccination in vivo

The mechanisms surrounding APC activation by inactivated influenza subunit vaccines presented in the body of this work were elucidated *in vitro*. Preliminary studies analyzing APC activation *in vivo* were also conducted. To determine the main APC type responding to influenza vaccination *in* vivo, six to eight-week-old BALB/c mice (n = 1 per treatment per time point) were vaccinated subcutaneously (SC) in the inguinal area with either the seasonal or non-adjuvanted HPAI vaccine with or without the addition of a 1:10 dilution of $FITC^+$ FluoSpheres. DCs and macrophages expressing FITC were considered to have internalized vaccine antigen. Popliteal lymph nodes were harvested 24 or 48 hours following vaccination and assessed for the quantity of DCs and macrophages present therein. DCs were identified as $CD11c^+$ /$CD11b^+$, and macrophages were identified as $CD11c^+$/$F4/80^+$ using flow cytometry. Following vaccination with either the seasonal or non-adjuvanted HPAI vaccines with the addition of $FITC^+$ FluoSpheres, a larger number of total and $FITC^+$ DCs was

found in the draining lymph node (DLN) compared to macrophages (Figure A2a and c). The results also demonstrated that a larger number of DCs may be responding to the seasonal vaccine compared to the HPAI vaccine. Additionally, an increased quantity of total and $FITC^+$ DCs was present in the DLN 24 hours following vaccination compared to 48 hours following vaccination (Figure A2b and d). These data suggest that DCs are the primary APCs responding to influenza vaccination, and that 24 hours is the better time point at which to analyze APC activity in the DLN.

In the experiment discussed above, the majority of cells isolated from the DLN at 24 hours following vaccination with either the seasonal or non-adjuvanted HPAI vaccine expressed FITC (Figure A3). Interestingly, 48 hours following vaccination, a much larger FITC negative cell population was present in the DLN of mice vaccinated with the seasonal vaccine compared to mice vaccinated with the non-adjuvanted HPAI vaccine. In the DLN of mice that received the non-adjuvanted HPAI vaccine, there remained a larger number of $FITC^+$ cells compared to mice that received the seasonal vaccine. This data suggests that 48 hours following vaccination, in mice treated with the seasonal vaccine, APCs have presented antigen to T cells and T cells are clonally expanding. However, a limitation to this study is that T cell markers were not utilized, so this conclusion is only speculation at this time and needs follow-up.

Antigen presenting cell activation in vivo

To further examine APC activation *in vivo,* expression of the co-stimulatory molecules CD40 and CD86 on DCs harvested from the popliteal lymph node was assessed. BALB/c mice (n = 3 per treatment) were vaccinated intramuscularly (IM) with either the seasonal or non-adjuvanted H5N1 vaccine. Popliteal lymph nodes were harvested 24 hours following vaccination and expression of the co-stimulatory molecules CD40 and CD86 was assessed by flow cytometry. DCs were identified as CD11c/CD11b positive. Significantly higher levels of CD40 (Figure A4a) and CD86 (Figure A4b) were detected on DCs isolated from mice that received the seasonal vaccine compared to mice that received the non-adjuvanted HPAI vaccine. These data demonstrate that the seasonal vaccine is able to more efficiently activate APCs *in vivo* compared to the non-adjuvanted HPAI vaccine. Interestingly, when comparing cell counts, the number of DCs present in the DLN did not vary between treatment groups as seen in Figure A2 (data not shown). This could be due to the difference in the route of immunization (IM vs. SC).

T cell responses in vivo

Preliminary studies assessed early T cell responses *in vivo*. BALB/c mice (n = 2 per treatment) were vaccinated SC in the inguinal area with either the inactivated subunit vaccine targeting the 2009 H1N1 pandemic virus or the non-adjuvanted HPAI vaccine. Popliteal lymph nodes were harvested 24 hours following vaccination and assessed for a $CD28^-/CD62L^+$ homing T cell

population. In the DLNs harvested from mice that received the 2009 H1N1 vaccine, a $CD28^{-}/CD62L^{+}$ homing T cell population was detected, whereas in mice that received the non-adjuvanted HPAI vaccine, this cell population was not detected (Figure A5). This data suggests differences in T cell responses to the 2009 H1N1 vaccine when compared to the non-adjuvanted HPAI vaccine.

Figure Legends

Figure A1. Cross-protective neutralizing antibodies in BALB/c mice. BALB/c mice received either a primary or a primary and secondary vaccination of the adjuvanted HPAI vaccine targeting VN1203. Sera were collected three weeks following primary vaccination or four weeks following secondary vaccination and neutralizing antibody titers to other strains of H5N1 were determined utilizing neutralization assays. The viruses analyzed include: HK156 (closed circles); HK483 (squares); HK486 (triangles); VN1194 (upside-down triangles); DH795 (diamonds); and CM645 (open circles). Data points represent individual animals.

Figure A2. DCs respond to influenza vaccine antigen *in vivo.* BALB/c mice received a SC vaccination of either the seasonal or non-adjuvanted HPAI vaccine with or without the addition of $FITC^+$ FluoSpheres. Popliteal lymph nodes were harvested 24 or 48 hours following vaccination and assessed for $CD11c^+/CD11b^+$ DC and $CD11c^+/F4/80^+$ macrophage populations by flow cytometry. **A and B.** Cell counts in the DLN following vaccination with either the seasonal vaccine (white bars) or non-adjuvanted HPAI vaccine (black bars) at 24 and 48 hours respectively. **C and D.** Cell counts representing $FITC^+$ APCs in the DLN following vaccination with seasonal vaccine (white bars) or non-adjuvanted HPAI vaccine (black bars) mixed with $FITC^+$ FluoSpheres at 24 and 48 hours respectively. The mice utilized in this experiment were separate from those in A and B.

Figure A3. Increased FITC negative cell population present in the DLN following seasonal vaccination. BALB/c mice received a SC vaccination of either the seasonal or non-adjuvanted HPAI vaccine with the addition of $FITC^+$ FluoSpheres. Popliteal lymph nodes were harvested 24 or 48 hours following vaccination and cells were assessed for the presence or absence of $FITC^+$ expression by flow cytometry.

Figure A4. Increased expression of co-stimulatory molecules on DCs treated with seasonal influenza vaccine. BALB/c mice were vaccinated intramuscularly with either the seasonal vaccine or non-adjuvanted HPAI vaccine. Popliteal lymph nodes were harvested 24 hours following vaccination and $CD11c^+/CD11b^+$ DCs were assessed for the expression of co-stimulatory markers by flow cytometry. **A.** Median fluorescence intensity (MFI) of CD40 expression on DCs isolated from the DLNs of mice vaccinated with the seasonal influenza vaccine (white bars) or non-adjuvanted HPAI vaccine (black bars). Error bars indicate mean ± SEM (*p<.05, Student's t-test). **B.** Median fluorescence intensity (MFI) of CD86 expression on DCs isolated from the DLNs of mice vaccinated with the seasonal influenza vaccine (white bars) or non-adjuvanted HPAI vaccine (black bars). Error bars indicate mean ± SEM (*p<.05, Student's t-test).

Figure A5. $CD28^-/CD62L^+$ homing T cell population present in the DLN of mice treated with seasonal but not HPAI vaccine. BALB/c mice were

vaccinated subcutaneously with either the 2009 H1N1 pandemic vaccine or the non-adjuvanted HPAI vaccine. Popliteal lymph nodes were harvested 24 hours following vaccination and cell populations were assessed by flow cytometry for $CD28^-/CD62L^+$ homing T cells. Cells were gated on $CD28^-$ cell populations followed by expression of CD62L.

Figures

Figure A1

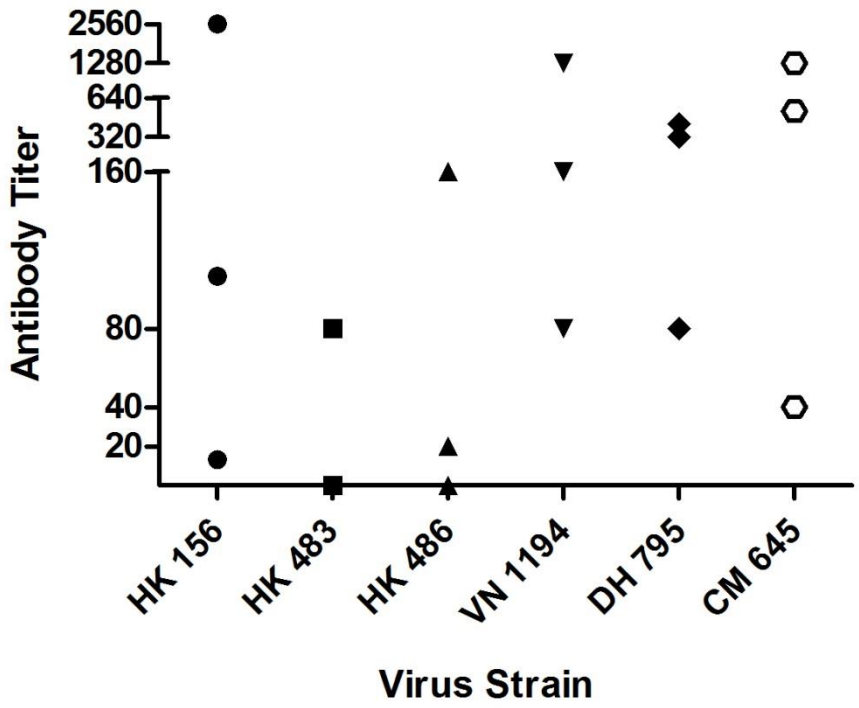

Figure A2

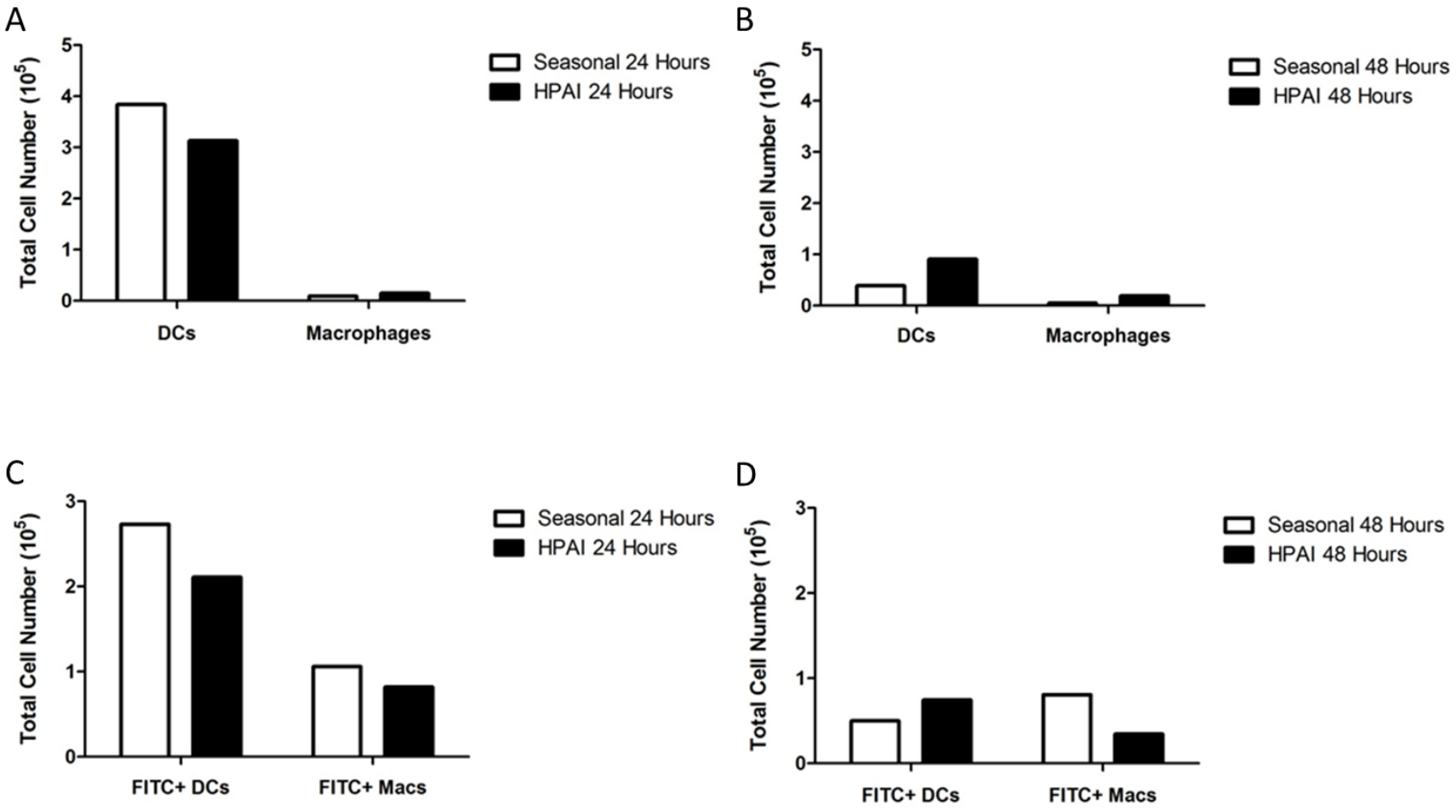
A
Total Cell Number (10^5)
5
4
3
2
1
0
DCs
Macrophages
Seasonal 24 Hours
HPAI 24 Hours
B
Total Cell Number (10^5)
5
4
3
2
1
0
DCs
Macrophages
Seasonal 48 Hours
HPAI 48 Hours
C
Total Cell Number (10^5)
3
2
1
0
FITC+ DCs
FITC+ Macs
Seasonal 24 Hours
HPAI 24 Hours
D
Total Cell Number (10^5)
3
2
1
0
FITC+ DCs
FITC+ Macs
Seasonal 48 Hours
HPAI 48 Hours

Figure A3

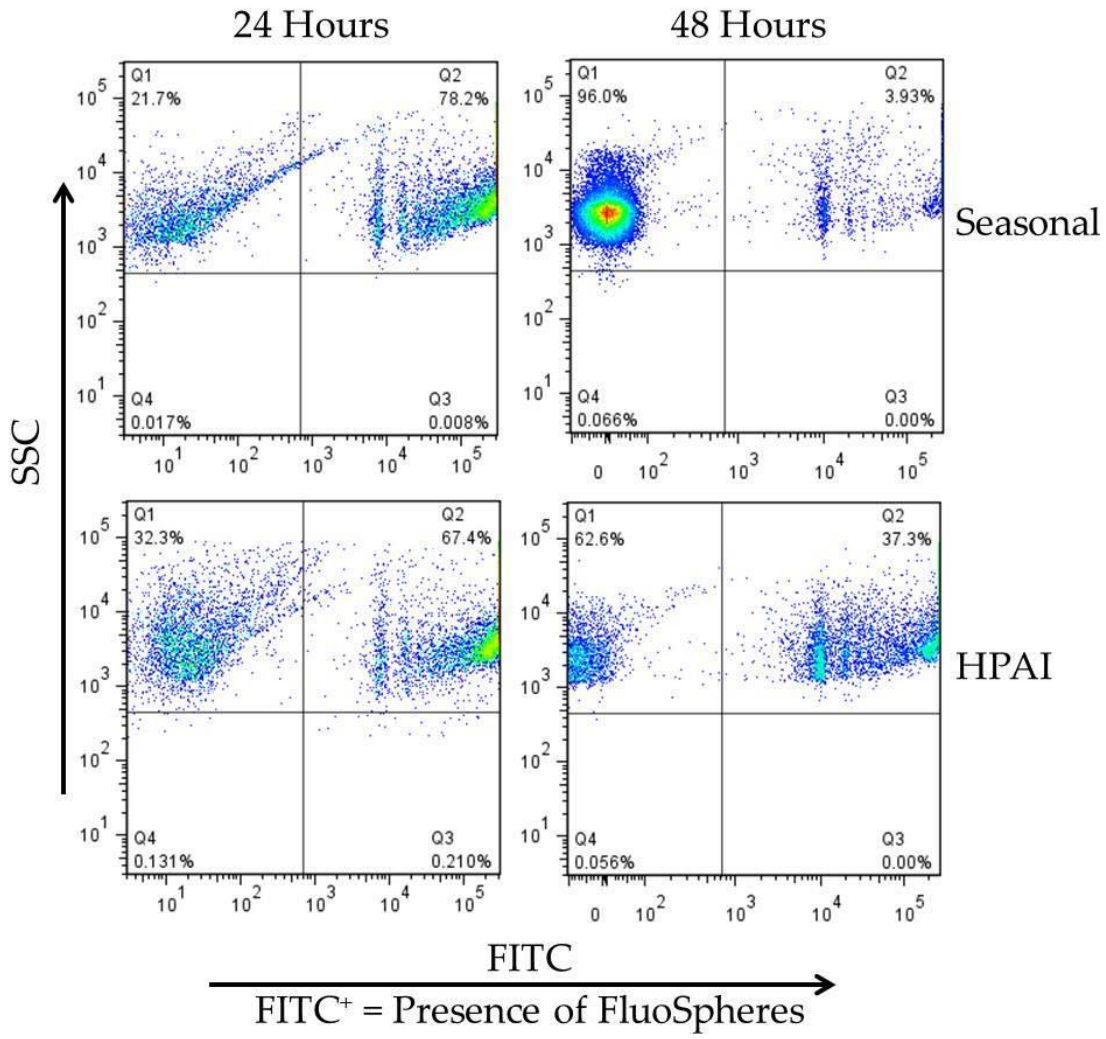
24 Hours
48 Hours
Q1
21.7%
Q2
78.2%
Q4
0.017%
Q3
0.008%
Q1
96.0%
Q2
3.93%
Q4
0.066%
Q3
0.00%
Seasonal
Q1
32.3%
Q2
67.4%
Q4
0.131%
Q3
0.210%
Q1
62.6%
Q2
37.3%
Q4
0.056%
Q3
0.00%
HPAI
SSC
FITC
FITC+ = Presence of FluoSpheres

Figure A4

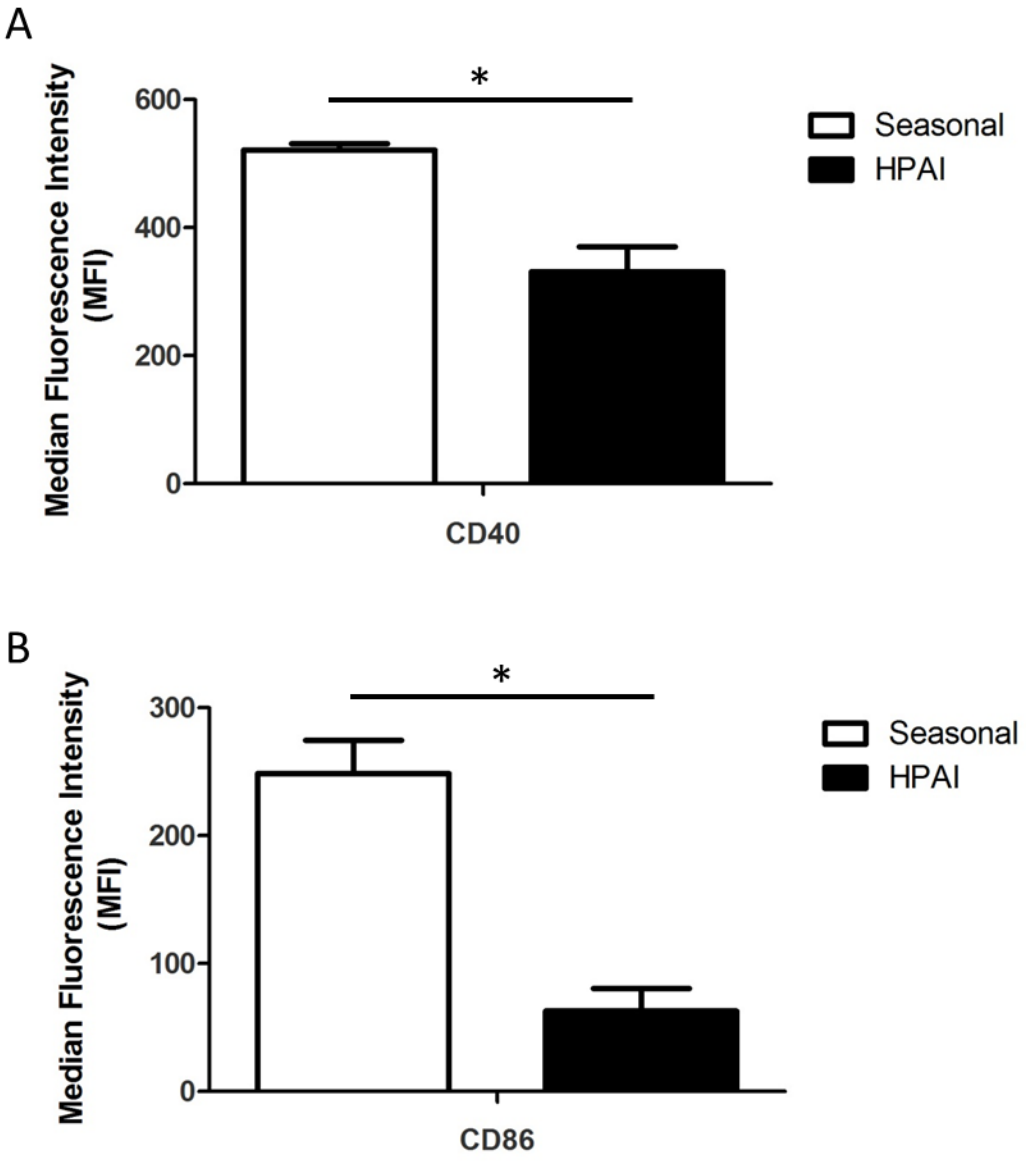
A
Median Fluorescence Intensity (MFI)
600
400
200
0
*
CD40
Seasonal
HPAI
B
Median Fluorescence Intensity (MFI)
300
200
100
0
*
CD86
Seasonal
HPAI

Figure A5