ANTIBODY-GUIDED COMPLEXES AND THEIR POTENTIAL APPLICATIONS IN POULTRY RESEARCH

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2017

Major Subject: Veterinary Pathobiology

ABSTRACT

Targeting the CD40 receptor displayed by antigen-presenting cells to deliver a specific immunogen has been successfully used to enhance immune responses, specifically increasing antibody production and enhancing antibody affinity. When tested in chickens, this platform induced specific IgG and IgA production within one week post-immunization. However, proof of conferred protective efficacy using the CD40-targeting vaccination method was still undetermined. Whole avian influenza virus was loaded onto the guided complex and immunized birds were challenged with highly pathogenic avian influenza (HPAI) to test efficacy. Furthermore, this research addresses the application of guided complexes as an alternative method for epitope mapping of microbial enzymes. Short peptide segments of the *Clostridium perfringens* alpha toxin were loaded onto the antibody-guided complex and immunized into chickens to induce antibody production for downstream use in neutralization assays to identify specific regions able to block the toxin's enzymatic functions. Lastly, to expand the antibody-guided system repertoire, monoclonal antibodies against a new receptor, specifically dendritic cell (DC) marker CD205, were developed for potential use to further enhance immune response activation. Anti-CD205 monoclonal antibodies were used to develop a new *in vitro* DC system obtained from peritoneal exudate cells.

In HPAI efficacy studies, functional antibody titers were detected up to six weeks after a single subcutaneous administration. When boosted, the antibody-guided complex conferred 100% protection in birds upon lethal H5N1 challenge. The guided system also

proved useful for rapid polyclonal antibody production in chickens, which can be used in epitope mapping studies. This system favors linear peptide targets for immunization in order to maintain cost-effectiveness and short turnover time, but can still be used with conformational epitopes. Monoclonal antibodies were successfully constructed against chicken CD205 and used in a variety of immunoassays, as well as magnetic bead isolation of DCs from peritoneal exudate cell populations. Overall, these data are the first to report protective efficacy using the CD40-targeting system in chickens, the first to propose the use of guided complexes in epitope mapping, and the first to isolate DCs from peritoneal exudate using the anti-CD205 monoclonal antibodies.

ACKNOWLEDGEMENTS

I would like to thank my committee chair Dr. Luc R. Berghman for his patience and support. As an advisor, he is beyond exemplary. Dr. Berghman offers a balanced mentoring style, innately knowing when to deliver direct supervision and when to provide students the freedom to formulate their own ideas (and make mistakes). Furthermore, he was able to adjust to my rather unconventional personality, which has unsurprisingly shifted many ordinary conversations to awkward and uncomfortable in mere moments. He has survived my acute emotional fluctuations and understood its inevitable correlation to the status of my research, and yet he remained unflustered and able to provide sound counsel to mitigate further distress. He has also provided thousands of liters of coffee through the years, because as any wise professor knows, a caffeinated graduate student is a productive graduate student.

I would also like to thank my committee members, Drs. Michael F. Criscitiello, Lisa R. Bielke, David J. Caldwell, Shuping Zhang, as well as unofficial member Dr. Billy M. Hargis, for over five years of encouragement and guidance in both life sciences and everyday life. They approach research seriously, but never forget to find the humor in a situation or in themselves. They have directed me through many scientific hurdles, opened up their homes to me, offered counsel on career planning, shared family recipes, overseen the progression of my research, and overall been wonderful people. I am very fortunate to know and work with this particular group of scientists and sincerely thank them for their support.

Completion of this graduate program would not have occurred without the support and competence of both the Veterinary Pathobiology and Poultry Science departmental staff members, as well as Texas A&M College of Veterinary Sciences' graduate research staff. Collaborative research was completed in cooperation with various members from Dr. Bielke and Dr. Hargis' laboratories; these colleagues have expanded my understanding to a wide scope of topics while being pleasant people to work with. I would specifically like to thank Dr. Wen-Ko (Ivan) Chou for years of scientific training and camaraderie in the Berghman laboratory. He has assisted me on long sampling days, sympathized with laboratory frustrations, defended me against aggressive students, and helped troubleshoot problematic assays. He has truly been the finest companion throughout this process. Finally, I would like to thank my siblings: Kim, Dan, and Ann, simply for their patience with me as the youngest offspring.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of committee chair Professor Luc R. Berghman of the Texas A&M University Departments of Veterinary Pathobiology and Poultry Science and committee members Professors Lisa R. Bielke of the Ohio State University Department of Animal Science, Michael F. Criscitiello of the Texas A&M University Department of Veterinary Pathobiology, David J. Caldwell of the Texas A&M Department of Poultry Science, and Shuping Zhang of the University of Missouri Department of Veterinary Pathobiology. Billy M. Hargis of the University of Arkansas Department of Poultry Science also provided a significant deal of collaboration, supervision, and training. Facilities and animal care were completed in collaboration; work, analysis, and interpretation for the dissertation were completed by the student.

The Diversity Fellowship from Texas A&M University's Association of Former Students supported graduate study for the first three years. Following years were funded by teaching assistantships provided by the Texas A&M University College of Veterinary Medicine and research assistantships under the sponsorship of Dr. Luc R. Berghman. Avian influenza and alpha-toxin epitope mapping trials were partially funded by Pacific GeneTech, Ltd. Pacific GeneTech was not involved in the study design or collection, analysis, or interpretation of the data. A PCT application with International Patent Publication No. WO2015/187969 has been filed for the antibody-guided vaccine delivery system.

NOMENCLATURE

Ab	antibody
Ad	adherent
AGP	antimicrobial growth promoters
AI	avian influenza
AIV	avian influenza virus
ANOVA	analysis of variance
APC	antigen presenting cell
APHIS	Animal and Plant Health Inspection Service
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CLECT	C-type lectin binding domain
Cpa	*Clostridium perfringens* alpha toxin
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DIVA	differentiation of infected from vaccinated animals
DMEM	Dulbecco's modified eagle medium
EID_{50}	50% embryo infectious dose
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum

FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte-monocyte colony-stimulating factor
HA	hemagglutinin
HAU	hemagglutination unit
HI	hemagglutination inhibition
His	histadine
HPAI	highly pathogenic avian influenza
iAIV	inactivated avian influenza virus
IEDB	Immune Epitope Database and Analysis Resource
IFA	immunofluorescent assay
IFN-γ	interferon gamma
Ig	immunoglobulin
IL-4	interleukin 4
KLH	keyhole limpet hemocyanin
LPAI	lowly pathogenic avian influenza
LPS	lipopolysaccharide
M2e	M2 ion channel extracellular region
MHC	major histocompatibility complex
NA	neuraminidase
NE	necrotic enteritis
NetB	necrotic enteritis toxin-like beta

Non-Ad	non-adherent
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEC	peritoneal exudate cells
PLC	phospholipase C
PRR	pattern recognition receptor
RIG-I	retinoic acid inducible gene I
RPMI	Roswell Park Memorial Institute medium
S.C.	subcutaneous
TLR	toll-like receptor
TMB	peroxide/tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
Tukey HSD	Tukey honest significant difference
USDA	United States Department of Agricultur

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CHAPTER I

INTRODUCTION

Brief Introduction to the Poultry Industry

Poultry production is an extensive global industry as birds are less costly to feed, require less space, and are quicker to mature than other domesticated animals used for meat production. In the US, over 54 billion pounds of broiler meat (1) and over 102 billion eggs (2) were produced in 2016, as reported by the USDA National Agricultural Statistics Service. As a relatively affordable source of high quality animal protein, more poultry has been consumed per capita than any other animal-based protein source (3). Due to the large-scale nature of the industry and high stocking density of birds, proper care and health management is integral to successful and humane rearing of birds.

Diseases and Other Challenges Faced by the Industry

Antimicrobial growth promoters (AGPs) were officially approved for use in animal feeds in 1951, with 32 antimicrobials available for use without veterinary prescription. Of the original 32 approved compounds, 15 possessed anti-coccidial properties and 11 served as growth promoters at sub-therapeutic doses (4). The exact mechanism by which AGPs were able to improve growth is largely unknown, but it has been suggested that the use of antimicrobials reduces overall gut inflammation and allows for more efficient nutrient absorption (5). The use of AGPs continued for decades until concern over the increase in antibiotic-resistant bacteria prompted the ban

of specific AGPs as a feed additive in Denmark, a regulation later implemented by other countries in the European Union (6, 7). Due to rising consumer concern over the use of AGPs in livestock, commercial producers in the United States have also limited or ceased their usage. Since the removal of AGPs from feed, increased incidences of coccidiosis and necrotic enteritis (NE) have been observed (8, 9). This is especially true with increasing consumer demand for free-range birds, which encounter more pathogens than their conventionally housed counterparts. Although the use of ionophore-based coccidiostats has not been banned, some producers have preemptively halted the use of these drugs, further increasing the potential threat of coccidiosis in their flocks. Numerous alternatives have been tested to replace AGPs, ranging from probiotics to novel feed additives, but none have proven as effective as AGPs and none have been universally adopted by the industry (10).

Necrotic Enteritis

Necrotic enteritis is a multifactorial disease affecting poultry, primarily causing damage in the gastrointestinal tract, and in severe cases, resulting in mortality rates ranging from 2-10% (8). The essential causative agent of NE is *Clostridium perfringens*, but infection with this bacterium alone does not by itself cause disease. *Clostridium perfringens* is a Gram-positive anaerobic species that is ubiquitous in soil and is normally present at low levels in the bird's enteric microflora. Dysbiosis of the gut environment, inducing increased mucoid viscosity, triggers the overgrowth of the normally low levels of *C. perfringens* (11). This imbalance can be caused by many

different factors, such as a diet with excess non-starch polysaccharides (barley, wheat, rye, *etc*.) or a preexisting gut infection (coccidiosis, salmonellosis, *etc*.) (12, 13). As producers are reluctant to use ionophores to control coccidiosis, use of coccidial vaccines has become more common. These vaccines are live-pathogen based and purposely designed to induce subclinical natural infection, but their use has been reported to inadvertently increase the incidence of NE (14). Previously, NE disease was controlled by the addition of antibiotics such as bacitracin and lincomycin to the feed, but efforts to limit the use of antibiotics have essentially removed these options as prophylactic tools to prevent the disease.

Clinical signs of NE include a sudden onset of depression, diarrhea, and potentially death. Upon necropsy, lesions can be observed in the small intestine and is the typical method of diagnosis by attending veterinarians, but definitive laboratory diagnosis requires histological staining or culture of the lesions for *C. perfringens*. The actual damage is not caused by replication of the bacteria itself, but by the resulting release of a cocktail of toxins, inducing an enterotoxemia (15). *Clostridium perfringens* toxins are mainly categorized as either alpha, beta, epsilon, or lambda; in addition, each major toxin class is micro-heterogeneous and consists of a number of toxin-like variants (16). In poultry, the primary cause of NE-induced intestinal damage primarily originates from the hemolytic and phospholipase C enzymatic activities of alpha toxin (17). Various attempts have been made to design vaccines against NE by targeting alpha toxin, but most of those candidate vaccines have only provided partial protection or have not been formulated for use in poultry. Immunogens tested include whole recombinant

toxin, partial recombinant toxin, short peptide regions of the toxin, subunits of toxin expressed in vectors, and whole toxin expressed in bacterial vectors (18-21). Despite the many attempts at vaccine formulation and improved knowledge of the domains required for enzymatic function, a specific epitope region that provides complete protection against the disease has yet to be recognized by the scientific community (22). More recently, data have stated that alpha toxin may be non-essential (23) and a necrotic enteritis toxin-like beta variant (*NetB*) was asserted to be the main cause of disease (24), but initial studies solely targeting *NetB* as a vaccine candidate have proven unsuccessful (25) and further sequencing of virulent *C. perfringens* strains revealed that many did not possess the *NetB* gene. Despite this lead, alpha toxin seems to remain to be the primary culprit of NE-associated morbidity. Some further attempts have been made to identify protective epitopes on the toxin, but this work has mainly relied on time-consuming, laborious site-directed mutagenesis and protein expression techniques (22, 26-28). Development of a new more cost-effective method for epitope mapping would not only be beneficial for the study of alpha toxin, but also for mapping other enzymes.

Avian Influenza

Another disease of economical importance to the poultry industry would be avian influenza (AI). Avian influenza virus (AIV) is a member of the *Orthomyxoviridae* virus family, possessing a segmented, negative-sense RNA genome capable of infecting both avian and mammalian hosts. Influenza viruses are classified by their hemagglutinin (HA) and neuraminidase (NA) proteins, which required for attachment and release of the

virion from the host cell (29). Due to the fact that both proteins are highly expressed on the surface of the virus, host antibody responses are primarily directed towards these external proteins. Protection against disease has been correlated to high circulating antibody titers against the HA protein, preventing initial attachment of the virus to the host cell and therefore preventing infection of host cells, and hence viral replication and establishment of disease symptoms. Although vaccines have been developed against AIV, efficacy issues arise due to lack of cross-protection against different AIV subtypes. There are 16 distinct antigenic HA subtypes (H1-H16) and possessing neutralizing antibodies against one subtype does not guarantee protection against another subtype.

Small mutations commonly occur in AIV, as this virus possesses a RNA genome and is more prone to error during normal replication events (30). These small mutations accumulate over time as the virus spreads from one population to the next and is referred to as antigenic drift (30). The accumulated mutations from one flu season to the next as the virus circulates around the globe is the main cause and requirement for annual flu vaccine reformulations in humans, as each year's formulation must be updated to the major circulating strain. Periodic strain updates occur in poultry vaccines against AI, as needed. Large genome mutations can also occur suddenly when two or more viruses infect the same cell and exchange genome segments during replication, which is referred to as antigenic shift. These larger, abrupt changes can result in pandemic outbreaks since the existing host populations do not possess any level of immunity against this newly produced virus, as was the case in the 2003 and 2009 H5N1 pandemics (31).

Highly pathogenic avian influenza (HPAI) is capable of inducing 75-100% mortality in birds within 4 days of infection, while low pathogenic avian influenza (LPAI) may cause mild respiratory issues and general lethargy, or even no symptoms at all. Though highly pathogenic strains can result from antigenic shift, a major disease threat can also result from the small mutations accumulated during antigenic drift. HA protein cleavage required for successful attachment and invasion of host cells by the virus is typically achieved by exogenous trypsin from the host's respiratory and gastrointestinal tract (32). If this cleavage site were altered to a polybasic sequence, this crucial cleavage could be performed by furin-like proteases ubiquitously expressed in the body, allowing the viral infection to become systemic and no longer limited to the respiratory or gastrointestinal tracts (32).

Avian influenza is a disease that, although not endemic to the United States, is becoming more of a threat. Outbreaks of HPAI have occurred within the United States, with disease and control efforts that resulted in the death of over 48 million poultry in the 2014-2015 outbreak and 400,000 poultry in the 2016 Indiana outbreak (33), both of which resulted in large economic losses and reduced food production in the country. The United States does not actively vaccinate against this disease as this would cause issues with differentiating infected from vaccinated animals (DIVA) and therefore interfere with the country's international trading status as an AI-free country (34). Outbreaks of AI immediately trigger a "stamping out" approach in which entire flocks of affected birds are killed and the corresponding housing and equipment vigorously disinfected (35). Despite these efforts, HPAI outbreaks still randomly occur within this

country. Some countries with endemic AI in their poultry flocks choose to vaccinate birds, primarily with a circulating strain of AIV. The circulating AIV strain is propagated in embryonic chicken eggs and collected from the allantoic fluid, then chemically inactivated before being mixed with a mineral oil emulsion based adjuvant. This killed virus-mineral oil component vaccine is the major vaccine formulation used in the industry. Although initially effective to induce some antibody production against homologous HA protein, studies show that current vaccine methods are only partially protective and also lack long-term efficacy (36, 37), further emphasizing the dire need for more modern and efficacious vaccine alternatives.

Avian Immune System

Avian species are phylogenetically distinct from mammals, which is clearly reflected in substantial differences in their immune systems. This phylogenetic divergence has a practical consequence in that antibodies or standard primer sets used in mammalian assay systems typically perform poorly, or not at all, in avian research. Protein-coding open reading frames are estimated to only share 60% homology between avian and humans (38). Anatomically, primary lymphoid organs in birds include the multi-nodular thymus, the site of T-cell maturation, and the bursa of Fabricius, the site of B-cell maturation, while mammals do not possess a bursa and implement B-cell maturation within the bone marrow (39). Oddly enough, chickens only produce three classes of immunoglobulins (Ig): IgM, IgY, and IgA, while lacking the IgD and IgE classes (39). Though, the IgY isotype has been found to possess a composite of IgG and

IgE functions, and is considered the avian equivalent to IgG (40). Furthermore, birds do not possess lymphotoxins or lymphotoxin receptors needed for development of organized lymph nodes like mammals (41-43), and instead carry out secondary lymphoid functions in the spleen and regions of multiple nodular lymphoid tissues, such as Peyer's patches, pyloric tonsils, or cecal tonsils (39). On a cellular level, chickens possess heterophils in the place of all poly-morphonuclear cells, such as neutrophils, basophils, and eosinophils, which are typically present in other vertebrate animals (44-46). Chickens also preferentially use γδ T-cells as opposed to the αβ phenotype favored in mammals (47). Upon release of the sequenced chicken genome in 2004, scientists were able to confirm the presence of all immune gene families in the chicken typically found in mammals, but chickens seem to take a more minimalist approach to immune gene quantity and possess reduced repertoires (43, 48). For example, chickens have only retained genes absolutely necessary to maintain the use of major histocompatibility complex (MHC) receptors (49) and a tumor necrosis factor alpha (TNF-α) homolog is absent from the avian genome, despite the detection of a potential corresponding TNF-α receptor (48). Chickens also lack retinoic acid inducible gene I (RIG-I) and toll-like receptor (TLR) 8 receptors used to detect single-stranded RNA, although the similarly functioning TLR7 is still present (50). Based on this information, it is not surprising that birds preferentially use gene conversion mechanisms during immune repertoire development, despite sharing the ability to also perform traditional somatic hypermutation mechanisms typically used by mammals (51-53).

Despite these differences, the fundamental steps required to mount an immune

response against a foreign pathogen transpire in the bird similarly to their mammalian counterparts (54). Most infectious disease agents enter through mucosal tissues, either through the respiratory or gastrointestinal routes. Pathogens enter these systems and establish, either by viral infection and replication in host cells, or bacterial use of host nutrients for replication. This self-promoting process may damage the host and lead to disease. During this process, the avian host's immune system normally responds by attempting to clear these invading pathogens from the system. Initially, this occurs through innate immunity processes that activate immediately upon detection of non-self, foreign entities via pattern recognition receptors (PRR) present on innate effector cells and B-cells. These PRRs bind and recognize common pathogen signature motifs called pathogen-associated molecule patterns (PAMP), which are not biologically present in the avian host. Common PAMPs include lipopolysaccharides of gram-negative bacterial cells walls, flagellin, or double-stranded RNA. Detection of these unwanted pathogens initiates recruitment of many innate immune cells to the affected site, including macrophages and heterophils, both possessing phagocytic and inflammatory functions. If this infection is not cleared quickly, the adaptive immune response will be triggered. This is initiated by antigen uptake of pathogen components by professional antigen presenting cells (APC), a population of cells consisting of dendritic cells (DC), macrophages, and B-cells. The DC will present pathogen-derived antigen to naïve T-cells in secondary lymphoid tissues in an attempt to activate a matching lymphocyte that is able to recognize the specific antigen. Should the presented antigen be recognized and bound by a T-lymphocyte, while receiving co-stimulatory signals, these T-lymphocytes

will become fully activated and undergo clonal expansion to multiply into daughter effector T-cells. Effector helper T-cells will go on to provide co-stimulatory signals to naïve B-cells, initiating a B-cell clonal expansion and consequent antibody production response to the specific antigen. In effect, all adaptive immune responses require professional APCs to initiate and mediate a reaction.

CD40

The CD40 co-stimulatory receptor is a member of the tumor necrosis factor (TNF) receptor superfamily. This 30kDa receptor is expressed on all professional APCs and interaction with this receptor mediates many downstream immune functions (55). CD40's ligand, CD154, also called CD40L, is expressed on helper T-cells and, upon CD40-CD154 ligation, recipient APCs undergo activation and enhancement of effector functions. B-cells, in particular, will begin clonal expansion and robust antibody production (Figure 1). This signal also aids in immunoglobulin isotype class switching from initial low affinity IgM to more effective isotypes (56) and drives affinity maturation (57). Supplementation with soluble CD154 *in vitro* has been shown to maintain chicken B-cells under cell culture conditions (58), further emphasizing the importance of CD40 engagement to B-cell survival and function. In human disease, patients deficient in CD40 expression suffer from hyper-IgM syndrome and lack the ability to produce other Ig classes and are, therefore, less able to clear pathogens which rely heavily on humoral immunity (59). Some preliminary research has been completed with avian CD40 receptor targeting, but has primarily focused on the inclusion of

CD154 ligand into some iteration of a vaccine to increase immunogenicity, mainly by expressing CD154 receptor onto bacterial vaccine vectors (60-62).

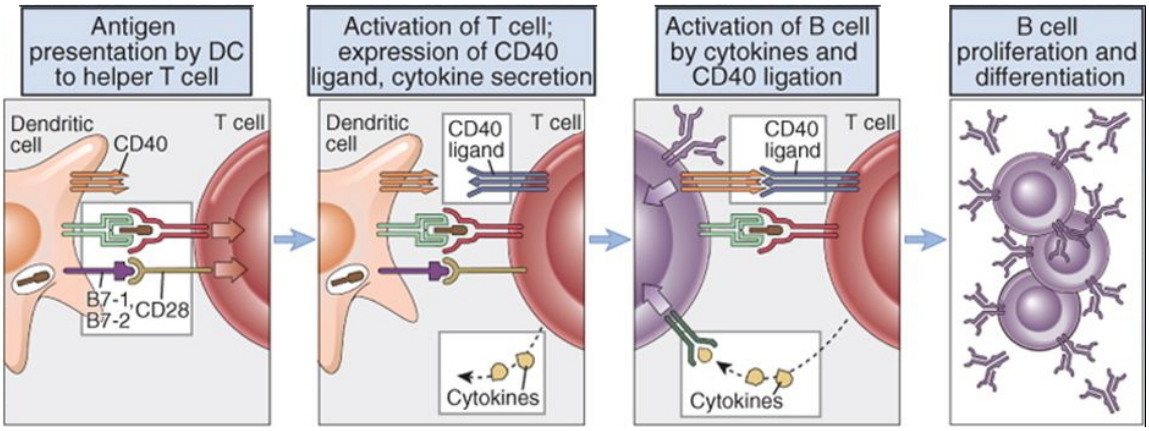

Figure 1. Abbas *et al.*'s diagram of basic steps to B-cell activation and proliferation.
Dendritic cells must activate naïve helper T-cells, which go on to activate naïve B-cells through cytokine stimulation and CD40 receptor engagement to allow B-cells to undergo proliferation and assume effector functions. Figure modified from Abbas *et al*., 2007 (54).

CD205

An additional receptor important to initiation of the adaptive immune response would be CD205. CD205 receptor, previously known as DEC205 or Lys75, is an endocytic receptor capable of binding mannose through its C-type lectin domains, but also binds oligonucleotides such as CpG (63). This 205kDa receptor is highly expressed on dendritic cells and thymic epithelial cells, and mediates antigen uptake and presentation. As shown in Figure 2, CD205-expressing DCs are capable of antigen

presentation in either MHC-I or MHC-II context, activating $CD8^+$ cytotoxic or $CD4^+$ helper T-cells respectively (64, 65). Higher CD205 expression on DCs is linked to increased antigen presentation in mice (66) and studies targeting CD205 during immunization were also able to increase induction of T-cell immunity (67).

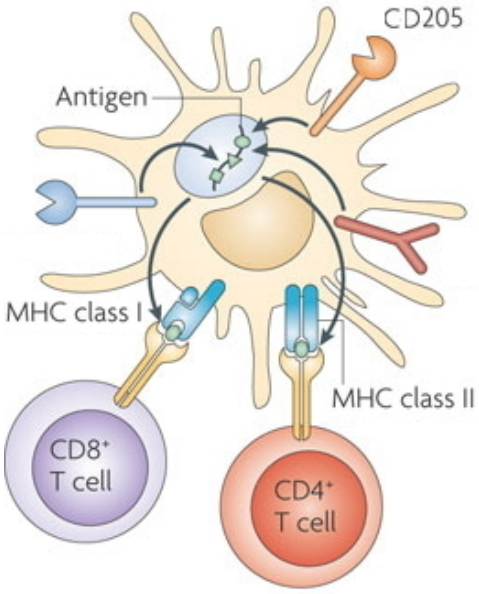

Figure 2. Geijtenbeek *et al.*'s diagram of dendritic cell presenting antigen on MHC-I and MHC-II.
$CD205^+$ DCs are capable of presenting antigen on MHC-II to activate $CD4^+$ helper T-cell and cross-presentation of antigen on MHC-I to activate $CD8^+$ cytotoxic T-cells. Figure modified from Geijtenbeek *et al*., 2009 (68).

Avian CD205 gene structure is highly conserved when compared to its mammalian counterpart (69). As expected, $CD205^+$ DCs are highly expressed in tissues which mediate T-cell activation, such as chicken bursa, thymus, and spleen (69). Very little research has been completed on CD205 in chickens, primarily due to the lack of

available reagents. Commercial antibodies are not available for chicken CD205, nor do transformed chicken DCs cell lines exist for *in vitro* testing. Although a method was described to collect bone marrow myeloid progenitor cells from the chicken to induce DCs differentiation with granulocyte-monocyte colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) cytokines, this procedure is labor intensive and the culture time required before usable mature DCs are available can take up as much as 2-3 weeks (70). DCs developed from this method also differentiate *ex vivo* and may not be representative of *in vivo* matured, activated primary DCs. In order to facilitate the study of chicken DCs and further understand the functions of the CD205 receptor, more reagents and methods needed to be developed for use in poultry.

Antibodies in Therapeutic Use

Antibodies have been used for therapeutic purposes since the use of antitoxins and antisera can counteract acute disease symptoms. Production of such antisera is performed in animals, typically large mammals such as horses and donkeys, by immunization against the toxin or target of interest to induce development of neutralizing antibodies, and the antisera collected for therapeutic uses. Immunosuppressive drugs also utilize antibodies as a method to bind and block inflammatory cytokines and are commonly dispensed medications for human use. Antibody-based medications licensed for human use include Humira (adalimumab), which targets TNF-α, and HuZAF (fontolizumab), which targets interferon gamma (IFNγ). Both utilize monoclonal antibodies to treat chronic inflammatory diseases such

as rheumatoid arthritis or Crohn's disease (71). These drugs have been highly successful in treating inflammatory diseases and various iterations of the anti-TNF-α monoclonal drugs have been manufactured (golimumab marketed at Simponi, infliximab marketed as Remicade, and certolizumab pegol marketed as Cimzia) (71, 72). This type of passive immunization primarily uses antibodies for their blocking and neutralizing functions. In contrast, some therapeutics utilize the ability of antibodies to imitate naturally occurring receptor-ligand interactions to induce specific downstream functions (73). The use of these so-called agonistic antibodies is not as well established as the use of neutralizing antagonistic antibodies, but this research field and its potential applications are vast.

Agonistic antibodies function by mimicking the action of a naturally binding ligand to a specific receptor to induce controlled activation and downstream functions specific to that receptor (74). This is typically achieved by creation and screening of antibody candidates during monoclonal antibody development to ensure the selected antibody both binds and activates - as opposed to merely bind. The majority of antibody therapy studies have been completed in murine and human systems (75), primarily as a method to treat and clear cancerous cells (76). The most extensively studied immune receptor studied for this type of therapy is the CD40 receptor (77, 78). Although activation of this receptor on APCs typically induces a pro-inflammatory immune response, this receptor is also highly expressed on cancerous cells which undergo apoptosis when engaged by agonistic anti-CD40 antibodies (79). Immunization with agonistic anti-CD40 antibodies combined with soluble cancer antigen target has recently been shown to stimulate development of cancer-specific T-cells reaching up to 60% of

the total T-cell population in mice (80). Antibody targeting and Fc receptor manipulation has been quite successful in this regard, with studies showing effective induction of antibody-dependent cell-mediated cytotoxicity or complement dependent cytotoxicity (81). A recent study has shown targeting with an IgE antibody against a specific cancer marker to be even more effective at clearing cancerous cells than IgG by harnessing the system originally designed for parasite clearance and therefore increasing macrophage phagocytic activity (82). This may be another avenue to pursue in disease research, particularly in designing methods to induce specific immune response pathways using agonistic antibody-mediated targeting.

Antibody-Guided Complexes

An initial mouse study by Barr *et al.* in 2003 illustrated that conjugation of antigen to anti-CD40 monoclonal antibodies was able to increase antibody responses against the specified antigen using less reagent (10μg) compared to immunization of a mixed solution of unconjugated anti-CD40 and antigen. Previous administration of anti-CD40 monoclonal antibodies mixed with antigen required delivery of higher dosages ranging from 100μg to 250μg of antibodies to increase immunogenicity against the antigen (83-85). Administration of anti-CD40 antibody without antigen conjugation has also been associated with several negative side effects, such as general polyclonal activation of B-cells and increased incidence of splenomegaly in mice (86). The transition to direct conjugation of antigen to CD40-targeting antibodies was able to

mitigate these negative side effects in mice while maintaining immunogenicity using a reduced amount of targeting antibody and antigen.

In response to this research, the Berghman laboratory developed an agonistic monoclonal antibody against chicken CD40 protein receptor, designated as 2C5, capable of stimulating proliferation of serum-starved B-cells and also activating chicken macrophages *in vitro* based on nitric oxide assays (87). These agonistic antibodies were used to present antigen directly to professional APCs in an effort to induce rapid immune responses (88). This was accomplished by biotinylation of the anti-CD40 antibodies and the synthetic peptide immunogen, and then using streptavidin as a scaffold to create a complex containing two anti-CD40 antibodies and two peptide immunogens held together by one streptavidin (Figure 3). This antibody-guided complex was administered subcutaneously into birds and serum collected to monitor subsequent immune response. Peptide-specific IgY antibodies, the avian equivalent of mammalian IgG, were detected in serum as early as 4 days post-immunization, continued to increase at 7 days, peaked at 10 days, and began to wane at 14 days post-immunization. This experiment demonstrated that the guided-antibody complex was capable of inducing robust antibody production, as well as rapid antibody isotype switching from less desirable IgM to IgY antibodies in chickens. When administered via different routes such as oral, eye drop, cloacal, or subcutaneous, this complex was able to induce statistically significant increased peptide-specific circulatory IgY levels, as well as measurable secretory IgA levels from tracheal wash samples when compared to the non-guided complex control groups (89).

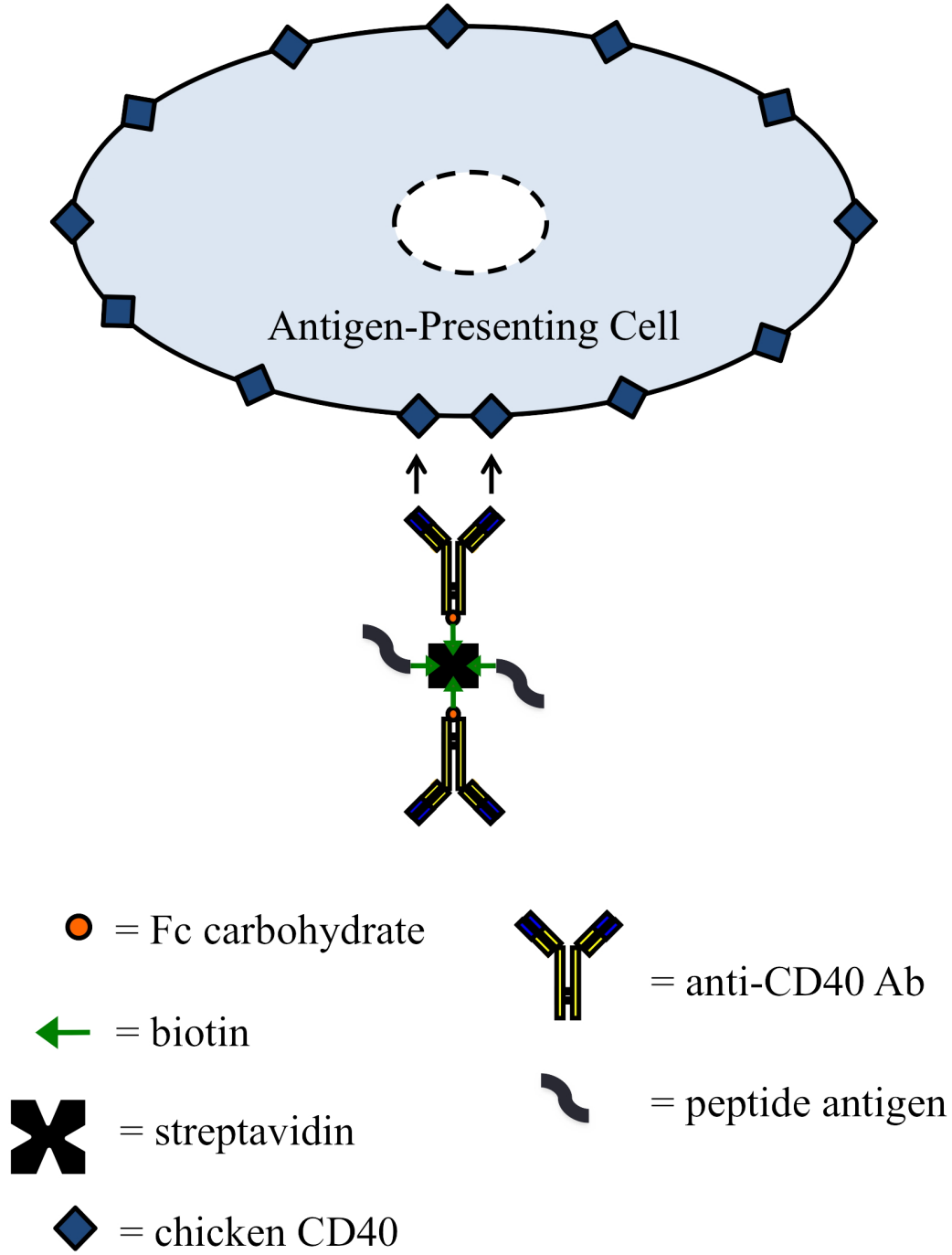

Figure 3. Schematic of antibody-guided complex loaded with peptide antigen. Anti-CD40 monoclonal antibodies (Ab) were biotinylated and attached to a streptavidin scaffold at a ratio of 2 anti-CD40 : 1 streptavidin : 2 peptide antigen. The resulting complex was used for immunization in chickens to provide both target antigen and co-stimulatory binding signals to antigen-presenting cells.

The CD40-targeting antibody-guided complex was shown to effectively induce antibody production in chickens, but the following questions remain to be fully investigated. Are there other potential uses for this system beyond a vaccine delivery platform? Can immunogens beyond short synthetic peptides be loaded onto the system without negatively impacting efficacy? And how efficacious is this platform as a vaccine? Furthermore, the potential of targeting other immune receptors for antibody-guided immunization in chickens has also not been fully examined. Ligation of different immune receptors would render different responses; depending on the desired response, this may be preferable as an alternative to solely targeting CD40.

CHAPTER II

A FAST AND INEXPENSIVE PROTOCOL FOR EMPIRICAL VERIFICATION OF NEUTRALIZING EPITOPES IN MICROBIAL TOXINS AND ENZYMES*

Introduction

Inducing antigen-presenting cell activation and effector responses requires both binding of a specific antigen by the APC and co-stimulatory signals received from helper T-cells. Binding and activation of the CD40 receptor on B-cells emulates the germinal center environment and triggers downstream rapid antibody production and isotype switching against a specific antigen (55). Manipulation of this system would be beneficial for controlled guidance of the immune system's reactivity against a defined target, specifically by using agonistic monoclonal antibodies against the CD40 receptor loaded with an antigen of interest. These CD40-targeting antibody-guided complexes have previously been tested for use as *in vivo* delivery systems for vaccines (91-93). In poultry, this CD40-targeting approach has been shown to induce robust and specific IgG serum antibody responses within one week (88), as well as secretory IgA production in the mucosal samples (89), essentially bypassing the weaker - chiefly IgM - initial immune response associated with primary immunizations. The application of this guided complex to induce rapid antibody production beyond its initial vaccine

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*Reprinted with permission from "A fast and inexpensive protocol for empirical verification of neutralizing epitopes in microbial toxins and enzymes" by Vuong CN, Chou WK. Kuttappan VA, Hargis BM, Bielke LR, and Berghman LR, 2017. *Front. in Vet. Sci.*, 4:91. Copyright 2017 by Christine N. Vuong (90).

designation has not been exploited. To assess the capabilities of the antibody-guided immunization system, *Clostridium perfringens* alpha toxin (Cpa) was used as a model microbial toxin for rapid antiserum production and downstream epitope mapping.

Alpha-toxin is one of many toxins produced by Clostridial bacteria and possesses both hemolytic and phospholipase C (PLC) enzymatic activities, making it an ideal model for epitope mapping. Neutralizing antibodies can be produced against specific regions of the toxin to test the antibody's ability to inhibit one or both of the toxin's enzymatic functions. In poultry, *C. perfringens* is the causative agent responsible for necrotic enteritis and continues to be an obstacle for the industry (94, 95). Although part of the commensal gut flora, *C. perfringens* can cause disease when an altered gut microenvironment or pre-established intestinal damage facilitates abnormal overgrowth and microbial dysbiosis in the gut (11). This imbalance results in intestinal lesions caused by the bacterium's multiple toxins and leaky gut syndrome in the bird (96, 97). Although alpha-toxin is no longer considered the sole toxin to target for vaccine development (23), a rapid method to determine the regions required to neutralize a toxin's activities would be of significant interest. Previous epitope mapping studies have primarily utilized site-directed mutagenesis, but this method requires specific base changes, molecular cloning, and downstream expression and purification before the altered toxin can be tested for change in function (22, 26-28). Introduction of a less expensive and more rapid epitope mapping method would be beneficial for researchers attempting to identify essential regions on a protein or candidate targets for therapeutics.

In this study, *C. perfringens* alpha-toxin was used as a model microbial toxin for epitope mapping to determine whether the antibody-guided immunization method has potential to be used for rapid identification of targets for downstream toxin neutralization or vaccine development. A panel of linear peptide epitopes spanning the majority of the Cpa's amino acid sequence was synthesized. The synthetic peptides were incorporated into the antibody-guided immunogen complex and administered in chickens for polyclonal IgG production. The peptide-specific antisera produced were used for downstream *in vitro* neutralization testing against the toxin's hemolytic and phospholipase C enzymatic functions, respectively. Using Cpa as a model toxin, this approach expands the function of antibody-guided immunization complexes beyond its initial use as a delivery system in poultry and highlights its potential as a method for rapid IgG production/reagent development, and as the fastest method to deliver proof of concept of potential toxin and enzyme neutralization strategies.

Materials and Methods

Peptide epitope design

Hydrophilic segments ranging from 9-23 amino acids in length were designed based from the 398 amino acid *Clostridium perfringens* alpha toxin sequence (GenBank Accession CAA35186.1) using Immune Epitope Database and Analysis Resource (IEBD) open-source predictive algorithms to construct a peptide library (98). The library consisted of 23 peptide epitopes in order to provide maximum coverage of the primary structure of the toxin while maintaining ease of synthesis (Table 1); peptides

were designated as numbers 1-23 based on starting position on the original Cpa toxin sequence (Figure 4). Hydrophobic stretches of the Cpa toxin were omitted to avoid peptide synthesis issues. Only consecutive linear regions of the alpha toxin were selected for synthesis to avoid the time and expense associated with the protein expression and purification required to produce conformational epitopes. Biotinylated commercially-synthesized peptides (Genscript, Piscataway, NJ) were incorporated stochiometrically in the antibody-guided immunization complex as described previously (88).

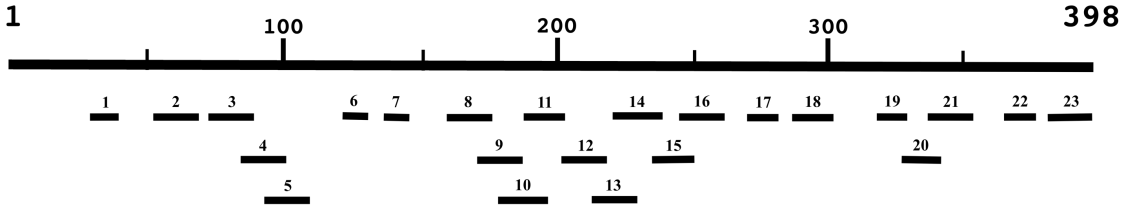

Figure 4. Schematic of 23 peptides generated based on *C. perfringens* alpha toxin amino acid sequence.
Linear peptides were selected based on ease of synthesis using IEDB publically available B-cell epitope prediction algorithms (98). (Figure not to scale.)

Table 1. *C. perfringens* alpha toxin-derived synthetic peptides.
Design based on Cpa GenBank Accession: CAA35186.1 amino acid sequence.

Peptide #	Start Position	Length	Sequence
1	31	9	GKIDGTGTH
2	51	15	ENDLSKNEPESVRKN
3	71	20	ENMHELQLGSTYPDYDKNAY
4	81	20	TYPDYDKNAYDLYQDHFWDP
5	91	20	DLYQDHFWDPDTDNNFSKDN
6	117	10	IPDTGESQIR
7	136	10	EWQRGNYKQA
8	158	23	DIDTPYHPANVTAVDSAGHVKFE
9	170	20	VDSAGHVKFETFAEERKEQY
10	181	20	TFAEERKEQYKINTAGCKTN
11	191	21	KINTVGCKTNEDFYADILKNK
12	200	20	EDFYADILKNKDFNAWSKEY
13	210	20	KDFNAWSKEYARGFAKTGKS
14	220	17	ARGFAKTGKSIYYSHAS
15	233	17	SHASMSHSWDDWDYAAK
16	240	20	SWDDWDYAAKVTLANSQKGT
17	270	16	DVSEGNDPSVGNNVKE
18	291	12	STSGEKDAGTDD
19	309	13	KTKDGKTQEWEMD
20	320	21	DNPGNDFMAGSKDTYTFKLKD
21	330	20	SKDTYTFKLKDENLKIDDIQ
22	354	16	RKRKYTAFPDAYKPEN
23	379	19	VVDKDINEWISGNSTYNIK

Antibody-guided immunogen complex

Immunization complexes were produced as previously described by Chen *et al.* (2012). Biotinylated CD40-targeting antibodies were complexed with each synthetic peptide using streptavidin as a scaffold. Antibody-guided complexes were

stoichiometrically produced to contain a molar ratio of 2 antibodies and 2 peptides to every 1 streptavidin. Non-targeting complexes were also produced by replacing CD40-targeting antibody with normal (non-targeting) mouse IgG and served as negative controls. Non-targeting control complexes incorporated either peptide #13 or #14 and were further designated as 13C and 14C, respectively.

Immunizations

Seventy-five six-week-old broilers were divided into sets of three, creating a total of 25 groups. Animal care and handling was approved by Texas A&M University Institutional Animal Care and Use Committee (permit #2013-0254). Because chickens are outbred animals and were expected to exhibit divergent immune response levels, each peptide candidate was administered to three birds to ensure at least one good responder. Pre-immune serum was collected from all birds and designated as Day1 samples. Each group of birds was subcutaneously immunized with 50ug of antibody-guided complex carrying one of the twenty-three peptides. Two extra groups of birds were immunized with non-targeting antibody complex (as negative controls), using either peptide #13 or #14, and are further referenced as control groups 13C or 14C. Serum was collected one week-post immunization and designated as Day7 samples; these samples were used for *in vitro* antibody titer measurements and toxin neutralization assays.

ELISA

Goat anti-biotin IgG and the biotinylated target peptides with pre-mixed at a 1:1 molar ratio. This pre-mix was coated onto 96-well microtiter plates at a concentration of 5ug/mL in carbonate-bicarbonate coating buffer, pH 9.6. Wells were blocked with 5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) and serum samples were applied at 1:50 dilution. Samples were incubated for 2 hours at 37°C before peroxidase-conjugated goat anti-chicken IgG detection antibody was applied (Jackson ImmunoResearch, West Grove, PA). Peroxide/tetramethylbenzidine (TMB) substrate system was used as the colorimetric endpoint and enzymatic reactions halted with 2M sulfuric acid. Absorbances were read at 450nm using a Perkin-Elmer Victor 2 plate reader (Waltham, MA). Antibody titers were reported as Day7:Day1 ratio to correct for interference from pre-existing cross-reactive antibodies in circulation. No statistical analysis was performed as only qualitative responses, production of any neutralizing antibodies for use in downstream assays, were needed for study.

Hemolytic neutralization and phospholipase C neutralization assays

Purified *C. perfringens* alpha toxin was obtained from the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) and used at a working dilution of toxin in sterile PBS for neutralization assays, as recommended by the manufacturer. Antisera from the two highest responders of each group, based on previously performed enzyme-linked immunosorbent assay (99), were used for hemolytic neutralization testing. Sera were titrated by 2-fold serial dilution

starting from an initial 1:10 starting dilution on a microtiter plate in a 50μL volume, and then mixed 1:1 v/v with the working stock of alpha-toxin. Toxin and sera were incubated at 37°C for 1 hour to allow potential binding/neutralization of the toxin. After initial incubation, 100μL of 5% (v/v) sheep red blood cells diluted in PBS were added and incubated for another hour at 37°C. After incubation, neutralization of hemolytic activity was observed. Phospholipase C neutralization assays were performed using the same procedure, but modified for the application of 10% (v/v) egg yolk emulsion as a source of phospholipids, in lieu of red blood cells. Neutralization titers are reported as the inverse of highest serum dilution factor capable of fully neutralizing the enzyme. Because each peptide candidate was only represented by two antisera samples and reported in inverse dilution factor, standard errors are not included. Statistics to compare between groups was not performed, as only qualitative data showing ability to neutralize were needed to determine whether a specific region is a suitable target or not; antibodies against any region able to neutralize both enzymatic activities would have been considered an indicator for potential candidate after epitope mapping.

Results

Peptide-specific polyclonal IgY rapidly produced using antibody-guided immunization

All groups of birds mounted humoral immune responses against their respective peptide immunogen within 7 days of immunization (Figure 5), as measured by peptide-specific IgG titers via ELISA. As expected, control groups receiving peptide loaded onto non-targeting complexes also mounted low level antibody responses against the

peptide, but these responses did not reach the overall robust levels induced by CD40-targeted peptide delivery. Individual immune responses varied, as anticipated from outbred birds. Statistical analysis to compare response between groups was not necessary as only qualitative responses, production of any neutralizing antibodies for use in downstream assays, were needed for this study.

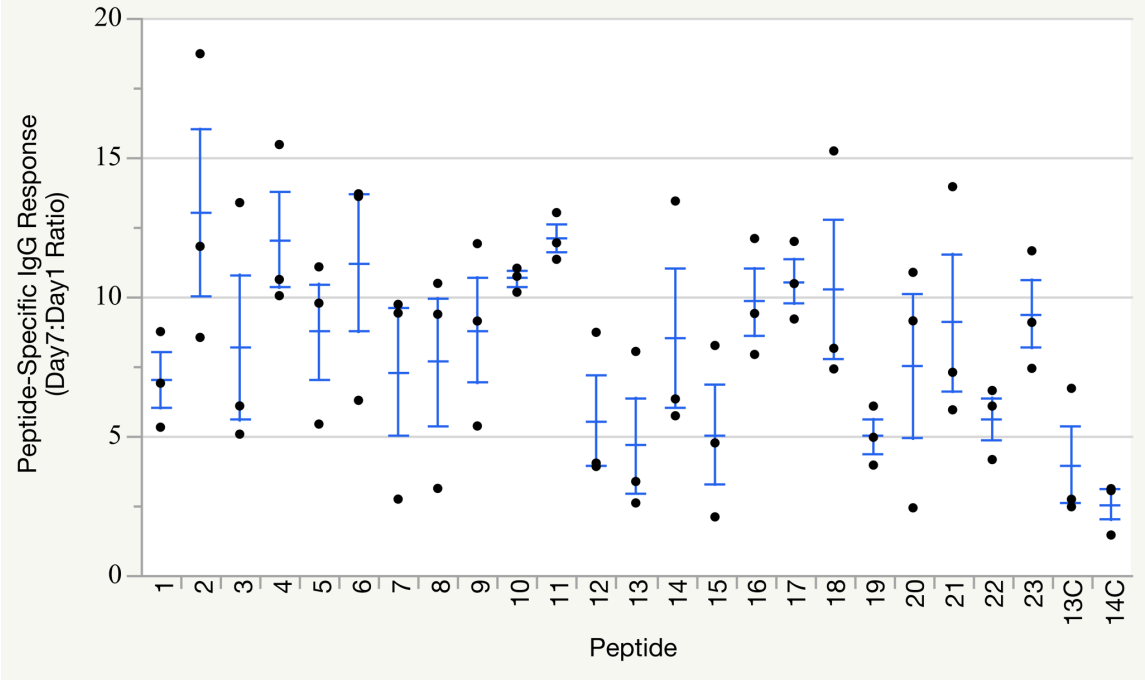

Figure 5. Peptide-specific IgG responses.
Individual peptide-specific antibody titers reported as Day7:Day1 ratio based on ELISA measurements against the matching peptide used for immunization. Groups 13C and 14C were immunized with non-targeting complexes loaded with either peptide #13 or #14 and served as non-targeting negative controls. Group means and standard error overlaid.

Blocking/binding Cpa linear epitopes sufficient for neutralizing hemolytic activity, but not PLC activity

As seen in Figure 6, all tested antiserum samples were able to neutralize *in vitro* Cpa hemolytic activity to varying degrees (individual titers ranging from 80 to 320), suggesting antibody binding of any accessible region on the toxin itself is sufficient to block hemolytic activity. Of note, groups 13C and 14C also produced some peptide-specific antibodies, as measured by ELISA, but these antibodies were unable to neutralize hemolytic activity. This suggests antibodies produced against epitopes loaded onto antibody-guided complexes have gone through some affinity maturation and bind more efficiently than the immunization using the non-targeting counterparts. In contrast to the hemolytic neutralization results, none of the serum samples from the experimental groups were able to neutralize Cpa's phospholipase C activity (Figure 7). Hyperimmunized chicken antisera against native *C. perfringens* obtained from USDA-APHIS were used as a positive control in neutralization assays and were capable of neutralizing PLC activity. The hyperimmune antisera would possess an assortment of antibodies against various regions and spatial conformations of Cpa, implying that the critical site responsible for PLC activity cannot be emulated by a synthetic linear peptide.

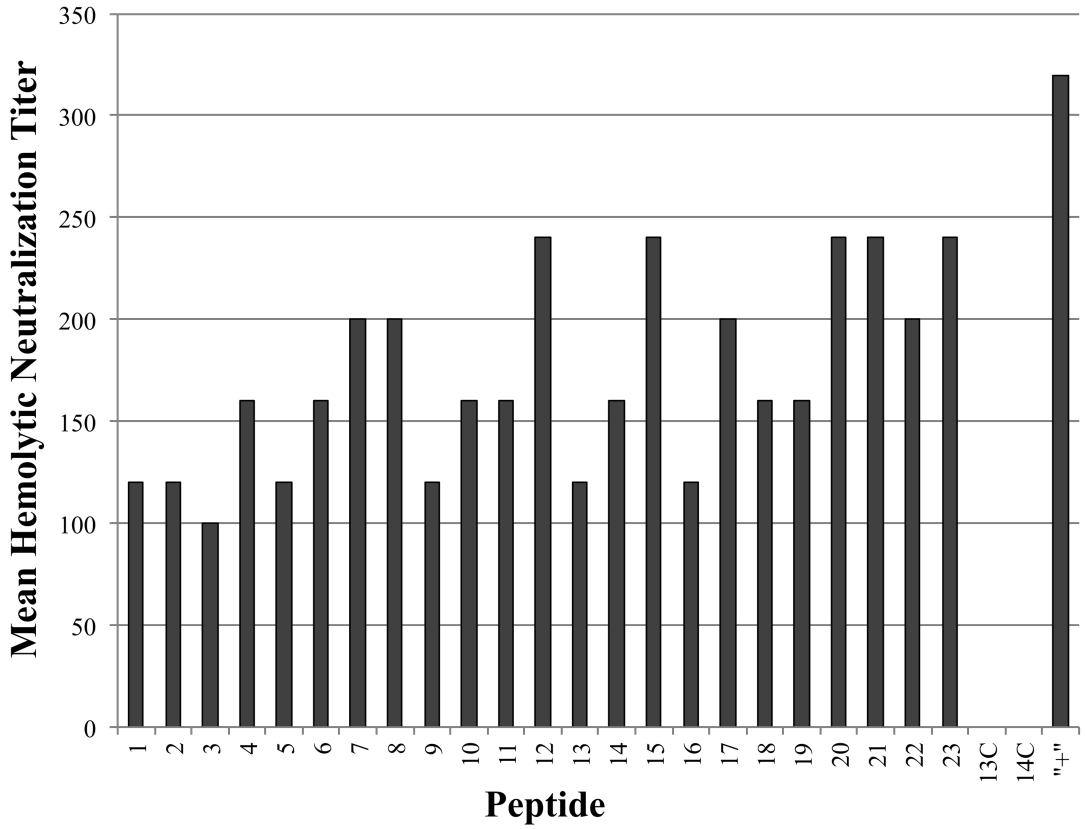

Figure 6. Mean hemolytic neutralization titers.
Mean hemolytic neutralization titers. Hemolytic neutralization titers reported as the inverse of the highest serum dilution factor capable of completely neutralizing Cpa toxin's hemolytic activity. Hyperimmune serum against alpha toxin obtained from USDA APHIS was used as positive control serum and the corresponding group was labeled as "+" on chart.

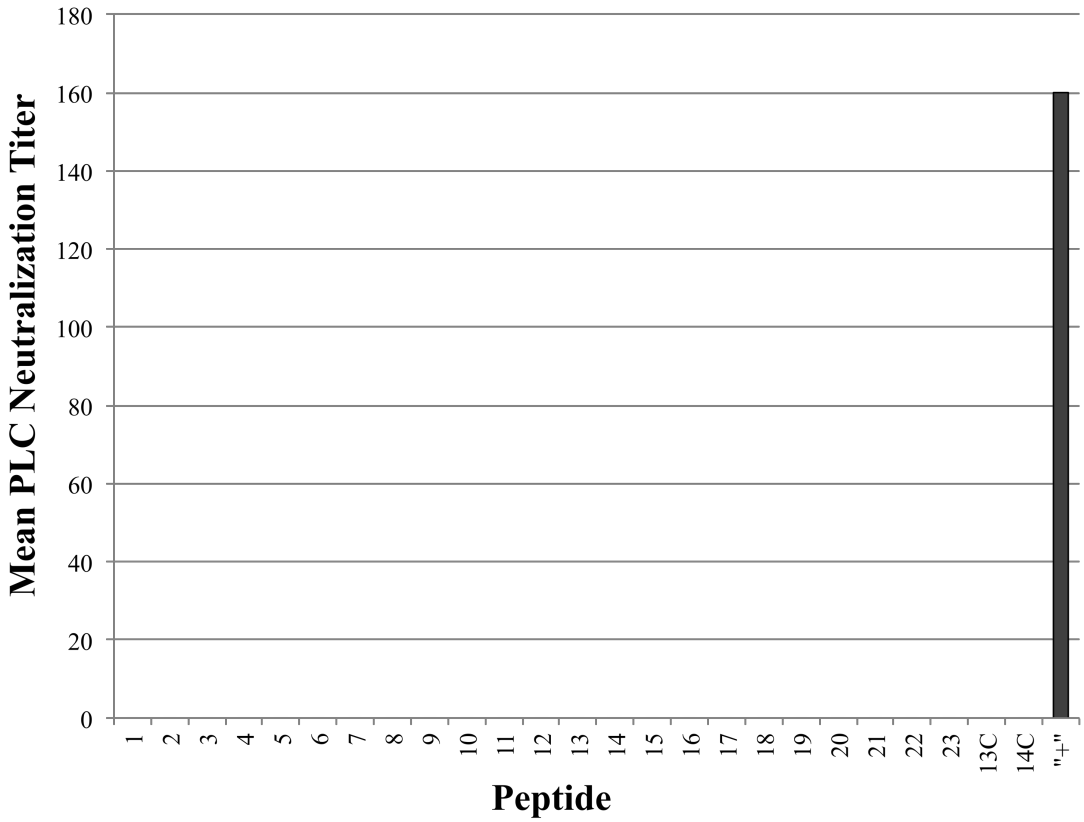

Figure 7. Mean phospholipase C neutralization titers.
PLC neutralization titers reported as the inverse of the highest serum dilution factor capable of completely neutralizing Cpa toxin's PLC activity. Hyperimmune serum against alpha toxin obtained from USDA APHIS was used as positive control serum and the corresponding group was labeled as "+" on chart.

Discussion

Antibody-guided complexes were initially designed for use as a vaccine delivery system, but this technique clearly has potential to also become an analytical tool to rapidly dissect molecules into their various active domains and pinpoint essential motifs underlying specific biological activities. This system has the added advantages of being both quicker and more cost-effective than standard site-directed mutagenesis procedures.

The antibody-guided method used in this experiment was purposely limited to presenting linear epitopes; it appeared that those did not produce antibodies capable of neutralizing PLC activity. Although unable to identify a PLC-neutralizing region on the Cpa, this procedure has proven useful for rapid polyclonal antiserum production/reagent development for research purposes. Because a multitude of antigen targets, linear or conformational, can be designed and readily incorporated into this system, the use of antibody-guided complexes beyond its original platform can be appreciated.

Interestingly, the results suggest that binding to any continuous epitope of the Cpa is sufficient to block its hemolytic activity, but not to neutralize its PLC activity. As only linear peptides were tested, this suggests neutralization requires binding and blocking of one or more conformation-dependent regions on the toxin itself to inhibit PLC functions (100). Removal of the toxins' enzymatic activities requires modification of the sequence during recombinant design or chemical inactivation of purified toxins in order to render them safe for previous vaccine efforts. These modifications may alter the conformation of the toxin itself, and therefore efforts to make the tested vaccines safer have actually caused them to be less efficacious (18, 101). Results from this study support previously reported data in which altered toxin used as a vaccine target was unable to induce production of neutralizing antibodies or fully protective immune responses. Due to these findings, targeting a single toxin may not be the answer for controlling NE. Preventing overgrowth of *C. perfringens* by preemptive nutritional and biosecurity control procedures to maintain gut health or developing therapeutics capable

to blocking overgrowth of the bacterium itself may, at least for the time being, be more effective solutions.

This antibody-guided immunization technique is designed to target chicken CD40, a unique concept for antiserum production instead of the commonly used mouse, rabbit, or goat hosts. The phylogenic divergence between avian and mammalian systems allows this method to be potentially used for antiserum production against commonly conserved mammalian target epitopes that have previously proven non-immunogenic in mammalian hosts. Avian host systems also permit the collection of eggs, which contain the specific antibody of interest within the yolk, decreasing blood collection requirements and associated stress on the animal. Avian IgG antibodies produced by antibody-guided immunization are suitable for laboratory research use, development of diagnostic assays, as well as epitope mapping using linear epitopes. Biotinylated peptides can be easily and inexpensively synthesized in as little as a week, incorporated into the antibody-guided complex, and birds can be immunized for an initial serum collection as early as one-week post immunization. Conformational epitopes can also be targeted with this system, but would require more time and expense to generate before proceeding to *in vivo* immunizations. Although antiserum production with this method is a viable option with conformational epitopes, it does not lend itself well for epitope mapping. This specific study has provided proof of principle for the use of antibody-guided immunogen complexes to quickly produce antibodies for epitope mapping verification.

CHAPTER III

DELIVERING INACTIVATED AVIAN INFLUENZA TO ANTIGEN PRESENTING CELLS BY TARGETING CD40 FOR ENHANCED PROTECTION AGAINST LETHAL CHALLENGE

Introduction

The CD40 receptor expressed on all professional APCs is responsible for mediating the release of co-stimulatory and pro-inflammatory cytokines enhancing immune response activity during infection. This includes activation and proliferation of macrophages, B-cell affinity maturation and isotype switching from low intrinsic affinity IgM to more effective IgG and IgA isotypes, and releasing chemokines to recruit other immune cells to effector sites (56, 57). This response typically requires 2-3 weeks to develop because the target antigen must make contact with the APC while the cell is also provided with helper T-cell co-stimulatory signals to induce downstream activation responses (55). Given the significance of its abilities, intentionally targeting CD40 to enhance immune responses during immunization is a reasonable approach. Agonistic anti-CD40 antibodies can act as surrogate co-stimulatory signal from the helper T-cell while physically loading antigens to the anti-CD40 antibody would allow direct delivery of the target immunogen to the APC, circumventing the time typically needed to initiate this response (86). Antibody-guided immunization methods have grown in popularity owing to their ability to directly present antigen to APCs, inducing more robust immune responses in a shortened time period (74, 102). CD40 receptor targeting in particular has

been the most extensively studied immune receptor targeting mechanism, primarily in human and murine systems (77, 78, 103). Agonistic CD40 antibody is likely to be used in live animal treatments given the availability to use the same antibody for different mammalian species (104). Previous research using CD40-targeted complexes for immunization in poultry has shown a surge in immunogenicity by inducing both systemic IgG and mucosal IgA responses after a single administration (88, 89). This is particularly important for respiratory diseases, as secretory IgA is the main element protecting birds during mucosal infection (105, 106) and the CD40 activation pathway is the singular mechanism capable of inducing immunoglobulin isotype-switching in birds (107).

Although AIV is not endemic to poultry within the United States, AIV remains a large issue for other countries and a near-constant biosecurity issue within the US, as emphasized by the recent 2014-2015 highly pathogenic avian influenza (HPAI) outbreak which caused an estimated loss of approximately 48 million birds and the 2016 Indiana outbreak which resulted in a loss of over 400,000 birds (33). Current AIV vaccines center their protection on development of protective antibodies against the virus' HA protein (35, 108, 109). This is problematic as there are 16 different subtypes of HA, requiring vaccine propagation and reformulation as different outbreaks occur (110). The M2 ion channel's extracellular (M2e) region of AIV has been identified as a conserved epitope among most Type A influenza strains and many attempts have been made to use this domain for development of a universal vaccine, but with variable results (111-113). An effective protective response correlates with sufficient antibody titer against the HA

protein, which relates with virus neutralization. Some vaccines against highly pathogenic AIV have proven to be only partially protective due to mismatched antigen compared to the field virus, and a lack long-term efficacy, leading to the need to investigate potential vaccine efficacy enhancers (36, 37, 114, 115).

The antibody-guided adjuvant complex delivery system has been previously tested for use in poultry, but has so far been limited to presentation of synthetic peptides and observation of immunogenicity (88, 89). Using this established chicken CD40-targeting/antibody-guided adjuvant complex in combination with anti-M2e monoclonal antibodies (Figure 8), this modified complex could potentially bind inactivated avian influenza virions (iAIV), regardless of HA type. Avian influenza virus was selected as the pathogen target due to its monofactorial disease causation and well-defined benchmark for functional protection (survival past 4 days post-challenge), making it an ideal candidate target for testing proof of concept. This approach in theory has the potential to overcome the weak response and short-term protection issues of some AIV vaccines (92). The novel use of anti-M2e antibodies in the complex in order to universally bind whole avian influenza viruses to the targeting-complex will permit the use of this enhanced delivery system regardless of the subtype of the circulating virus. In this study, the antibody-guided adjuvant system, specifically targeting the chicken CD40 receptor of antigen presenting cells, was tested for the ability to present inactivated HPAI virus and confer functional protection upon homologous challenge in chickens.

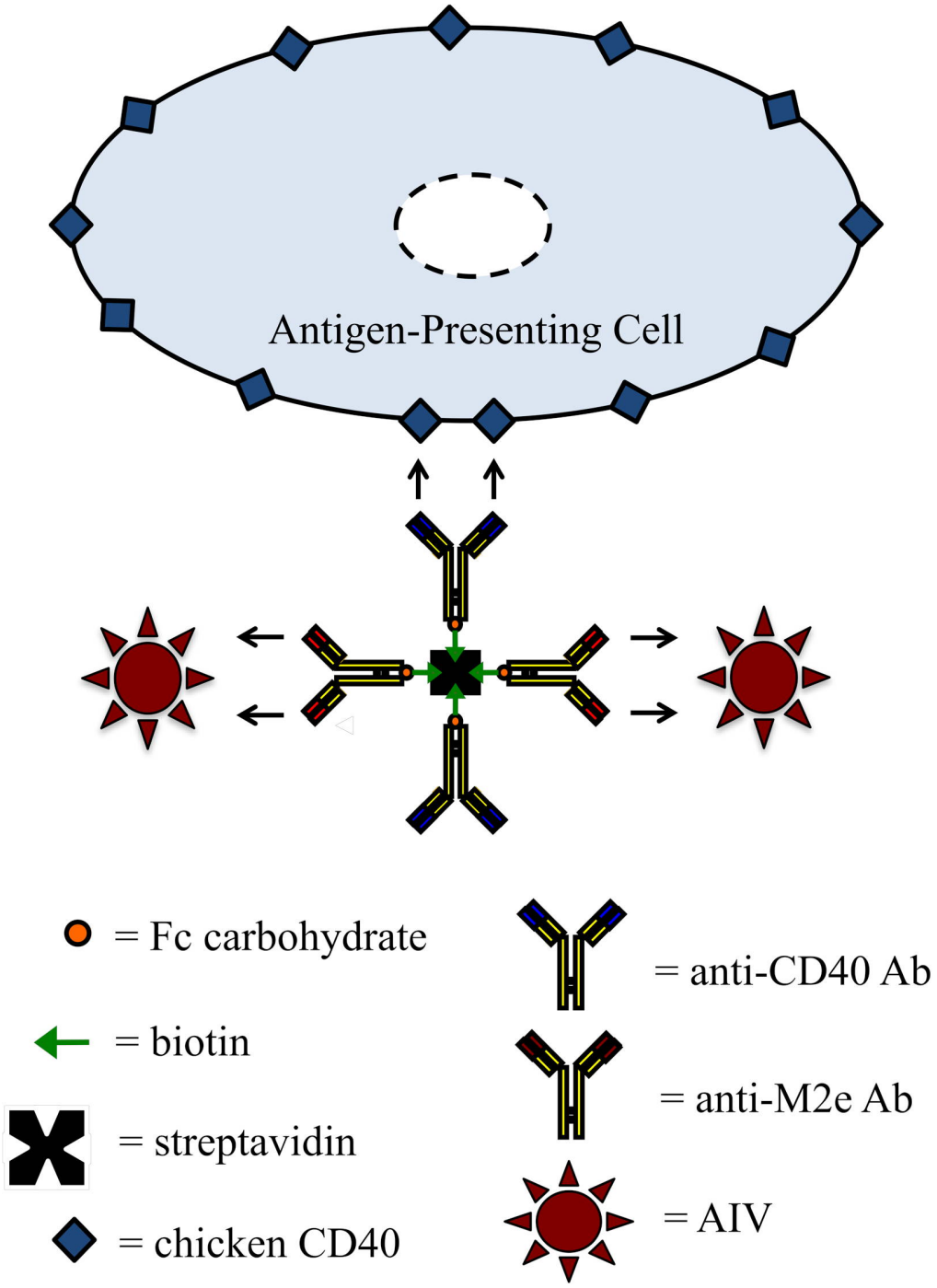

Figure 8. Antibody-guided adjuvant complex schematic.
Anti-cCD40 monoclonal antibodies (Ab) and anti-AIV M2e monoclonal antibodies were directionally biotinylated on their carbohydrate moieties and attached to a streptavidin scaffold at a ratio of 2 anti-CD40 : 1 streptavidin : 2 anti-M2e. The resulting complex was mixed with 384 HAUs of inactivated AI virus, which incorporates the virus into the vaccine complex. This complex was used for immunization in chickens.

Materials and Methods

Viruses

Inactivated A/Turkey/Virginia/158512/2002 H7N2 low pathogenic avian influenza (LPAI) virus was used to coat ELISA plates during anti-M2e monoclonal antibody screening. A/Egret/Hong Kong/757.2/2002 H5N1 HPAI was used for initial *in vivo* testing of three different anti-M2e monoclonal antibody candidates on modified antibody-guided complex. Immunogenicity trials used inactivated A/Turkey/Wisconsin/1968/H5N9 LPAI virus. The challenge trial also used Egret/02 H5N1 HPAI virus. All viruses were propagated in chicken embryo allantoic fluid. Viruses utilized for vaccination or hemagglutination inhibition (HI) assays were beta-propiolactone inactivated (116). Vaccines were formulated to contain 384 hemagglutination units (HAU) of virus per dose. HI assays were performed with the identical virus used during corresponding immunization.

Anti-M2e monoclonals

Monoclonal antibodies were produced against the extracellular domain of avian influenza virus' M2 ion channel protein. Hybridomas were created with Sp2/0 mouse myeloma cells using standard electrofusion protocols (87). Splenocytes were harvested from mice hyper-immunized against synthetically produced peptide conjugated to keyhole limpet hemocyanin (KLH) (GenScript, Piscataway, NJ) containing the M2e amino acid protein sequence (EVETPTRN). The M2e peptide sequence selected had 100% consensus with over 50 different strains of avian influenza in the GenBank protein

sequence database. Mouse work procedures were carried out in accordance with permit 2014-0013, as approved by the Texas A&M University Institute of Animal Care and Use Committee. Hybridomas were screened and selected based on production of IgG antibodies specific against the synthetic M2e peptide via enzyme-linked immunosorbent assay (99) as well as against whole inactivated Turkey/02 H7N2 LPAI virus (117). From the original 15 double positive (against peptide as well as whole virus) parent hybridomas, three parents were selected for further subcloning. Subclones were screened again against the peptide and whole AIV. From the pool of hybridoma subclones which remained double positive, one subclone from each parent was selected (creating a panel of three potential candidates which were designed clones A, B, and C) for further screening and use in downstream adjuvant complex formation and trials.

Anti-M2e monoclonal selection

Upon cloning by limiting dilution, three monoclonals were selected as potential candidates for immunization complex formulation, designated as clones A, B, and C. The respective monoclonal antibodies were incorporated into the CD40-targeting complex as previously described (88), with minor modifications in that biotinylated anti-M2e monoclonal antibodies were substituted for previously described biotinylated peptide antigens. This created a complex comprising four biotinylated monoclonal antibodies (two against the chicken CD40 receptor and two against AIV M2e, see Figure 8) on a central streptavidin scaffold molecule. This antibody-guided complex was then mixed with a fixed amount of iAIV. The initial virion to complex incorporation ratios

tested included 250, 500, 1000, 2000, 4000, 8000, and 16000 virion units per complex (n=20 birds per dosage per candidate monoclonal). Each vaccine dose consistently contained 384 HAU of inactivated Egret/02 H5N1 HPAI virus, propagated in allantoic fluid. The resulting adjuvant complex was mixed 1:1 v/v with sterile saline containing 5% (v/v) squalene and 0.4% (v/v) Tween 80 for subcutaneous administration. Virion-loaded complex was administered to two-week-old layers obtained from Medion Vaccine Company (Bandung, Indonesia). Serum was collected one week post-immunization to measure serum hemagglutination inhibition (HI) titers against homologous iAIV. HI titer data were converted to Log_2. For immunogenicity comparisons, each clone's dosage (viral particle : complex ratio) data were combined and averaged prior to comparison with the other clones, making results reported for each monoclonal candidate from an n=140. ANOVA and post-hoc Tukey HSD analysis were performed using JMP statistical software, version 12. P-values ≤ 0.05 were considered statistically different. This study was carried out in accordance with the Indonesian government's biosafety level 3 animal use regulations.

Vaccine preparation

Further adjuvant complex studies were performed using clone C anti-AIV M2e monoclonal antibodies. This vaccine was mixed with iAIV at a 1000X virion to complex ratio, with each vaccine dose containing 384 HAU of iAIV as previously tested. Guided complex was administered via either subcutaneous, oral, or eye drop (oculo-nasal) routes, depending on the trial. Subcutaneously administered guided complex was

prepared as previously stated, while the orally-administered complex was alginate-encapsulated in sterile saline before administration as described in detail in (89). Eye drop administered complex was used directly without any additional preparation. A control group receiving mineral oil emulsion based vaccine containing 384 HAU of iAIV used as a reference control during initial screening for anti-M2e monoclonal screening and during the final challenge trial.

Immunogenicity optimization

Male Leghorn chickens (n=10/group) were either subcutaneously, orally, or via eye drop vaccinated at two weeks of age. Beta-propiolactone-inactivated Turkey/68 H5N9 LPAI virus was used for immunization as well as downstream HI assays. Pre-immune sera were collected at the start of the study and post-immunization sera were collected for up to 4 weeks. Serum samples were used in HI assays against the same iAIV used during immunization. The HI assays were performed as follows: a 2-fold serial dilution of the serum samples was mixed 1:1 v/v with 8 HAU of the inactivated AIV used in formulation of the vaccine complex followed by the addition of 5% red blood cells. Reciprocal of the highest serum dilution capable of hemagglutination inhibition was considered the final HI titer; these HI titers were converted to Log_2 values and used for statistical analysis. Analysis of variance (ANOVA) and post-hoc Tukey HSD analysis performed using JMP statistical software, version 12. P-values ≤ 0.05 were considered statistically different. Bird procedures followed standard practices set by the University of Arkansas Institutional Animal Care and Use Committee.

Challenge trial

Specific pathogen-free Leghorn chickens were obtained from Medion Vaccine Company for use in this study and animal care and procedures conducted in accordance with Indonesian government biosafety level 3 animal regulations. Inactivated Egret/03 H5N1 HPAI virus was used for guided complex preparation and the live virus was used during challenge. Birds were immunized with antibody-guided complex loaded with inactivated H5N1 HPAI virus, final volume 0.5ml, at two weeks of age, either subcutaneously at the nape of the neck or orally (n=25 birds per group). Groups were boosted at four weeks of age. One group received commercial oil emulsion based vaccine at 10 days of age (no boost) and was used as a point of reference for the experimental groups. All groups were challenged with 200µL of $1x10^6$ 50% embryo infectious dose (EID_{50})/mL homologous HPAI through the intranasal route at five-weeks-of-age. Percentage of group survival 4days post-challenge was calculated and used as an indicator for protective efficacy, as the standard mortality rate of HPAI challenge in birds is 75-100% within 4 days (118).

Results

Antibody-guided inactivated virion is able to induce neutralizing IgG antibody titers against AI

Each candidate anti-M2e monoclonal antibody was used to form an antibody-guided complex (two anti-M2e antibodies along with two anti-cCD40 antibodies onto a central streptavidin scaffold molecule). Each candidate adjuvant complex was mixed

with 384 HAU of inactivated virus and administered to birds for immunogenicity comparison. This test would also confirm that the addition of the anti-M2e antibody-AI virus to the original complex does not sterically hinder the binding and activation of the CD40 receptor. Based on anti-AI titers measured by HI titer one week post-immunization, clone C induced statistically greater responses than clones A and B, as well as the traditional mineral oil emulsion vaccine formulation (Figure 9). Based on this evidence, all further testing was conducted using clone C anti-AIV monoclonal antibody. No clear immunogenicity differences were observed between compositions with different virion:complex ratio), thus a 1,000X ratio was used for subsequent studies. (Individual dosage ratio response data are not shown, but averaged titers for all dosages are presented as mean response per candidate clone in Figure 9.)

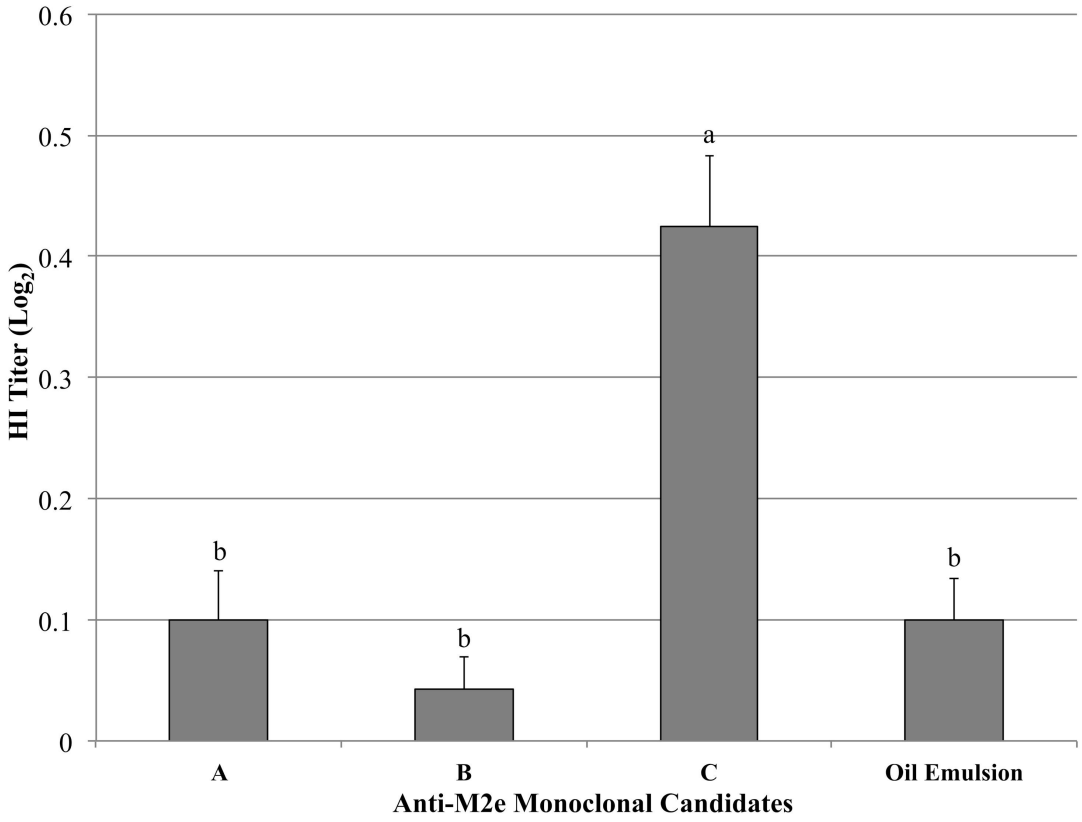

Figure 9. Clone C was the most immunogenic of the three candidate anti-M2e monoclonal antibodies.

Each candidate monoclonal antibody (clones A, B, or C) was combined with 384 HAU inactivated Egret/02 H5N1 HPAI AIV. Different ratios of antibody complex mixed with fixed 384 HAU of iAIV were tested, with dosages ranging from 250 to 16000 times virus particle to guided-complex ratios. AIV-loaded complex was used for immunization of chickens and sera were collected one week post-immunization for HI assays. A group receiving oil emulsion based vaccine was used as a reference. As no dose-dependent effects were observed in the viral particle:complex dosage ratios tested (250-16000), HI titers of each candidate (clones A, B, or C) are presented as the mean of all ratio ranges for each clone. Significantly different HI titers are indicated with non-matching letters. Clone C induced significantly higher HI titer compared to all other groups.

Subcutaneous prime required for induction of AIV neutralizing antibodies

Optimization of adjuvant complex administration used inactivated Turkey/68 H5N9 LPAI with a combination of subcutaneous, oral, or eye drop routes in a two-week interval prime/boost schedule. Based on serum HI titer data collected 1, 2, and 4 weeks post-boost (Figure 10), subcutaneous prime is required to induce immunogenicity and produced statistically higher HI titers compared to non-subcutaneous counterparts. Groups receiving only oral/eye drop administration of the vaccine complex did not produce neutralizing antibodies against the virus and were essentially non-responsive. The group receiving both subcutaneous prime and boost mounted the greatest immune response, with HI titers statistically higher than all other groups. Interestingly, the group receiving a subcutaneous prime only (no boost received) retained measurable levels of neutralizing antibodies up until the latest time point, *i.e.* 6-weeks post immunization for this group.

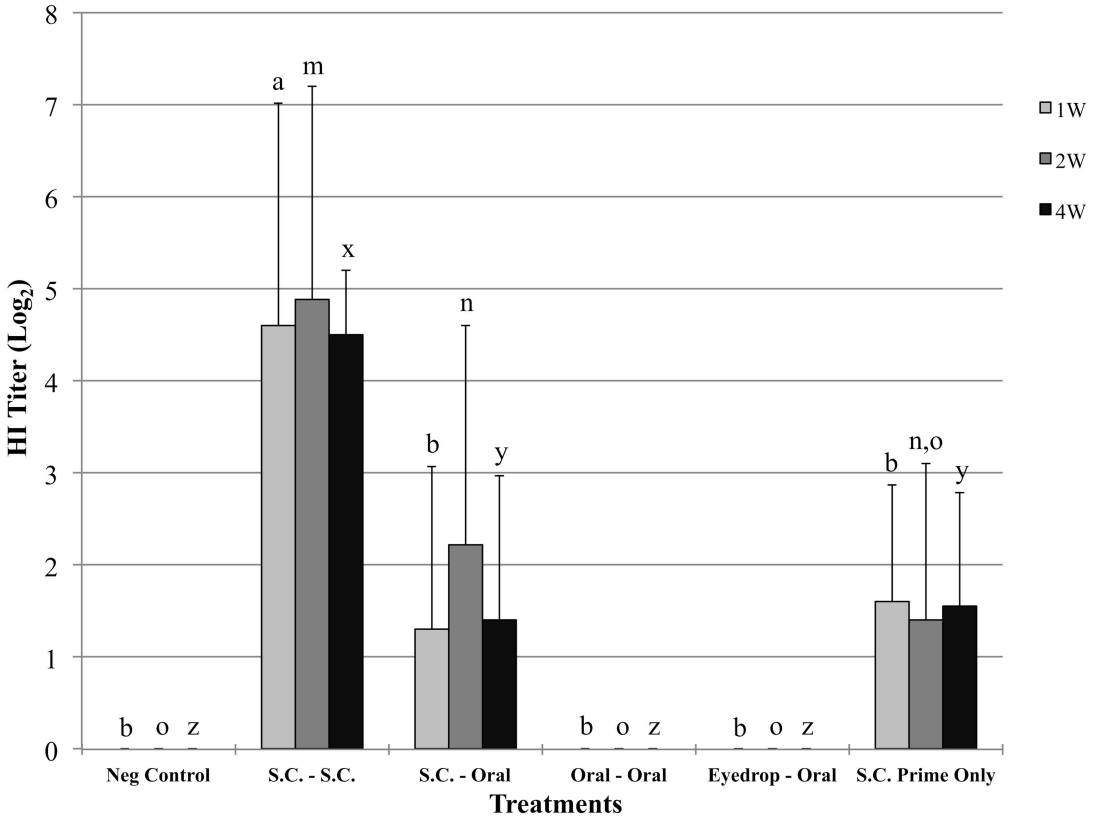

Figure 10. Subcutaneous administration induces the highest immune response. Immunogenicity trials tested subcutaneous (S.C.), oral, or eye drop administration of the vaccine complex (prime at 2 weeks of age and boost at 4 weeks of age). Serum HI titers were measured at 1, 2, and 4 weeks (W) post-boost. Significantly different HI titers between groups at each time point are indicated with non-matching letters. Results show that subcutaneous administration is required to induce a robust response. Birds primed and boosted subcutaneously had significantly higher HI titers than birds receiving only one subcutaneous injection. No response was observed in birds receiving non-subcutaneous administrations.

Subcutaneously administered guided complex is able to protect birds against HPAI challenge

Antibody-guided complex was able to confer protective immunity following lethal H5N1 HPAI challenge when birds were immunized subcutaneously (Table 2).

Protection was measured as survival 4 days post-challenge with the homologous Egret/03 H5N1 HPAI. The group immunized subcutaneously with both prime and boost yielded a 100% survival rate after challenge, matching survival response conferred by the mineral oil emulsion vaccinated group. Groups receiving only one subcutaneous administration demonstrated partial protection, exhibiting 56-64% survival after challenge, depending on the group. Groups receiving only oral administration of the complex did not acquire any protective immunity against the HPAI challenge and 0% survival was observed. As expected, no survival was obtained in the sham vaccinated control group.

Table 2. HPAI-loaded adjuvant complex challenge trial treatment groups and survival data.
Birds were immunized with the guided-complex loaded with Egret/02 H5N1 HPAI virus either subcutaneously (S.C.) or orally. Prime occurred at 14 days of age and boosts were administered at 28 days of age. A group received iAIV without adjuvant to serve as a comparison. Another control group received an oil emulsion based vaccine subcutaneously at Day 10 without boost. Birds were challenged at 35 days of age with homologous AIV. Percent survival was calculated at 4 days post-challenge.

Prime (Day 14)	Route	Boost (Day 28)	Route	Challenge (Day 35)	% Survival (Day 40)
--	--	--	--	No	100
--	--	--	--	Yes	0
Oil Emulsion	S.C.	--	--	Yes	100
iAIV	S.C.	iAIV	S.C.	Yes	36
Complex	S.C.	Complex	S.C.	Yes	100
Complex	S.C.	Complex	Oral	Yes	56
Complex	Oral	Complex	Oral	Yes	0
Complex	S.C.	--	--	Yes	64

Discussion

With these data, the antibody-guided complex has been successfully used to present whole virus, beyond its initial testing with short, synthetic peptides. The M2e region was targeted as a method to load the virus onto the complex, but the immune response was evaluated by HI assays, which primarily measures neutralizing antibodies against the viral hemagglutinin. Use of anti-M2e antibody to load the virus onto the complex did not diminish antibody production against HA, but enhanced the speed of the immune response, and potentially also its duration, as the virus was guided to the CD40 receptor using this complex. Interestingly, neutralizing antibody titers were still detected six weeks after a single prime injection. Previous immunogenicity studies had not measured sustained response beyond a couple weeks post-immunization (88). The data presented not only prove this delivery method is capable of inducing a sustained antibody response, but also that the antibodies maintain their functionality and are still capable of neutralizing virus. This method was also able to match efficacy, based on survival, of a traditional oil emulsion-based AI vaccine produced by the company and is the first study to confirm protective efficacy using the antibody-guided complex in poultry, specifically against HPAI.

Many adjuvant studies have been completed in an effort to enhance AI vaccine immunogenicity and efficacy beyond the standard mineral oil emulsion formulation, either by expressing HA proteins on a recombinant vector (119), by the addition of immunopotentiators to the formulation (120), or switching to a DNA vaccine platform (121). These studies have reported varying results, ranging from only slightly increased

immunogenicity, to reduction of viral shedding, and from partial to full protection, but none of these vaccine candidates have been implemented as a replacement to mineral oil emulsion based AI vaccines. As a potential vaccine component against HPAI, this antibody-guided adjuvant has proven to dramatically enhance both immunogenicity and protection of inactivated virus. Because the complex was developed using anti-M2e antibodies, a conserved region on all Type A AI viruses, the modified complex can be mass-produced and stockpiled prior to use, to then be mixed with crude allantoic fluid containing any inactivated AI virus prior to vaccine administration. The complex used in this study was tested with both LPAI and HPAI viruses, both of which demonstrated highly positive immune responses (immunogenicity measured by HI titer and/or protection against challenge). In countries that only allow the use of vaccination as an emergency program in the event of a highly pathogenic AI outbreak, induction of a rapid immune response is necessary to prevent mortality and the guided adjuvant complex accommodates this need. In countries with endemic AI implementing a vaccination program, the addition of the antibody-guided complex to the existing vaccine formulations or to replace the initial priming immunization would allow for enhanced immunogenic efficacy and reduced induction time.

In its current incarnation, use of this system would not be economically feasible for general poultry vaccination programs because it is too costly to produce, with AI as its target or otherwise. Further research using the antibody-guided complex needs to be completed in order to optimize the administration route to accommodate ease of use within the poultry industry. Subcutaneous immunizations require individual bird

handling and experienced personnel to administer the vaccine, which is cost-prohibitive for the industry (although it is not an unusual practice in low wage developing countries). If formulated as a stable feed additive or spray while maintaining efficacy, the guided complex would become more accessible for the industry. This would require optimization to a more efficient and stable version for oral or mucosal delivery. Dosage studies will also need to be completed in order to reduce the amount of complex and inactivated virus needed while maintaining efficacy. Reducing cost per dose would be an important factor for an industry that maintains billions of birds. Time course studies will also need to be performed to monitor lasting protection beyond initial immunization. The present study monitored antibody titers up to six weeks after a single injection, but the extent of time the birds are protected beyond this period would be a very important consideration to gauge effectiveness and to schedule a booster immunization program in longer-living birds, such as layers or breeders. Converting from a monoclonal antibody system to a recombinant expression system would also be advantageous during production and consequently make each dose less expensive after initial set-up. Recombinant production is easier to scale up for mass production (*e.g.* in a plant expression system) and allows more control over design/manipulation and quality assurance testing. This would potentially allow all components used in this design (4 antibodies incorporated onto a streptavidin scaffold) to be expressed as one single entity, reducing preparation steps and time, and therefore potential mistakes, currently required to formulate the adjuvant complex before use. Degree of cellular immunity involvement has also yet to be confirmed using this system in chickens; however, it can be reasonably

assumed that helper T-cell responses are stimulated because this is required for downstream recurrent B-cell activation, class switching and affinity maturation responses. The extent of cytotoxic T-cell involvement remains unknown. Although in its current form this vaccine adjuvant system requires optimization of production and administration regimen, this system may nevertheless be cost effective, even in the United States, to protect high value animals, such as breeder stocks.

CHAPTER IV

DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST DENDRITIC CELL MARKER CD205 AND ITS USE TO ISOLATE DENDRITIC CELLS FROM PERITONEAL EXUDATE CELLS

Introduction

Dendritic cells serve as the primary antigen-presenting cell for the immune system, the only professional APC capable of initiating activation of T-cells for downstream adaptive immune responses (122). In order to obtain chicken DCs, current protocols require the isolation of bone marrow myeloid progenitor cells and the induction of dendritic cell differentiation by the supplementation of GM-CSF and IL-4 cytokine growth factors into the cell culture (70). Maturation of these cells is performed completely within *in vitro* settings, which may not be fully representative of the *in vivo* environment nor the naturally maturing host DCs itself. A verified, standard isolation method to recover fully matured dendritic cells from chickens has yet to be employed by the poultry research field.

Reagent development for DC-specific research is also limited in chickens. Because of the challenges with providing a native cell for screening purposes, development of monoclonal antibodies against chicken DCs has been difficult. The CD205 receptor is a large surface receptor that serves as a dendritic cell marker and a potential target for antibody development (123). CD205, previously designated DEC205 or Lys75, is a mannose receptor expressed primarily on cortical thymic epithelial cells

and dendritic cells in mice (66). In the chicken, this 1732 amino acid/199.62 kDa protein contains one ricin B-type lectin domain at its N-terminus, followed by ten C-type lectin-like domains, and is trailed by a transmembrane anchoring domain (Figure 11) (69). Recombinant expression of the protein has also proven problematic due to the protein's large size, profuse glycosylation sites, and numerous repeat regions. Recently, monoclonal antibodies against chicken CD205 were developed by targeting specific C-type lectin domains at the center of the protein (70), bypassing use of the whole protein.

Figure 11. Diagram of CD205 domains.
CD205 contains a ricin B-type lectin at its N-terminus and is followed by ten C-type lectin (CLECT) binding domans and is trailed by a transmembrane anchoring domain (indicated by a blue block). Domain prediction analysis and diagram obtained from ExPASy protein analysis resources (124).

Chicken peritoneal exudate cells (PEC) have traditionally been a source of various immune cells for *ex vivo* studies, primarily to isolate heterophils and macrophages (125). In this study, we observe the presence of dendritic cells within the PEC population and propose this cell collection method as an alternative method to isolate and study chicken primary, mature DCs. Concurrent experiments were performed on PECs to develop monoclonal antibodies against the CD205 receptor and were successfully used to isolate $CD205^{+}$ cells from PECs, further expanding the reagents available for chicken DC research.

Materials and Methods

Antibodies utilized

Anti-chicken MHCII and Bu-1 monoclonal antibodies used for immunofluorescent assay (IFA) staining were obtained commercially and used to stain all APCs or B-cells, respectively (Southern Biotech, Birmingham, AL). Anti-CD40 antibody was a non-commercial monoclonal antibody developed in the Berghman lab and used as a second marker for all professional APCs during IFA staining (87). The anti-CD205 antibodies used were developed in this study and not commercially obtained. Secondary antibodies, non-specific mouse IgG antibodies used for blocking, and streptavidin conjugates were obtained from Jackson Immunoresearch (West Grove, PA). Anti-histadine (His) tag monoclonal antibodies were used to verify recombinant CD205 protein expression and purification (Thermo Scientific, Rockford, IL).

Collection and culture of peritoneal exudate cells (PEC)

Spent hens were injected intraperitoneally with 8 ml of 3% (w/v) Sephadex G50 (Sigma-Aldrich, St. Louis, MO) in sterile PBS. After 42 hours, birds were euthanized by CO_2 asphyxiation and a small incision was made into the abdomen. The peritoneal cavity was flushed with 35 ml of PBS to recover peritoneal exudate cells. Initial collection sample was centrifuged to pellet down cells and then washed once with PBS. The cell pellet was resuspended in PBS and mononuclear cells purified using Histopaque-1077 (Sigma-Aldrich) following manufacturer instructions. Mononuclear cells recovered after density gradient separation were used as a source of PECs in

following experiments. Initial studies removed macrophages by plastic adherence. This was completed by culturing the PECs in tissue culture treated flasks in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% (v/v) chicken serum, 8% (v/v) fetal bovine serum (FBS), 1mM sodium-pyruvate, 2mM L-alanyl-L-glutamine, 100 units/ml penicillin, and 100ug/ml streptomycin for 4 hours. Cells attached to the flask were considered adherent (Ad) PECs and cells remaining in suspension were considered non-adherent (Non-Ad) PECs. Subsequent flow cytometry and morphological studies utilized affinity-purified $CD205^+$ cells using the developed monoclonal antibodies, omitting plastic-adherence separation. Peripheral blood mononuclear cells (PBMC) and spleen mononuclear cells were also collected by Histopaque-1077 polysucrose gradient and used as control samples for comparison.

Generation of anti-chicken CD205 monoclonal antibodies

Previous development of anti-CD205 monoclonal antibodies by Wu *et al.* targeted the C-type lectin domains 4-6. The nucleotide sequence for C-type lectin domains 4-6 was obtained from NCBI gene databases and codon optimized for bacterial protein expression. The redesigned, truncated chicken CD205 gene was synthesized into a pUC57 cloning vector by a commercial vendor (Genscript, Piscataway, NJ). The gene synthesis product was used for cloning and expression of the truncated CD205 protein by transformation into BL21 Star *E. coli* of the Invitrogen Champion TOPO cloning system (Life Technologies, Grand Island, NY). Protein expression was induced using 1mM isopropyl β-D-1-thiogalactopyranoside when bacterial culture reached an OD_{600} of

0.5-0.8. After 6 hours, bacterial cultures were washed five times with PBS and bacterial pellets were frozen at -20°C to aid in cell wall lysis. B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) was used to extract bacterial protein from the cytoplasm, and recombinant CD205 protein was purified from the extract using Nickel-NTA affinity chromatography. Full-length expression was verified by western blot against the C-terminal His-tag marker. Purified recombinant chicken CD205 was quantified by Bradford assay and was used as the immunogen for monoclonal antibody development or as coating protein for screening ELISAs.

Female Balb/c mice (Charles Rivers, USA) were subcutaneously immunized with 50 μg of recombinant chicken CD205 (C-type lectin domains 4-6) protein per mouse in RIBI buffer. Three subcutaneous boosts with 25 μg/mouse were performed at three-week intervals. Plasma was collected 1 week after each immunization to monitor the specific IgG response against the recombinant CD205 protein based on ELISA and western blot. Once mice were hyper-immunized, based on the antibody titer plateaus, mice were sacrificed and splenocytes harvested. Splenocytes were used for electrofusion with mouse Sp2/0 myeloma cells to produce B-cell hybridomas using an Electro Cell Manipulator ECM 2001 (BTX, Holliston, MA). Hybridoma cultures were maintained at 37°C at 5% CO_2 and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 15% (v/v) FBS (Atlanta Biologicals, Lawrenceville, GA). Hybridoma supernatants were screened for CD205-specific antibody production via ELISA against recombinant CD205 protein and immunofluorescence assays against non-adherent peritoneal exudate cells. Based on these criteria, parent hybridomas were

chosen, and subsequently sub-cloned by limiting dilution. Sub-cloned hybridomas were screened again following the same methods before the final monoclonal sub-clones were selected for ascites production (Antagene, Santa Clara, CA) and cryogenic storage. Antibodies were purified from ascites using Protein G affinity columns (Thermo Scientific) following manufacturer instructions. For downstream flow cytometry or magnetic sorting applications, the anti-CD205 antibodies were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) following manufacturer provided procedures.

Flow cytometric analysis of CD205$^+$ cells from PEC

Anti-CD205 monoclonal antibodies were used to estimate DC percentage in chicken blood and spleen cell populations via flow cytometric analysis. Cells were collected from birds 1 week after vaccination with commercial Newcastle's Disease Virus vaccine to ensure activation of the immune system. Cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 5% FBS and 0.1% (w/v) sodium azide) and aliquoted to $1x10^6$ cells portions. Cell surface Fc receptors were be blocked by incubation with 1µg of unlabeled mouse IgG for 15 minutes at 4°C prior to staining. Primary incubation with biotinylated mouse anti-chicken CD205 monoclonal antibodies was performed for 1 hour at 4°C. Washes were performed with 0.5mL FACS buffer. Secondary incubation occurred with streptavidin-Pacific Blue conjugate. Cells were fixed with 2% paraformaldehyde in PBS and filtered through 40µm cell strainers to ensure single-cell suspension. Flow cytometric analysis

was performed using the BD FACSAriaII System (BD Biosciences, San Jose, CA) at the TAMHSC College of Medicine - Cell Analysis Facility and data were analyzed using FlowJo software.

Magnetic-sorting and culture of CD205^{+} cells from PEC.

A 50%-50% mix of two anti-CD205 monoclonal antibodies, designated 3B6-2B1 and 2F1-1G3 was biotinylated and used to functionalize streptavidin-coated magnetic beads (BioLegend, San Diego, CA). After collection of PECs, the anti-CD205 functionalized beads were mixed with PECs at a ratio of 100µl of beads to $1x10^{8}$ cells and incubated on ice for 30 minutes. Cell isolation procedures followed the manufacturer's recommendations. CD205^{+} sorted cells were further cultured in growth media supplemented with 50ng/mL each of GM-CSF and IL-4 growth factors (Kingfisher Biotech, St. Paul, MN). Cells were cultured for 48 hours before activation using 1µg/ml lipopolysaccharide (LPS) from *Salmonella enterica* serotype enteritidis (Sigma-Aldrich).

Live animal work and permits.

Mouse and bird handling procedures were performed under animal use permit #2013-0254. Mice were obtained from Charles Rivers Laboratories and general care provided through the College of Veterinary Medicine Comparative Medicine Program.

Results

Presence of dendritic cells observed in PECs

Initial observation of PECs after removal of highly adherent macrophages by plastic adherence indicated APCs remained in the non-adherent population based on IFA staining against MHC-II (Figure 12). This population of cells was also stained with anti-Bu-1 (a B-cell marker) and anti-CD40 (marker for all APCs). Staining for Bu1 showed fewer immunopositive cells than revealed with general APC markers (anti-MHC-II and anti-CD40). Since the majority of the macrophages have been removed by plastic adherence, the difference in staining could theoretically be explained by the presence of dendritic cells. Once the Non-Ad population was activated with LPS for 24 hours, the CD40-positive staining cells increased in frequency and intensity within this Non-Ad PEC population (Figure 12). Total RNA was extracted from Ad and Non-Ad PEC populations and further reverse-transcribed to complementary DNA (cDNA) to measure the gene expression of the CD205 receptor, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the house-keeping control gene. Amplification of CD205 receptor cDNA can be seen on the resulting gel electrophoresis of the polymerase chain reaction (PCR) product at the theoretically expected length of 299 bases in the Non-Ad PEC population (Figure 13), but not in the Ad population. Results strongly suggested that $CD205^+$ DCs were/are a subset of the Non-Ad cell population and confirmed that peritoneal exudate cell collection might indeed be a feasible method to obtain primary DCs.

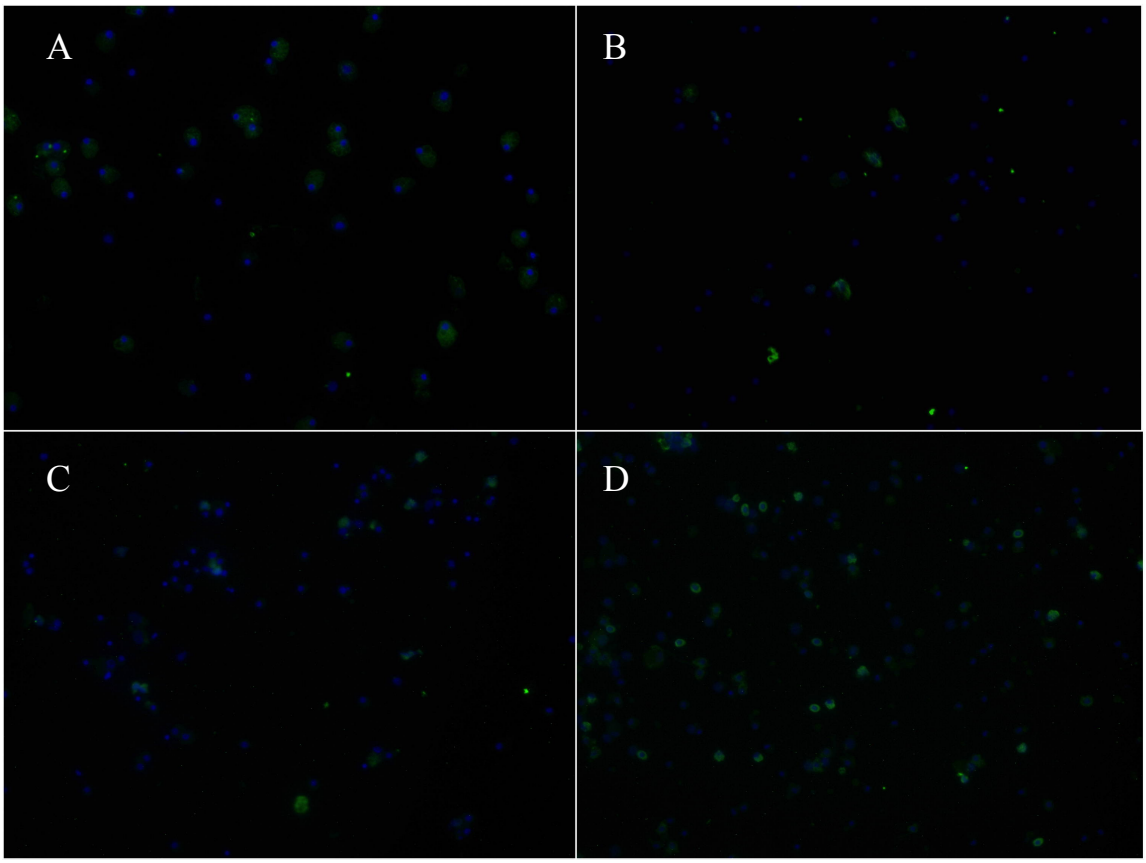

Figure 12. Immunofluorescent staining of non-adherent PECs with APC marker antibodies.
Adherent PECs removed by plastic adherence and non-adherent cells fluorescein isothiocyanate (FITC)-stained (green) against A) MHC-II, B) Bu-1, C) CD40, and D) CD40 after activation with LPS for 24 hours. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (seen in blue) in all images.

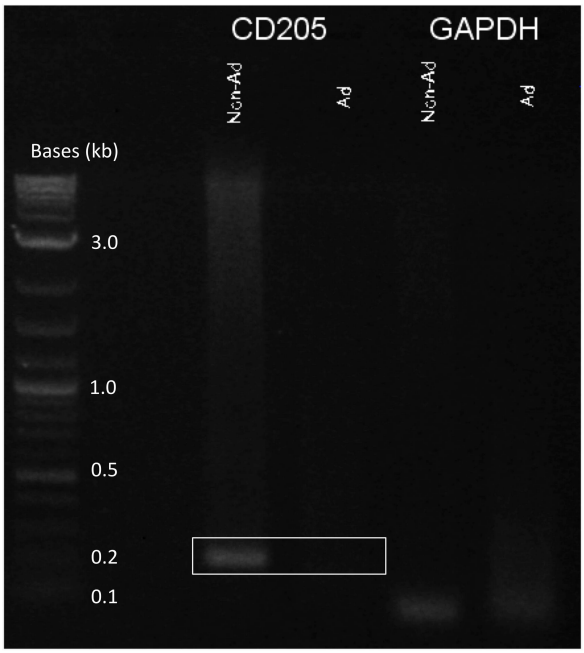

Figure 13. Expression of CD205 mRNA in non-adherent PECs but not in adherent PECs.

Anti-CD205 monoclonal antibodies stain PECs

After presence of $CD205^+$ cells was confirmed, a truncated version of the CD205 protein receptor was designed for recombinant protein expression, specifically to be used as a target immunogen for monoclonal antibody development. Only C-type lectin domains 4-6 were used for protein expression in *Escherichia coli*, following the initial design as described by Wu et *al.* (2010) to avoid previous whole CD205 protein expression issues. This recombinant, truncated version contained a C-terminal His-tag marker and the identity of the resulting protein was verified by western blot (Figure 14). This protein preparation with an estimated purity of approximately 85% was used at to immunize and screen mice for anti-CD205 monoclonal antibody development.

Resulting subcloned monoclonal hybridomas were screened for anti-CD205 antibody production by IFA on Non-Ad PECs. Results for three of the positive monoclonal candidates are shown in Figure 15, all of which stained a subpopulation of the Non-Ad PECs. Further applications of the monoclonal antibodies, including flow cytometry or magnetic bead cell sorting, used monoclonals 3B6-2B1 and 2F1-1G3, either alone or in a mixed manner.

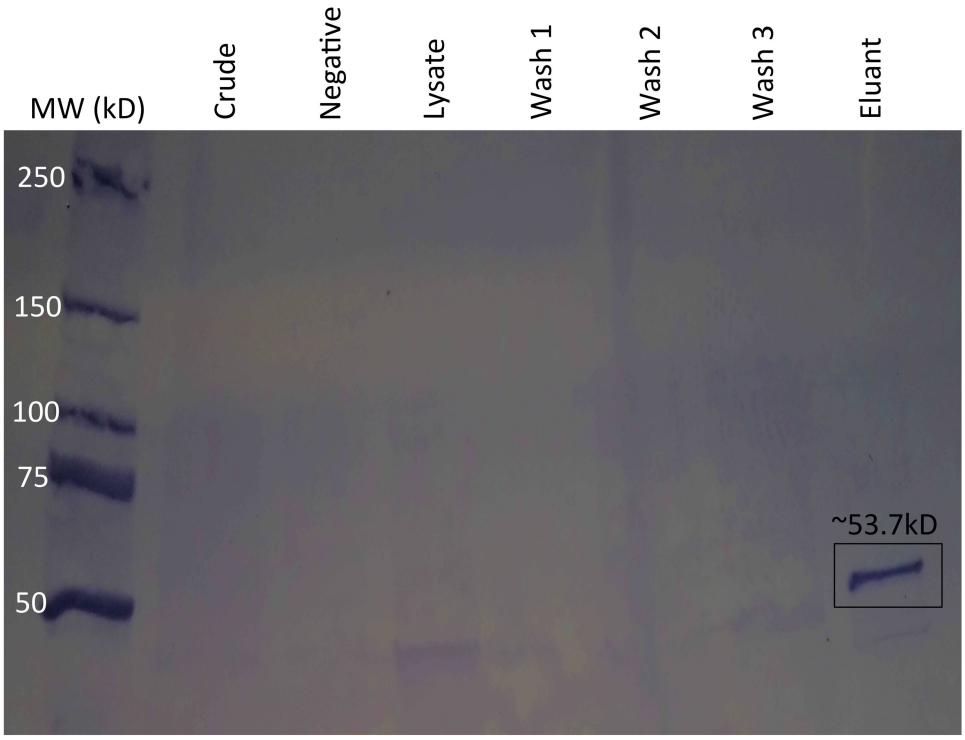

Figure 14. Truncated CD205 protein expressed in *E. coli* system based on His-tag staining.
Anti-His-tag stained western blot after Ni-NTA purification of the protein.

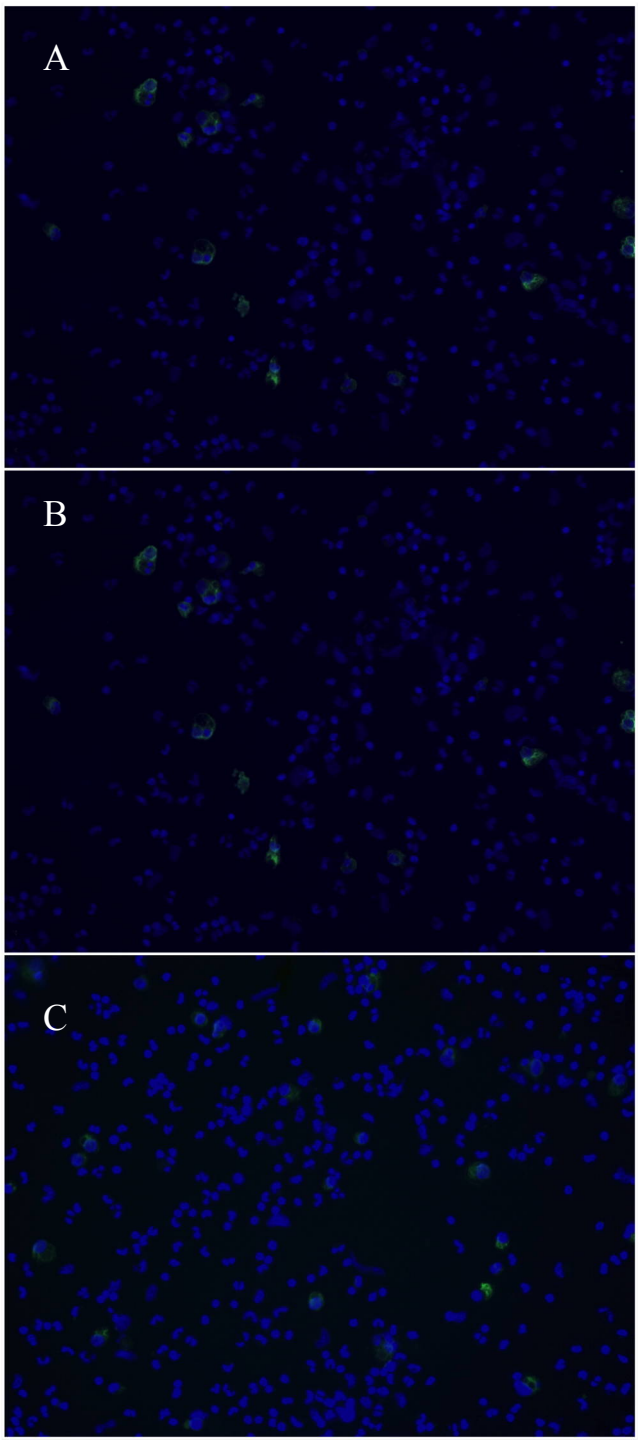

Figure 15. Screening of different candidate monoclonal antibodies against CD205 on non-adherent PECs.
Non-Ad PECs screened for anti-CD205 staining (green) with three different candidate hybridomas A) 3B6-2B1, B) 2F1-1G3, and C) 2F3-1D6. Nuclear DNA was stained blue with DAPI in blue.

Percentage of CD205$^+$ DCs in PECs

Purified monoclonal antibodies against CD205 receptor were biotinylated and used with a streptavidin-conjugated Pacific Blue fluorophore for flow cytometry screening. Spleen and blood samples were collected (one week after vaccination with a commercially available Newcastle's Disease Virus vaccine to ensure immune response activation) for analysis of CD205$^+$ staining cells. Mononuclear cells were collected from each sample type and stained with individual monoclonal antibody candidates, 3B6-2B1 or 2F1-1G3. Resulting CD205$^+$ staining percentage of the mononuclear cells ranged from 6.7-9.8% (Table 3). As dendritic cells typically range from 5-10% of the immune cells (126), these data correlate with previously described DC literature. PECs were not analyzed by flow cytometry as the cell population is naturally sticky when collected from the peritoneal cavity and prone to clogging the flow cell during flow cytometric analyses. Magnetic purification of CD205$^+$ cells was performed by binding biotinylated anti-CD205 antibodies to streptavidin-coated magnetic beads. A 50%-50% mix of both monoclonal antibodies was used to functionalize the magnetic beads. This procedure was performed on spleen mononuclear cells and PEC samples, recovering 7.15×10^6 and 2.15×10^7 cells from each respective source (Table 4).

Table 3. Flow cytometry using anti-CD205 mAb on spleen and PBMC samples. Birds were vaccinated with commercial Newcastle's Disease Virus to activate the immune system. Spleen mononuclear cells and PBMCs were stained using anti-CD205 monoclonal antibodies and percent stained were analyzed by flow cytometry.

Source (age)	CD205+ (3B6)	CD205+ (2F1)
Spleen (Day 7)	7.2%	9.8%
Blood (Day 7)	8.7%	6.4%
Spleen (Day 24)	6.7%	6.8%

Table 4. Magnetic sorting of $CD205^+$ cells.
Monoclonal anti-CD205 antibodies were mixed 50%-50% and loaded onto magnetic beads and used to sort $CD205^+$ cells from spleen mononuclear cells or PECs.

Source	Starting Total # Cells	$CD205^+$ Purified	% $CD205^+$ Purified
Spleen	$1x10^9$	$7.15x10^6$	7.15%
PECs	$1.5x10^8$	$2.15x10^7$	19.4%

Morphology of cultured PEC-DCs

Initial isolation of $CD205^+$ cells from PECs exhibit no morphological differences post-purification procedure (Figure 16), but observation of PECs after activation show distinct development of appendages, or arms, in cultures of both whole PECs (Figure 17) and $CD205^+$ sorted PECs (Figure 18). Culture of PECs itself seems to induce low levels of activation based on slight arm development (Figure 17), while the sorting procedure induces a greater level of activation (Figure 18). These background activation responses do not reach the superior levels of appendage development and cellular aggregation induced by LPS activation. Additionally, immunofluorescent staining of sorted and

activated PECs against CD40 and CD205 show positive expression of both receptors (Figure 19). In contrast, $CD205^+$ isolated cells from spleen exhibit low reactivity after the sorting procedure and only slight appendage development after LPS activation (Figure 20). These cells also do no engulf or clear the magnetic beads bound to the cellular surface during the isolation procedure as efficiently as PEC-derived cells once placed in culture.

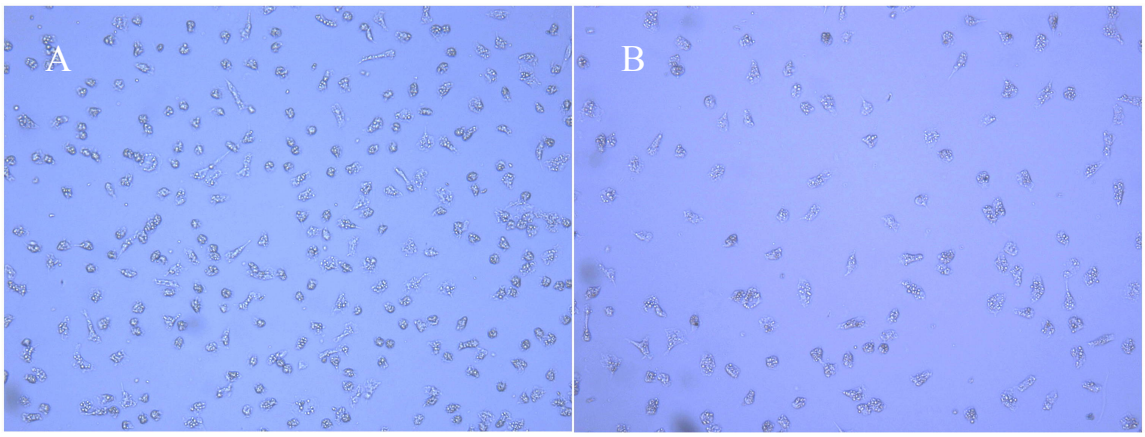

Figure 16. Crude *vs.* $CD205^+$ sorted PECs.
PECs A) before and B) after sorting by magnetic isolation for $CD205^+$ cells using the monoclonal antibodies.

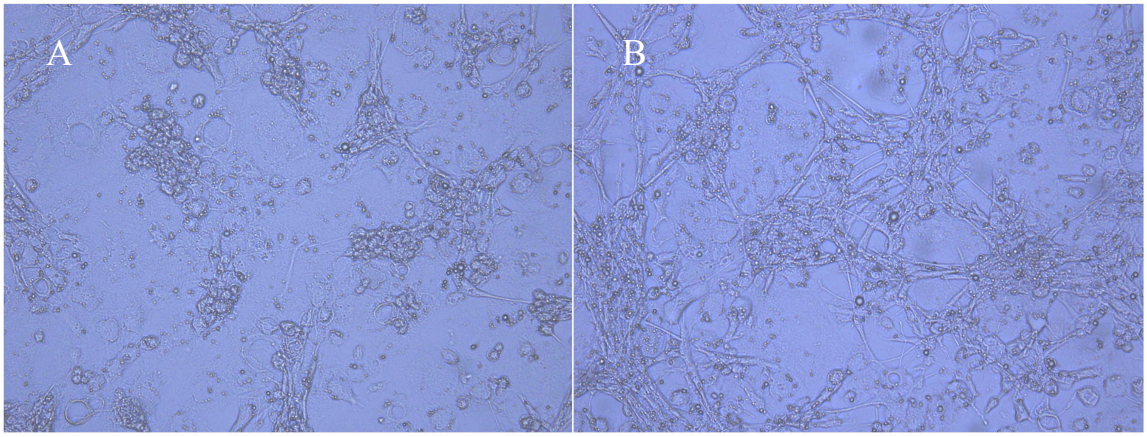

Figure 17. Unsorted PECs cultured and LPS-activated/stimulated.
Crude, unsorted PECs were cultured with growth factors for 48 hours and then left A) non-activated or B) activated with 1µg/ml LPS for 48 hours.

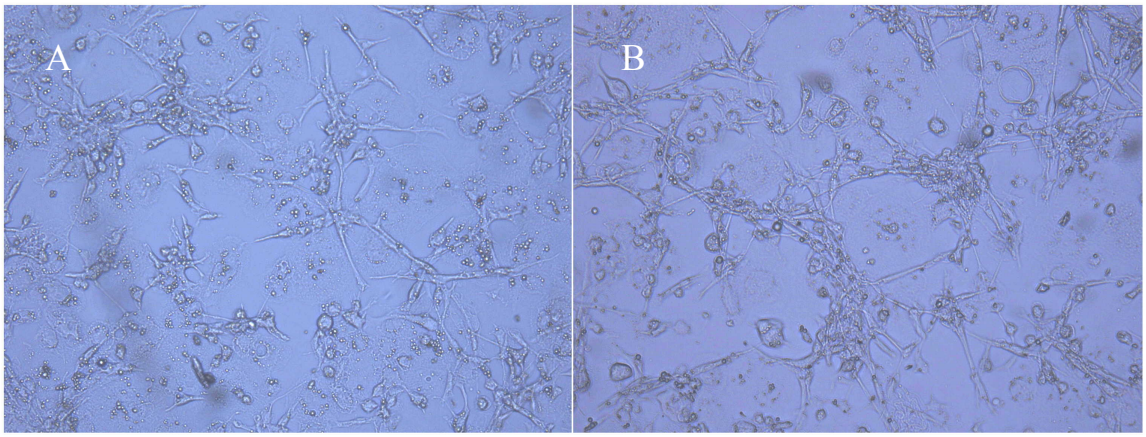

Figure 18. PECs sorted for CD205^{+} cells cultured and activated.
CD205^{+} PECs cultured for 48 hours with growth factors and left A) non-activated or B) activated with 1µg/ml LPS for 48 hours.

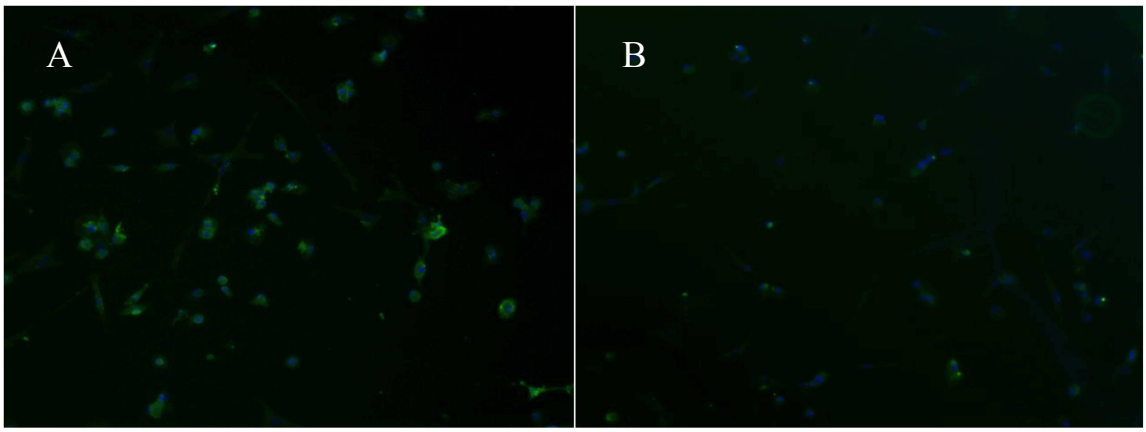

Figure 19. Immunofluorescent staining of CD205^{+} sorted and activated PECs against APC markers.
Sorted CD205^{+} PECs stained against A) CD40 or B) CD205.

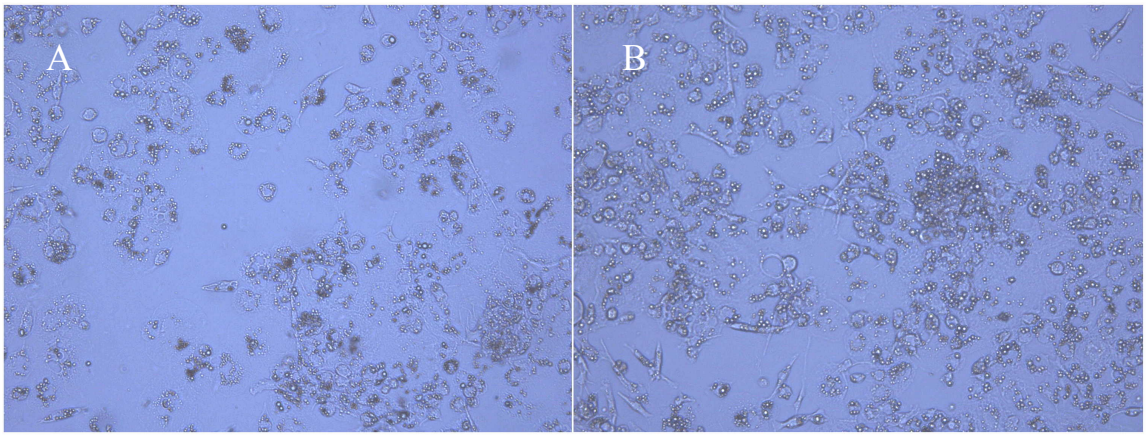

Figure 20. CD205^{+} sorted spleen cells.
Sorted splenocytes were cultured with growth factors for 48 hours before cells were left A) non-activated or B) activated by 1µg/ml LPS.

Discussion

Based on this study, peritoneal exudate cells are a viable source for primary mature dendritic cells from chickens. These dendritic cells can be recovered from the bird fully matured or placed into culture for further activation and manipulation. The

PEC collection method requires less labor than bone marrow myeloid progenitor collection, as well as less time in culture before use since mature DCs are already in the recovered PEC population and do not require additional differentiation. These cells also exhibit normal branched projection/dendrite, development and cellular aggregation upon activation stimulus, indicative of the dendritic cell response (126). Isolation of $CD205^+$ cells from PECs by magnetic sorting produced a higher percentage of recovered cells compared to splenic origin. In theory, a higher percentage of DC recovery could be expected from PEC, as this population of cells was specifically activated for induced migration of immune cells into the area of interest, while the spleen houses multiple immune cells awaiting activation upon influx of circulating lymph which skews the starting populations before purification. Monoclonal antibodies were also generated against the chicken CD205 receptor, the first to be reported in the United States. These antibodies were successfully used in multiple applications, including ELISA, western blot, immunofluorescence, flow cytometry, and magnetic bead purification for downstream cell culture.

Targeting CD205 is known to initiate downstream induction of type II MHC antigen presentation and type I MHC antigen cross-presentation (65, 67, 127, 128). Induction of both MHC antigen presentation pathways allows for the subsequent activation of both $CD4^+$ and $CD8^+$ T-cells, respectively (122). Theoretically, targeted activation of a dendritic cells with a specified immune target should induce a stronger and more rapid immune response in comparison to traditional unguided immunization methods that require longer induction times (129). Murine studies have also indicated

targeting CD205 during immunization is capable of inducing an even stronger immune response than CD40-targeting, while decreasing the required antigen load (130, 131). This research has yet to be completed in avian systems as DCs are the least characterized immune cell within the chicken, but may be the next step in characterizing the anti-CD205 antibodies developed. Antigen presentation and subsequent immune mechanisms induced by CD205 receptor activation also differ from CD40 downstream responses, thus targeting of one specific receptor may be better than another depending of the pathogen's mode of infection. Combining CD40 and CD205-targeting within the same vaccine would also be an option, increasing coverage of different antigen-presentation pathways and downstream responses, therefore ensuring chances for inducing protection.

Further analysis of the PEC-sourced primary dendritic cells is necessary to understand where these DCs originate before migration into the peritoneal cavity. Functional characterization and comparison to bone marrow origin DCs would also be of interest. Different subsets of DCs perform different roles, and therefore vary in immune response (65, 132). This is particularly important to distinguish, as certain assays may be more suited to a specific source, and therefore subset, of DC. Analysis of the monoclonal antibodies developed in this study also requires additional research. Beyond its use to bind and purify $CD205^{+}$ cells, these antibodies have not been tested to confirm whether they possess antagonistic functions, agonistic functions, purely recognize and bind the receptor, or induce development of immune tolerance. Published literature targeting CD205 in murine models has induced both pro-inflammatory and anti-

inflammatory effects, but a consensus has yet to be reached on the controlled effects from targeting dendritic cells (67, 133, 134). Despite the additional research required to further characterize the PEC-sourced DCs and anti-CD205 antibodies, the report of both has provided an additional resource of reagents for research and development in chickens.

CHAPTER V

CONCLUSIONS

The use of antibody-guided complexes in poultry is still relatively new, and its prospective applications still being discovered. The work presented herein was able to further extend the use of guided-complexes, primarily as a technique for rapid polyclonal sera production in chickens and as a potential epitope mapping method for target identification of microbial toxins. These data have also advanced the potential of antibody-guided complexes as vaccine adjuvant platforms. With the AIV immunogenicity and efficacy trials, this research is the first to report successful adaptation and application of the guided complex with whole pathogen instead of a synthetic peptide, measure fast and long-lived functional antibody responses up to six weeks after a single immunization, and the first reported study to transition from immunogenicity data to protective efficacy using the guided-complex system in poultry.

Now that efficacy has been reported using the antibody-guided system in poultry, optimization is the next step to making the system more efficacious and implementable for the poultry industry. Antibody-guided complexes are more expensive and complicated to produce than traditional vaccines and development of a less expensive complex, and perhaps a recombinant bi-specific single-chain protein, would be more cost effective and user-friendly for the industry. A unified, single complex would not require preliminary processing and manipulation, which would also decrease the probability of errors during manufacturing. Administration through feed or spray

immunization is also favored by the industry, as those methods do not require handling of individual birds by trained personnel. Studies to increase efficacy of mucosally-administered complex would be a reasonable next step and its successful production would clear a major obstacle hindering introduction to mass application. Our completed studies currently described protocols with the priming administration at two weeks of age to avoid interference by maternal antibodies, but broilers only live to 6-7 weeks before processing once the target weight has been achieved and require established protection against disease immediately after maternal antibodies naturally degrade from their systems. Despite establishing a response within a matter of days, the current guided-complex vaccination timeline leaves the bird vulnerable to infection during this gap in time, after maternal antibodies diminish and before vaccine-induced protection is conferred. Vaccination *in ovo* or at day-of-hatch to provide protection at an earlier age, preferably without requiring a boost, would be more relevant for this industry and presents another opportunity for further research. Proper targeting and manipulation of the immune response will also need to be addressed in *in vivo* antigen targeting research. Although there are many murine and human studies which report successful induction of pro-inflammatory immune responses when targeting the CD40 receptor, studies with conflicting results have also been described. Some studies assert CD40-targeting is sufficient to induce memory T-cell development (135), while other studies claim CD40-targeting is only able to induce $CD8^+$ cytotoxic effector T-cells without further development of memory T-cells (136). Thus, studies of the CD40-targeting system's ability to induce cytotoxic T-cells and T-cell memory will also need to be conducted in

poultry to confirm inducible T-cell involvement. Additionally, the information gathered from these studies would help elucidate and confirm T-cell effector and memory induction mechanisms for avian immunologists in general.

Our studies also report the introduction of additional reagents that will advance the field of poultry immunology research, namely with the development of anti-CD205 receptor monoclonal antibodies and a novel technique to obtain primary mature DCs. With these new reagents, further research and characterization of the anti-CD205 antibodies as well as the isolated PEC-DC population can be initiated. It would be interesting to discern what type of function the anti-CD205 antibodies possess, such as whether these antibodies merely bind or whether they are able to neutralize or activate their targeted DCs. This study could be completed by collecting bone marrow derived DCs to use as a naïve population and *in vitro* testing of the cellular functions after application of the antibody. Beyond being a primary antibody for use in laboratory assays, the ideal utilization of the anti-CD205 antibodies would be its inclusion in the antibody-targeting systems to present antigen directly to DCs. With this in mind, some challenges will be faced when using CD205-targeting. Manipulation of the immune response by targeting CD205 has shown to be more complicated than CD40 targeting. Engagement of the CD40 receptor provides the co-stimulation needed to provoke APC activation, a single pathway with many outcomes, but all within the same scope of pro-inflammatory response. In contrast, DCs control all downstream responses to antigens by stimulating naïve T-cells to differentiate into different helper subsets, ultimately resulting in either activating or tolerizing environments. Because DCs initiate and

dictate the appropriate immune response during antigen presentation and activation of lymphocytes, controlled manipulation of DCs reactions is more difficult to predict and regulate. This further emphasizes the need to conduct studies using the recently developed anti-CD205 antibodies to test their ability to activate and induce immunogenic responses against specific antigens. For preliminary *in vivo* trials, this can be accomplished by using the established system of loading synthetic peptide onto a CD205-targeting complex and testing for inducible antigen-specific immunogenicity. $CD205^+$ DCs are also capable of presenting antigen on both MHC-I and MHC-II complex (64, 65), and targeting these cells has become very desirable to scientists who want to specifically stimulate $CD8^+$ cytotoxic T-cell responses, particularly against diseases which cannot be controlled by humoral immune response with high neutralizing antibody levels. Unfortunately, to direct antigen towards a particular antigen presentation context, it is not sufficient to simply conjugate the antigen onto a CD205-targeting antibody. Studies have discovered that $CD205^+$ receptor expression levels, speed of internalization, as well as amount of delivered antigen during CD205-targeting all govern preferential presentation on MHC-I or MHC-II (131). Research will need to be conducted to identify the specific requirements to control preferential presentation of antigen in the context of specific MHC complexes, a study which would help explain the mechanism for those attempting to manipulate this system and clarify the system to the field immunology of. DCs also determine if the eventual outcome of antigen-presentation will be either immunity or tolerance, a mechanism that is currently not fully understood and thus cannot be efficiently manipulated until more knowledge is

generated on this subject (75, 137). Antigen conjugated to anti-CD205 antibodies has been able to induce $CD8^+$ cytotoxic T-cell activation (138), but $CD205^+$ DCs have also been described to prompt development of $Foxp3^+$ regulatory T-cells (139). In addition, some studies state that targeting CD205 *per se* is sufficient to induce an immune response, while others state that solely targeting CD205 induces tolerance (140). Most studies seem to agree that combined targeting of both CD40 and CD205 can overcome tolerization responses and push the DC response towards immune cell activation (141). This data suggest that future anti-CD205 targeting investigations should be completed alongside anti-CD40 targeting to confirm whether the combination of the two targeting systems is able to provoke increased immunogenicity compared to singular CD205 or CD40-targeting administration, with perhaps even a synergistic effect when used in combination. Whether the desired goal is to induce activation or tolerance, the exact mechanism to fine-tune targeted control of the immune response using antibody-guided approach has yet to be clearly defined, providing opportunities for future researchers to uncover the many facets involved in avian immunological manipulation.

The research presented in this dissertation has substantially contributed to the development of antibody-guided complexes for poultry use by 1) introducing the use of the guided system for epitope mapping of microbial enzymes, 2) providing proof of principle for loading whole pathogen onto the complex, 3) proving protective efficacy against a high profile disease, and 4) developing new anti-CD205 antibodies to specifically target DCs while also presenting another method to isolate DCs from chickens. Although it is also clear that the current prototype is far from perfect, this

research has significantly assisted with the advancement of the antibody-guided systems in poultry. Literature clearly demonstrates antibody-guided immunization is more nuanced and convoluted than originally assumed and considerable research efforts are still necessary to elucidate the critical elements in immune response manipulation with antibody-guided complexes, but the prospective applications are vast and worth the effort both in terms of unveiling further basic scientific knowledge of the immune response and in terms of offering novel resources for applied veterinary or biomedical use.

REFERENCES

1. USDA National Agricultural Statistics Service (2017). *Broilers: Production and value of production by year, US* [Online]. United States Department of Agriculture. Available: https://www.nass.usda.gov/Charts_and_Maps/Poultry/brprvl.php [Accessed Feb 28 2017].
2. USDA National Agricultural Statistics Service (2017). *Eggs: Production and value of production by year, US* [Online]. United States Department of Agriculture. Available: https://www.nass.usda.gov/Charts_and_Maps/Poultry/ [Accessed Feb 28 2017].
3. National Chicken Council *Per capita consumption of poultry and livestock, 1965-2016, in pounds* [Online]. National Chicken Council. Available: http://www.nationalchickencouncil.org/about-the-industry/statistics/per-capita-consumption-of-poultry-and-livestock-1965-to-estimated-2012-in-pounds/ [Accessed May 23 2017].
4. Jones FT, and Ricke SC. Observations on the history of the development of antimicrobials and their use in poultry feeds. *Poult Sci* (2003) 82**:**613-617.
5. Niewold TA. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poult Sci* (2007) 86**:**605-609.
6. Taylor DJ. EU ban on four antibiotic growth promoters. *Vet Rec* (1999) 144**:**158.

7. Castanon JIR. History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science* (2007) 86**:**2466-2471. doi: 10.3382/ps.2007-00249.

8. Van Immerseel F, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F, and Ducatelle R. Clostridium perfringens in poultry: an emerging threat for animal and public health. *Avian Pathol* (2004) 33**:**537-549. doi: 10.1080/03079450400013162.

9. Williams RB. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. *Avian Pathol* (2005) 34**:**159-180. doi: 10.1080/03079450500112195.

10. Huyghebaert G, Ducatelle R, and Van Immerseel F. An update on alternatives to antimicrobial growth promoters for broilers. *Vet J* (2011) 187**:**182-188. doi: 10.1016/j.tvjl.2010.03.003.

11. Tellez G, Latorre JD, Kuttappan VA, Hargis BM, and Hernandez-Velasco X. Rye affects bacterial translocation, intestinal viscosity, microbiota composition and bone mineralization in Turkey poults. *PLoS One* (2015) 10**:**e0122390. doi: 10.1371/journal.pone.0122390.

12. Moore RJ. Necrotic enteritis predisposing factors in broiler chickens. *Avian Pathol* (2016) 45**:**275-281. doi: 10.1080/03079457.2016.1150587.

13. Annett CB, Viste JR, Chirino-Trejo M, Classen HL, Middleton DM, and Simko E. Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of

Clostridium perfringens type A. *Avian Pathol* (2002) 31**:**598-601. doi: 10.1080/0307945021000024544.

14. Chapman HD, Cherry TE, Danforth HD, Richards G, Shirley MW, and Williams RB. Sustainable coccidiosis control in poultry production: the role of live vaccines. *Int J Parasitol* (2002) 32**:**617-629.

15. Prescott JF, Parreira VR, Mehdizadeh Gohari I, Lepp D, and Gong J. The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review. *Avian Pathol* (2016) 45**:**288-294. doi: 10.1080/03079457.2016.1139688.

16. Popoff MR, and Bouvet P. Clostridial toxins. *Future Microbiol* (2009) 4**:**1021-1064. doi: 10.2217/fmb.09.72.

17. Sakurai J, Nagahama M, and Oda M. Clostridium perfringens alpha-toxin: characterization and mode of action. *J Biochem* (2004) 136**:**569-574. doi: 10.1093/jb/mvh161.

18. Cooper KK, Trinh HT, and Songer JG. Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with Clostridium perfringens. *Vet Microbiol* (2009) 133**:**92-97. doi: 10.1016/j.vetmic.2008.06.001.

19. Moreira GM, Salvarani FM, da Cunha CE, Mendonca M, Moreira AN, Goncalves LA, et al. Immunogenicity of a Trivalent Recombinant Vaccine Against Clostridium perfringens Alpha, Beta, and Epsilon Toxins in Farm Ruminants. *Sci Rep* (2016) 6**:**22816. doi: 10.1038/srep22816.

20. Schoepe H, Neubauer A, Schlapp T, Wieler LH, and Baljer G. Immunization with an alphatoxin variant 121A/91-R212H protects mice against Clostridium perfringens alphatoxin. *Anaerobe* (2006) 12**:**44-48. doi: 10.1016/j.anaerobe.2005.06.003.

21. Zekarias B, Mo H, and Curtiss R, 3rd. Recombinant attenuated Salmonella enterica serovar typhimurium expressing the carboxy-terminal domain of alpha toxin from Clostridium perfringens induces protective responses against necrotic enteritis in chickens. *Clin Vaccine Immunol* (2008) 15**:**805-816. doi: 10.1128/CVI.00457-07.

22. Nagahama M, Mukai M, Morimitsu S, Ochi S, and Sakurai J. Role of the C-domain in the biological activities of Clostridium perfringens alpha-toxin. *Microbiol Immunol* (2002) 46**:**647-655.

23. Keyburn AL, Sheedy SA, Ford ME, Williamson MM, Awad MM, Rood JI, et al. Alpha-toxin of Clostridium perfringens is not an essential virulence factor in necrotic enteritis in chickens. *Infect Immun* (2006) 74**:**6496-6500. doi: 10.1128/IAI.00806-06.

24. Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, et al. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. *PLoS Pathog* (2008) 4**:**e26. doi: 10.1371/journal.ppat.0040026.

25. Keyburn AL, Portela RW, Sproat K, Ford ME, Bannam TL, Yan X, et al. Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis. *Vet Res* (2013) 44**:**54. doi: 10.1186/1297-9716-44-54.

26. Guillouard I, Garnier T, and Cole ST. Use of site-directed mutagenesis to probe structure-function relationships of alpha-toxin from Clostridium perfringens. *Infect Immun* (1996) 64**:**2440-2444.

27. Nagahama M, Mukai M, Ochi S, and Sakurai J. Role of tryptophan-1 in hemolytic and phospholipase C activities of Clostridium perfringens alpha-toxin. *Microbiol Immunol* (2000) 44**:**585-589.

28. Nagahama M, Nakayama T, Michiue K, and Sakurai J. Site-specific mutagenesis of Clostridium perfringens alpha-toxin: replacement of Asp-56, Asp-130, or Glu-152 causes loss of enzymatic and hemolytic activities. *Infect Immun* (1997) 65**:**3489-3492.

29. Rott R, Klenk HD, Nagai Y, and Tashiro M. Influenza viruses, cell enzymes, and pathogenicity. *Am J Respir Crit Care Med* (1995) 152**:**S16-19.

30. Webster RG, Bean WJ, Gorman OT, Chambers TM, and Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* (1992) 56**:**152-179.

31. Rajagopal S, and Treanor J. Pandemic (avian) influenza. *Semin. Respir. Crit. Care Med.* (2007) 28**:**159-170. doi: 10.1055/s-2007-976488.

32. Steinhauer DA. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* (1999) 258**:**1-20. doi: S0042-6822(99)99716-7 [pii] 10.1006/viro.1999.9716.

33. USDA Animal and Plant Health Inspection Service (2017). *Highly Pathogenic Avian Influenza* [Online]. United States Department of Agriculture. Available:

https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/emergency-management/fadprep-hpai [Accessed May 15 2017].

34. Moore T, and Morgan N (2006). "Avian influenza: trade issues", in: *CAST Commentary.* (eds.) C. Beard & B. Gardner. (Ames, IA: Council for Agricultural Science and Technology).

35. Swayne DS, and B.L. Akey (2005). "Avian influenza control strategies in the United States of America," in *Avian influenza: prevention and control,* ed. R.S.S.a.G. Koch. (Dordrecht: Springer), 113-130.

36. Sun Y, Pu J, Fan L, Sun H, Wang J, Zhang Y, et al. Evaluation of the protective efficacy of a commercial vaccine against different antigenic groups of H9N2 influenza viruses in chickens. *Vet Microbiol* (2012) 156**:**193-199. doi: 10.1016/j.vetmic.2011.10.003.

37. Pfeiffer J, Suarez DL, Sarmento L, To TL, Nguyen T, and Pantin-Jackwood MJ. Efficacy of commercial vaccines in protecting chickens and ducks against H5N1 highly pathogenic avian influenza viruses from Vietnam. *Avian Dis* (2010) 54**:**262-271. doi: 10.1637/8715-031909-Reg.1.

38. International Chicken Genome Sequencing C. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* (2004) 432**:**695-716. doi: 10.1038/nature03154.

39. Schat KA, Kaspers B, Kaiser P, ProQuest (Firm), and ebrary Inc. (2014). *Avian immunology.* San Diego, Calif.: Academic Press.

40. Warr GW, Magor KE, and Higgins DA. IgY: clues to the origins of modern antibodies. *Immunol Today* (1995) 16**:**392-398. doi: 10.1016/0167-5699(95)80008-5.

41. Fu YX, and Chaplin DD. Development and maturation of secondary lymphoid tissues. *Annu Rev Immunol* (1999) 17**:**399-433. doi: 10.1146/annurev.immunol.17.1.399.

42. Ruddle NH. Lymphoid neo-organogenesis: lymphotoxin's role in inflammation and development. *Immunol Res* (1999) 19**:**119-125. doi: 10.1007/BF02786481.

43. Kaiser P. The avian immune genome--a glass half-full or half-empty? *Cytogenet Genome Res* (2007) 117**:**221-230. doi: 10.1159/000103183.

44. Kaiser P. Advances in avian immunology--prospects for disease control: a review. *Avian Pathol* (2010) 39**:**309-324. doi: 10.1080/03079457.2010.508777.

45. Daimon T, and Caxton-Martins A. Electron microscopic and enzyme cytochemical studies on granules of mature chicken granular leucocytes. *J Anat* (1977) 123**:**553-562.

46. Genovese KJ, He H, Swaggerty CL, and Kogut MH. The avian heterophil. *Dev Comp Immunol* (2013) 41**:**334-340. doi: 10.1016/j.dci.2013.03.021.

47. Arstila TP, and Lassila O. Androgen-induced expression of the peripheral blood gamma delta T cell population in the chicken. *J Immunol* (1993) 151**:**6627-6633.

48. Kaiser P, Poh TY, Rothwell L, Avery S, Balu S, Pathania US, et al. A genomic analysis of chicken cytokines and chemokines. *J Interferon Cytokine Res* (2005) 25**:**467-484. doi: 10.1089/jir.2005.25.467.

49. Kaufman J, Milne S, Gobel TW, Walker BA, Jacob JP, Auffray C, et al. The chicken B locus is a minimal essential major histocompatibility complex. *Nature* (1999) 401**:**923-925. doi: 10.1038/44856.

50. Magor KE, Miranzo Navarro D, Barber MR, Petkau K, Fleming-Canepa X, Blyth GA, et al. Defense genes missing from the flight division. *Dev Comp Immunol* (2013) 41**:**377-388. doi: 10.1016/j.dci.2013.04.010.

51. Reynaud CA, Anquez V, Dahan A, and Weill JC. A single rearrangement event generates most of the chicken immunoglobulin light chain diversity. *Cell* (1985) 40**:**283-291.

52. Reynaud CA, Anquez V, Grimal H, and Weill JC. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell* (1987) 48**:**379-388.

53. Reynaud CA, Dahan A, Anquez V, and Weill JC. Somatic hyperconversion diversifies the single Vh gene of the chicken with a high incidence in the D region. *Cell* (1989) 59**:**171-183.

54. Abbas AK, Lichtman AH, and Pillai S (2007). *Cellular and molecular immunology.* Saunders Elsevier.

55. Abbas AK, Lichtman AH, and Pillai S (2014). "Humoral Immune Responses," in *Basic Immunology*. 4 ed (Philadelphia, PA: Elsevier Saunders), 131-150.

56. Strom L, Laurencikiene J, Miskiniene A, and Severinson E. Characterization of CD40-dependent immunoglobulin class switching. *Scand J Immunol* (1999) 49**:**523-532.

57. Monson NL, Foster SJ, Brezinschek HP, Brezinschek RI, Dorner T, and Lipsky PE. The role of CD40-CD40 ligand (CD154) interactions in immunoglobulin light chain repertoire generation and somatic mutation. *Clin Immunol* (2001) 100**:**71-81. doi: 10.1006/clim.2001.5049.

58. Kothlow S, Morgenroth I, Tregaskes CA, Kaspers B, and Young JR. CD40 ligand supports the long-term maintenance and differentiation of chicken B cells in culture. *Dev Comp Immunol* (2008) 32**:**1015-1026. doi: 10.1016/j.dci.2008.01.012.

59. Notarangelo LD, Duse M, and Ugazio AG. Immunodeficiency with hyper-IgM (HIM). *Immunodefic Rev* (1992) 3**:**101-121.

60. Yin G, Lin Q, Qiu J, Qin M, Tang X, Suo X, et al. Immunogenicity and protective efficacy of an Eimeria vaccine candidate based on Eimeria tenella immune mapped protein 1 and chicken CD40 ligand. *Vet Parasitol* (2015) 210**:**19-24. doi: 10.1016/j.vetpar.2015.03.012.

61. Pose AG, Gomez JN, Sanchez AV, Redondo AV, Rodriguez ER, Segui RM, et al. Subunit influenza vaccine candidate based on CD154 fused to HAH5 increases the antibody titers and cellular immune response in chickens. *Vet Microbiol* (2011) 152**:**328-337. doi: 10.1016/j.vetmic.2011.05.033.

62. O'Meara KM, Kremer CJ, Layton SL, Berghman LR, Hargis BM, and Cole K. Evaluation of recombinant Salmonella expressing CD154 for persistence and enhanced antibody response in commercial turkeys. *Poult Sci* (2010) 89**:**1399-1405. doi: 10.3382/ps.2010-00697.

63. Lahoud MH, Ahmet F, Zhang JG, Meuter S, Policheni AN, Kitsoulis S, et al. DEC-205 is a cell surface receptor for CpG oligonucleotides. *Proc Natl Acad Sci U S A* (2012) 109**:**16270-16275. doi: 10.1073/pnas.1208796109.

64. Platt CD, Ma JK, Chalouni C, Ebersold M, Bou-Reslan H, Carano RA, et al. Mature dendritic cells use endocytic receptors to capture and present antigens. *Proc Natl Acad Sci U S A* (2010) 107**:**4287-4292. doi: 10.1073/pnas.0910609107.

65. Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, et al. Differential antigen processing by dendritic cell subsets in vivo. *Science* (2007) 315**:**107-111. doi: 10.1126/science.1136080.

66. Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A, Steinman RM, et al. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* (1995) 375**:**151-155. doi: 10.1038/375151a0.

67. Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H, et al. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* (2004) 199**:**815-824. doi: 10.1084/jem.20032220.

68. Geijtenbeek TB, and Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol* (2009) 9**:**465-479. doi: 10.1038/nri2569.

69. Staines K, Young JR, and Butter C. Expression of chicken DEC205 reflects the unique structure and function of the avian immune system. *PLoS One* (2013) 8**:**e51799. doi: 10.1371/journal.pone.0051799.

70. Wu Z, Rothwell L, Young JR, Kaufman J, Butter C, and Kaiser P. Generation and characterization of chicken bone marrow-derived dendritic cells. *Immunology* (2010) 129**:**133-145. doi: 10.1111/j.1365-2567.2009.03129.x.

71. Ecker DM, Jones SD, and Levine HL. The therapeutic monoclonal antibody market. *MAbs* (2015) 7**:**9-14. doi: 10.4161/19420862.2015.989042.

72. US Food and Drug Administration (2017). *FDA Approved Drug Products* [Online]. Available: https://www.accessdata.fda.gov/scripts/cder/daf/ [Accessed July 6 2017].

73. Carter PJ. Potent antibody therapeutics by design. *Nat Rev Immunol* (2006) 6**:**343-357. doi: 10.1038/nri1837.

74. Smyth MJ, and Kershaw MH. Immunology. The adjuvant effects of antibodies. *Science* (2011) 333**:**944-945. doi: 10.1126/science.1210801.

75. Keler T, He L, Ramakrishna V, and Champion B. Antibody-targeted vaccines. *Oncogene* (2007) 26**:**3758-3767. doi: 10.1038/sj.onc.1210375.

76. Peggs KS, Quezada SA, and Allison JP. Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. *Clin Exp Immunol* (2009) 157**:**9-19. doi: 10.1111/j.1365-2249.2009.03912.x.

77. Khalil M, and Vonderheide RH. Anti-CD40 agonist antibodies: preclinical and clinical experience. *Update Cancer Ther* (2007) 2**:**61-65. doi: 10.1016/j.uct.2007.06.001.

78. Khong A, Nelson DJ, Nowak AK, Lake RA, and Robinson BW. The use of agonistic anti-CD40 therapy in treatments for cancer. *Int Rev Immunol* (2012) 31**:**246-266. doi: 10.3109/08830185.2012.698338.

79. Vonderheide RH, Flaherty KT, Khalil M, Stumacher MS, Bajor DL, Hutnick NA, et al. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *J Clin Oncol* (2007) 25**:**876-883. doi: 10.1200/JCO.2006.08.3311.

80. Nimanong S, Ostroumov D, Wingerath J, Knocke S, Woller N, Gurlevik E, et al. CD40 Signaling Drives Potent Cellular Immune Responses in Heterologous Cancer Vaccinations. *Cancer Res* (2017) 77**:**1918-1926. doi: 10.1158/0008-5472.CAN-16-2089.

81. Kellner C, Derer S, Valerius T, and Peipp M. Boosting ADCC and CDC activity by Fc engineering and evaluation of antibody effector functions. *Methods* (2014) 65**:**105-113. doi: 10.1016/j.ymeth.2013.06.036.

82. Karagiannis SN, Josephs DH, Bax HJ, and Spicer JF. Therapeutic IgE Antibodies: Harnessing a Macrophage-Mediated Immune Surveillance Mechanism against Cancer. *Cancer Res* (2017) 77**:**2779-2783. doi: 10.1158/0008-5472.CAN-17-0428.

83. Ninomiya A, Ogasawara K, Kajino K, Takada A, and Kida H. Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice. *Vaccine* (2002) 20**:**3123-3129.

84. Ferlin WG, von der Weid T, Cottrez F, Ferrick DA, Coffman RL, and Howard MC. The induction of a protective response in Leishmania major-infected BALB/c mice with anti-CD40 mAb. *Eur J Immunol* (1998) 28**:**525-531. doi: 10.1002/(SICI)1521-4141(199802)28:02<525::AID-IMMU525>3.0.CO;2-M.

85. Sotomayor EM, Borrello I, Tubb E, Rattis FM, Bien H, Lu Z, et al. Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through in vivo ligation of CD40. *Nat Med* (1999) 5**:**780-787. doi: 10.1038/10503.

86. Barr TA, McCormick AL, Carlring J, and Heath AW. A potent adjuvant effect of CD40 antibody attached to antigen. *Immunology* (2003) 109**:**87-92.

87. Chen CH, Abi-Ghanem D, Njongmeta L, Bray J, Mwangi W, Waghela SD, et al. Production and characterization of agonistic monoclonal antibodies against chicken CD40. *Dev Comp Immunol* (2010) 34**:**1139-1143. doi: 10.1016/j.dci.2010.06.014.

88. Chen CH, Abi-Ghanem D, Waghela SD, Chou WK, Farnell MB, Mwangi W, et al. Immunization of chickens with an agonistic monoclonal anti-chicken CD40 antibody-hapten complex: rapid and robust IgG response induced by a single subcutaneous injection. *J Immunol Methods* (2012) 378**:**116-120. doi: 10.1016/j.jim.2012.02.006.

89. Chou WK, Chen CH, Vuong CN, Abi-Ghanem D, Waghela SD, Mwangi W, et al. Significant mucosal sIgA production after a single oral or parenteral administration using in vivo CD40 targeting in the chicken. *Res Vet Sci* (2016) 108**:**112-115. doi: 10.1016/j.rvsc.2016.08.013.

90. Vuong CN, Chou WK, Kuttappan VA, Hargis BM, Bielke LR, and Berghman LR. A Fast and Inexpensive Protocol for Empirical Verification of Neutralizing Epitopes in Microbial Toxins and Enzymes. *Front Vet Sci* (2017) 4**:**91. doi: 10.3389/fvets.2017.00091.

91. Demangel C, Palendira U, Feng CG, Heath AW, Bean AG, and Britton WJ. Stimulation of dendritic cells via CD40 enhances immune responses to Mycobacterium tuberculosis infection. *Infect Immun* (2001) 69**:**2456-2461. doi: 10.1128/IAI.69.4.2456-2461.2001.

92. Hatzifoti C, and Heath AW. CD40-mediated enhancement of immune responses against three forms of influenza vaccine. *Immunology* (2007) 122**:**98-106. doi: 10.1111/j.1365-2567.2007.02617.x.

93. Parviainen S, Ahonen M, Diaconu I, Hirvinen M, Karttunen A, Vaha-Koskela M, et al. CD40 ligand and tdTomato-armed vaccinia virus for induction of antitumor immune response and tumor imaging. *Gene Ther* (2014) 21**:**195-204. doi: 10.1038/gt.2013.73.

94. Al-Sheikhly F, and Al-Saieg A. Role of Coccidia in the occurrence of necrotic enteritis of chickens. *Avian Dis* (1980) 24**:**324-333.

95. Riddell C, and Kong XM. The influence of diet on necrotic enteritis in broiler chickens. *Avian Dis* (1992) 36**:**499-503.

96. Uzal FA, Freedman JC, Shrestha A, Theoret JR, Garcia J, Awad MM, et al. Towards an understanding of the role of Clostridium perfringens toxins in human and animal disease. *Future Microbiol* (2014) 9**:**361-377. doi: 10.2217/fmb.13.168.

97. Uzal FA, Vidal JE, McClane BA, and Gurjar AA. Clostridium Perfringens Toxins Involved in Mammalian Veterinary Diseases. *Open Toxinology J* (2010) 2**:**24-42.

98. Vita R, Overton JA, Greenbaum JA, Ponomarenko J, Clark JD, Cantrell JR, et al. The immune epitope database (IEDB) 3.0. *Nucleic Acids Res* (2015) 43**:**D405-412. doi: 10.1093/nar/gku938.

99. Thanh-Son T, Catherine B, Nigel S, Mark F, Pete K, Elisabeth le BD, et al. A maximum likelihood QTL analysis reveals common genome regions controlling resistance to Salmonella colonization and carrier-state. *BMC Genomics* (2012) 13**:**198. doi: 10.1186/1471-2164-13-198.

100. Schoepe H, Wieler LH, Bauerfeind R, Schlapp T, Potschka H, Hehnen HR, et al. Neutralization of hemolytic and mouse lethal activities of C. perfringens alpha-toxin need simultaneous blockade of two epitopes by monoclonal antibodies. *Microb Pathog* (1997) 23**:**1-10. doi: 10.1006/mpat.1996.0123.

101. Goossens E, Verherstraeten S, Valgaeren BR, Pardon B, Timbermont L, Schauvliege S, et al. The C-terminal domain of Clostridium perfringens alpha

toxin as a vaccine candidate against bovine necrohemorrhagic enteritis. *Vet Res* (2016) 47**:**52. doi: 10.1186/s13567-016-0336-y.

102. Barr TA, Carlring J, and Heath AW. Co-stimulatory agonists as immunological adjuvants. *Vaccine* (2006) 24**:**3399-3407. doi: 10.1016/j.vaccine.2006.02.022.

103. Barr TA, Carlring J, and Heath AW. CD40 antibody as a potent immunological adjuvant: CD40 antibody provides the CD40 signal to B cells, but does not substitute for T cell help in responses to TD antigens. *Vaccine* (2005) 23**:**3477-3482. doi: 10.1016/j.vaccine.2004.12.025.

104. Martin C, Waghela SD, Lokhandwala S, Ambrus A, Bray J, Vuong C, et al. Characterization of a Broadly Reactive Anti-CD40 Agonistic Monoclonal Antibody for Potential Use as an Adjuvant. *PLoS One* (2017) 12**:**e0170504. doi: 10.1371/journal.pone.0170504.

105. Strugnell RA, and Wijburg OL. The role of secretory antibodies in infection immunity. *Nat Rev Microbiol* (2010) 8**:**656-667. doi: 10.1038/nrmicro2384.

106. Brandtzaeg P. Role of secretory antibodies in the defence against infections. *Int J Med Microbiol* (2003) 293**:**3-15. doi: 10.1078/1438-4221-00241.

107. Cerutti A. The regulation of IgA class switching. *Nat Rev Immunol* (2008) 8**:**421-434. doi: 10.1038/nri2322.

108. Swayne DE, and Suarez DL. Current developments in avian influenza vaccines, including safety of vaccinated birds as food. *Dev. Biol.* (2007) 130**:**123-133.

109. Kapczynski DR, and Swayne DE. Influenza vaccines for avian species. *Curr Top Microbiol Immunol* (2009) 333**:**133-152. doi: 10.1007/978-3-540-92165-3_6.

110. Wright P, Neumann, G., Kawaoka, Y. (2007). "Orthomyxoviruses," in *Fields Virology,* ed. D. Knipe, Howley, P. 5 ed (Philadelphia, PA: Lippincott Williams & Wilkins).

111. Liu W, Zou P, and Chen YH. Monoclonal antibodies recognizing EVETPIRN epitope of influenza A virus M2 protein could protect mice from lethal influenza A virus challenge. *Immunol Lett* (2004) 93**:**131-136. doi: 10.1016/j.imlet.2004.03.003.

112. Liu W, Zou P, Ding J, Lu Y, and Chen YH. Sequence comparison between the extracellular domain of M2 protein human and avian influenza A virus provides new information for bivalent influenza vaccine design. *Microbes Infect.* (2005) 7**:**171-177. doi: S1286-4579(04)00349-1 [pii] 10.1016/j.micinf.2004.10.006.

113. Wu F, Huang JH, Yuan XY, Huang WS, and Chen YH. Characterization of immunity induced by M2e of influenza virus. *Vaccine* (2007) 25**:**8868-8873. doi: S0264-410X(07)01108-5 [pii] 10.1016/j.vaccine.2007.09.056.

114. Rudolf M, Poppel M, Frohlich A, Breithaupt A, Teifke J, Blohm U, et al. Longitudinal 2 years field study of conventional vaccination against highly pathogenic avian influenza H5N1 in layer hens. *Vaccine* (2010) 28**:**6832-6840. doi: 10.1016/j.vaccine.2010.08.038.

115. Shany SA, El-Kady MF, Eid BT, Hassan ER, and Abdel-Moneim AS. Humoral antibody responses to different H5N1 and H5N2 vaccination regimes:

implications for the development of autogenously based vaccines. *Vet Microbiol* (2011) 153**:**398-402. doi: 10.1016/j.vetmic.2011.06.006.

116. Budowsky EI, Smirnov Yu A, and Shenderovich SF. Principles of selective inactivation of viral genome. VIII. The influence of beta-propiolactone on immunogenic and protective activities of influenza virus. *Vaccine* (1993) 11**:**343-348.

117. Tumpey TM, Kapczynski DR, and Swayne DE. Comparative susceptibility of chickens and turkeys to avian influenza A H7N2 virus infection and protective efficacy of a commercial avian influenza H7N2 virus vaccine. *Avian Dis* (2004) 48**:**167-176. doi: 10.1637/7103.

118. Hooper PT, Russell GW, Selleck PW, and Stanislawek WL. Observations on the relationship in chickens between the virulence of some avian influenza viruses and their pathogenicity for various organs. *Avian Dis* (1995) 39**:**458-464.

119. Steensels M, Rauw F, van den Berg T, Marche S, Gardin Y, Palya V, et al. Protection Afforded by a Recombinant Turkey Herpesvirus-H5 Vaccine Against the 2014 European Highly Pathogenic H5N8 Avian Influenza Strain. *Avian Dis* (2016) 60**:**202-209. doi: 10.1637/11126-050615-Reg.1.

120. Singh SM, Alkie TN, Abdelaziz KT, Hodgins DC, Novy A, Nagy E, et al. Characterization of Immune Responses to an Inactivated Avian Influenza Virus Vaccine Adjuvanted with Nanoparticles Containing CpG ODN. *Viral Immunol* (2016) 29**:**269-275. doi: 10.1089/vim.2015.0144.

121. Shan S, Fenwick S, Ellis T, Poinern E, Edwards J, Le X, et al. Evaluation of different chemical adjuvants on an avian influenza H6 DNA vaccine in chickens. *Avian Pathol* (2016) 45**:**649-656. doi: 10.1080/03079457.2016.1195488.

122. Shrimpton RE, Butler M, Morel AS, Eren E, Hue SS, and Ritter MA. CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self. *Mol Immunol* (2009) 46**:**1229-1239. doi: 10.1016/j.molimm.2008.11.016.

123. Staines K, Hunt LG, Young JR, and Butter C. Evolution of an expanded mannose receptor gene family. *PLoS One* (2014) 9**:**e110330. doi: 10.1371/journal.pone.0110330.

124. Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, de Castro E, et al. ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Res* (2012) 40**:**W597-603. doi: 10.1093/nar/gks400.

125. Sabet T, Wen-Cheng H, Stanisz M, El-Domeiri A, and Van Alten P. A simple method for obtaining peritoneal macrophages from chickens. *J Immunol Methods* (1977) 14**:**103-110.

126. Paul WE (2013). *Fundamental immunology.* Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

127. Bordon Y. T cell responses: a dendritic cell designed for two. *Nat Rev Immunol* (2013) 13**:**844-845. doi: 10.1038/nri3560.

128. Mahnke K, Guo M, Lee S, Sepulveda H, Swain SL, Nussenzweig M, et al. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance

antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J Cell Biol* (2000) 151**:**673-684.

129. Matos I, Mizenina O, Lubkin A, Steinman RM, and Idoyaga J. Targeting Antigens to Dendritic Cells In Vivo Induces Protective Immunity. *PLoS One* (2013) 8**:**e67453. doi: 10.1371/journal.pone.0067453.

130. Caminschi I, and Shortman K. Boosting antibody responses by targeting antigens to dendritic cells. *Trends Immunol* (2012) 33**:**71-77. doi: 10.1016/j.it.2011.10.007.

131. Reuter A, Panozza SE, Macri C, Dumont C, Li J, Liu H, et al. Criteria for dendritic cell receptor selection for efficient antibody-targeted vaccination. *J Immunol* (2015). doi: 10.4049/jimmunol.1402535.

132. Heath WR, and Carbone FR. Dendritic cell subsets in primary and secondary T cell responses at body surfaces. *Nat Immunol* (2009) 10**:**1237-1244. doi: 10.1038/ni.1822.

133. Fukaya T, Murakami R, Takagi H, Sato K, Sato Y, Otsuka H, et al. Conditional ablation of CD205+ conventional dendritic cells impacts the regulation of T-cell immunity and homeostasis in vivo. *Proc Natl Acad Sci U S A* (2012) 109**:**11288-11293. doi: 10.1073/pnas.1202208109.

134. Hurwitz AA, and Watkins SK. Immune suppression in the tumor microenvironment: a role for dendritic cell-mediated tolerization of T cells. *Cancer Immunol Immunother* (2012) 61**:**289-293. doi: 10.1007/s00262-011-1181-5.

135. Lefrancois L, Altman JD, Williams K, and Olson S. Soluble antigen and CD40 triggering are sufficient to induce primary and memory cytotoxic T cells. *J Immunol* (2000) 164**:**725-732.

136. Mathieu M, Cotta-Grand N, Daudelin JF, Boulet S, Lapointe R, and Labrecque N. CD40-activated B cells can efficiently prime antigen-specific naive CD8+ T cells to generate effector but not memory T cells. *PLoS One* (2012) 7**:**e30139. doi: 10.1371/journal.pone.0030139.

137. Steinman RM, and Banchereau J. Taking dendritic cells into medicine. *Nature* (2007) 449**:**419-426. doi: 10.1038/nature06175.

138. Kreutz M, Giquel B, Hu Q, Abuknesha R, Uematsu S, Akira S, et al. Antibody-antigen-adjuvant conjugates enable co-delivery of antigen and adjuvant to dendritic cells in cis but only have partial targeting specificity. *PLoS One* (2012) 7**:**e40208. doi: 10.1371/journal.pone.0040208.

139. Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, Bonito AJ, Inaba K, et al. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol* (2008) 181**:**6923-6933.

140. Cruz LJ, Rosalia RA, Kleinovink JW, Rueda F, Lowik CW, and Ossendorp F. Targeting nanoparticles to CD40, DEC-205 or CD11c molecules on dendritic cells for efficient CD8(+) T cell response: a comparative study. *J Control Release* (2014) 192**:**209-218. doi: 10.1016/j.jconrel.2014.07.040.

141. Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* (2001) 194**:**769-779.