FIRST CHARACTERIZATION OF AVIAN MEMORY T LYMPHOCYTE RESPONSES TO AVIAN INFLUENZA VIRUS PROTEINS

A Dissertation

by

SHAILBALA SINGH

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

DOCTOR OF PHILOSOPHY

December 2009

Major Subject: Veterinary Microbiology

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Approved by:

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ABSTRACT

First Characterization of Avian Memory T Lymphocyte

Responses to Avian Influenza Virus Proteins. (December 2009)

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Co-Chairs of Advisory Committee: Dr. Ellen W. Collisson
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Although wild birds are natural hosts of avian influenza viruses (AIVs), these viruses can be highly contagious to poultry and a zoonotic threat to humans. The propensity of AIV for genetic variation through genetic shift and drift allows virus to evade vaccine mediated humoral immunity. An alternative approach to current vaccine development is induction of $CD8^+$ T cells which responds to more conserved epitopes than humoral immunity and targets a broader spectrum of viruses. Since the memory $CD8^+$ T lymphocyte responses in chickens to individual AIV proteins have not been defined, the modulation of responses of the memory $CD8^+$ T lymphocytes to H5N9 AIV hemagglutinin (HA) and nucleocapsid (NP) proteins over a time course were evaluated. $CD8^+$ T lymphocyte responses induced by intramuscular inoculation of chickens with AIV HA and NP expressing cDNA plasmids or a non-replicating human adenovirus vector were identified through ex vivo stimulation with virus infected, major histocompatibility complex (MHC) matched antigen presenting cells (APCs). The IFNγ production by activated lymphocytes was evaluated by macrophage production of nitric oxide and ELISA. MHC-I restricted memory T lymphocyte responses were determined

at 10 days and 3, 5, 7 and 9 weeks post-inoculation (p.i). The use of non-professional APCs and APC driven proliferation of cells with $CD8^+$ phenotype correlated with the activation of $CD8^+$ T lymphocytes. The responses specific to nucleocapsid protein (NP) were consistently greater than those to the hemagglutinin (HA) at 5 weeks when the $CD8^+$ T cell responses were maximum. By 8 to 9 weeks p.i., responses to either protein were undetectable. The T lymphocytes also responded to stimulation with a heterologous H7N2 AIV infected APCs. Administration of booster dose induced secondary effector cell mediated immune responses which had greater magnitudes than primary effector responses at 10 days p.i. Flow cytometric analysis (FACS) of the T lymphocytes demonstrated that memory $CD8^+$ T lymphocytes of chickens can be distinguished from naïve lymphocytes by their higher expression of CD44 and CD45 surface antigens. CD45 expression of memory lymphocytes further increases upon ex vivo stimulation with APCs expressing AIV. This is the first characterization of avian memory responses following both primary and secondary expression of any individual viral protein.

DEDICATION

For her love, sacrifice, support and unwavering faith in my abilities, this dissertation is dedicated to ma.

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I wish to thank my mentor, Dr. Ellen W. Collisson, who gave me the opportunity to be associated with this exciting and first of its kind study. I will always be indebted to her for her great and much sought after mentorship, both professionally and personally. This work would not have been accomplished without her expert guidance and incredible patience.

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NOMENCLATURE

AIV	Avian Influenza Virus
HA	Hemagglutinin
NP	Nucleocapsid Protein
APC	Antigen Presenting Cells
NO	Nitric Oxide
IFNγ	Interferon γ
PBS	Phosphate Buffered Saline
HI	Hemaggluntination Inhibition
MHC	Major Histocompatibility Complex

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

CLASSIFICATION AND ECOLOGY OF AVIAN INFLUENZA VIRUSES

Influenza viruses belong to the *Orthomyxoviridae* family of segmented RNA viruses. There are 3 genera of influenza viruses; type A, B and C (2, 21, 22). The avian influenza viruses (AIV) are type A influenza viruses (21, 22, 138). Influenza A viruses are classified into subtypes based on their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (2, 3). Serological studies have demonstrated the presence of 16 distinct HA and 9 distinct NA subtypes of AIV (21, 22, 36,105,131). Waterfowl and shorebirds (Orders Anseriformes and Charadriiformes) are considered the primary reservoirs of AIV, however on occasions these viruses can be transmitted to other species such as domestic poultry, including chickens and turkeys (Order Galliformes), humans, pigs, feral cats and seals (16,43). Classical AIVs replicate in the gastrointestinal tract of the waterfowl without causing disease (3, 21, 22, 60). The AIVs are shed in the feces of these birds thereby contaminating the water habitats and promoting the spread of virus (60, 81).

Depending on their virulence in domestic poultry AIVs are classified as low pathogenic (LP) or highly pathogenic (HP) viruses (3, 21, 22). LPAIV strains can cause asymptomatic to mild respiratory and enteric tract infections while infection with a

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This dissertation follows the style of Journal of Virology.

HPAIV strain causes clinical illness and systemic disease with 100% mortality in some cases (2). While LPAIV strains of all 16 HA subtypes have been isolated, HPAIV are restricted to only H5 and H7 subtypes (3, 21, 22). All HPAIV emerge only after introduction of AIV to poultry (3, 16, 18). Infections of poultry by the highly pathogenic strains result in severe economic losses in domestic poultry operations both due to disease and culling of suspected flocks (27, 47). Additionally since 1996, there has been a direct transmission of the highly pathogenic H5N1 AIV from poultry to humans in many Asian countries (2, 16, 31, 136). The zoonotic infection of this highly pathogenic virus has resulted in fatality in 60% of the human cases (3).

BIOLOGY OF THE VIRUS

AIV, like all Influenza A viruses are enveloped with single stranded, negative sense, segmented RNA genomes (17, 21, 22). Morphologically the viral particles are pleomorphic and may have spherical to filamentous shape with a diameter of 80-120 nm. The genomes of all type A influenza viruses, including AIV, are composed of eight negative sense RNA segments encoding for upto 12 different viral proteins (18, 21, 22, 143) (FIG. 1-1).

The mRNAs transcribed from the first 6 segments are monocistronic with the exception of segment 2 which in some viruses has overlapping reading frames and leaky ribosomal scanning leads to expression of three different proteins (21, 22, 143). The three largest genomic segments encode for proteins of the viral RNA polymerase complex. While genomic RNA segment 1 and 3 encode for polymerase basic protein (PB2) and polymerase acidic protein (PA) respectively segment 2 encodes for polymerase basic protein 1 (PB1) in all viruses along with PB1-F2 and PB1N40 in some strains (21, 22, 143) . Segments 4, 5 and 6 encode for hemagglutinin (HA), nucleocapsid protein (NP) and neuraminidase (NA) respectively (21, 22, 87). The last two small segments 7 and 8 encode for 2 proteins each by undergoing splicing. Matrix protein (M1) and the ion channel protein (M2) are encoded by segment 7 while segment 8 encodes for non-structural viral protein 1 (NS1) and nuclear export protein (NEP/NS2) (21, 22). Non coding sequences are present at the 5' and 3' ends of all viral RNA segments. These sequences are conserved amongst all the RNA segments of Influenza A viruses. The replication cycle and mRNA synthesis of all influenza viruses occurs in the nucleus of the host cell. The viral polymerase complex utilizes the cellular transcription and splicing machinery for synthesis and modification of mRNA (87).

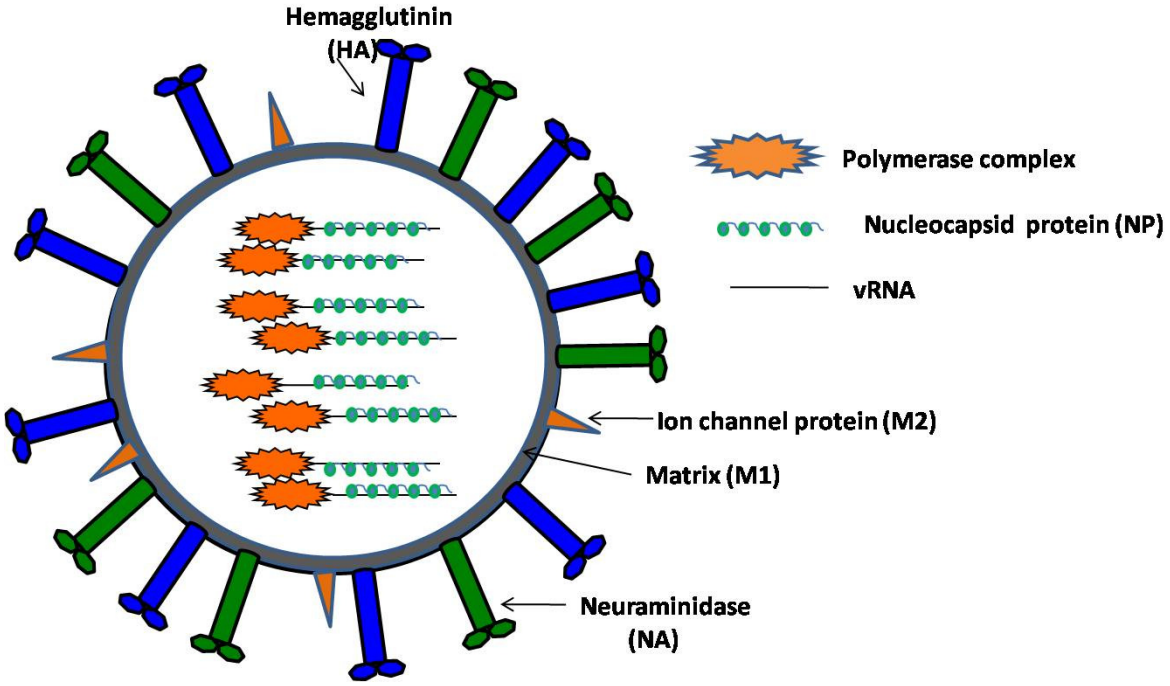

FIG. 1-1. Schematic of an avian influenza virus particle

The lipid envelope of the viral particle is derived from the cellular membrane of the host cell and anchors the viral transmembrane glycoproteins HA and NA, along with M2. PB1, PA, PB2 constitute the viral RNA polymerase complex, and along with NP, form the viral ribonucleoprotein (RNP) complex (87). The viral RNPs, in association with the viral genome, form the core of the virus (21, 22). The M1, with strong affinity for RNP complex, lies beneath the envelope in association with the viral core and functions as a bridge between the envelope and viral RNP complex (21, 22). The NEP/NS2 is critical for the export of M1 associated RNP complex from the nucleus to the cytoplasm during viral assembly (21, 22). The non-structural viral protein 1 NS1 is absent in the viral particle but is found in infected cells where it functions as a type I interferon antagonist (8, 32, 63). PB1-F2 is a pro-apoptotic protein and is found in the mitochondria of infected cells (143). The function of PB1-N40 has not been defined (143).

Viral attachment to the host cell is mediated by HA which recognizes sialic acid on the cell surface. AIV HA mainly recognizes the α-2, 3 linked sialic acid present throughout the respiratory tract of poultry but can also bind to α-2, 3 linked sialic acid found in the lower respiratory tract of humans enabling the virus to infect humans (73). Therefore, the nature of the sialic acid linkage is an important determinant of the viral host and tissue tropism (73). Precursor protein HA0 is cleaved post-translationally by host proteolytic enzymes into external HA1 and membrane anchored HA2 subunits to generate functional HA protein. The cleavage site of HA has been found to be an important determinant of the tissue tropism and hence the pathogenicity or virulence of

the virus (93, 105). LP viruses have a single basic amino acid at the cleavage site and require specific proteases like trypsin, found in the respiratory and enteric tracts, for activation of the virus (21, 22). Highly pathogenic viruses, in contrast, have a multi-basic cleavage site that can be cleaved by ubiquitous proteases, like furin, hence enabling the viruses to become systemic (21, 22, 105, 103). HA1 binds to the cellular receptor with sialic acid moieties and the viral particle enters the host cell by endosomal uptake (21, 22). The acidic pH of endocytic vesicles result in the conformational change in HA2 enabling the viral fusion with the host membrane (21, 22). The conformational change in the viral particle required for uncoating and release of RNP into the cytoplasm is mediated by the ion channel M2 dependent lowering of the pH (21, 22).

Viral transcription and replication occurs in the host cell nucleus by the viral RNA polymerase complex. Following the uncoating of the virus in the cellular endosome, the viral RNP complex is released in the cytoplasm and transported into the nucleus. This transport is mediated by NP which carries a nuclear localization signal (87). A 5'capped RNA fragment is required as a primer to initiate the transcription of mRNA (21, 22). PB2 protein of the polymerase complex uses its endonuclease activity to cleave the capped 5' end from cellular mRNA and makes it available for the transcription of viral mRNA (21, 22). The mRNA of influenza viruses are polyadenylated at their 3' end. These modifications are essential for the nuclear export and stability of viral mRNA. The translation machinery of the host cell is used for protein synthesis (21, 22).

Full length copy RNA (cRNA) of the viral genomic RNA (vRNA) is required for the replication of the virus. cRNA serves as the template for primer independent de novo synthesis of vRNA (21, 22). Without the proofreading capacity of DNA polymerases, the mutation rate of RNA polymerases is very high, resulting in a high degree of viral genome mutations and thus viral protein variability (20, 21). Although HA and NA are the most variable proteins, mutations can also occur in other genomic segments (21, 22). However, fewer variations are observed in the internal proteins, such as those constituting the viral RNP complex than in viral glycoproteins (21, 22). This conserved nature of the internal proteins could be attributed to lesser exposure to selective pressure and/or their limited ability to function with alterations (21, 22).

Besides the potential to mutate rapidly, the segmented nature of the viral genome allows for variation through reassortment of segments from different strains of viruses infecting the same cell (21, 22). The phenomenon of reassortment leads to the emergence of new viruses following co-infection of humans and pigs with influenza A viruses from other species, such as those of swine, human and avian origins (43). The influenza pandemics of 1918, 1957 and 1968 were caused by human influenza viruses encoding genes of avian, swine and human origins (2, 18, 31, 43). Genetic variation may readily occur through either reassortment or point mutations, providing the virus with the capacity to adapt to a range of species and even become zoonotic (43).

IMMUNE RESPONSES AGAINST INFLUENZA VIRUS

Innate Immunity: Influenza virus specific immune responses have been studied extensively in mammals, especially mice and humans. Initial defense against viral infection includes the ciliary epithelium and the mucus membrane of the respiratory tract which inhibit the attachment of the virus. The protease inhibitors present in the respiratory tract may hinder the cleavage of HA and hence its activation, impeding the uncoating of virus (59). In addition to these barriers, the host innate immune system is activated when the pattern recognition receptors (PPRs) interact with the pathogen (virus) associated molecular patterns (PAMP) (21, 22, 32, 135). Toll-like receptors (TLRs) 3 and 7 (both present in the chicken) recognize the viral double-stranded (ds) RNA complex and single-stranded (ss) RNA, respectively, initiating a cascade of events leading to activation of the type I interferon pathway (IFN) (45,135). TLR3 recognize the duplex of positive and negative sense RNA formed during viral replication while recognition by TLR7 does not require viral replication (45). The presence of viral elements in the cytoplasm is also detected by proteins like retinoic acid –inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) which also induce type I IFN production (32, 46, 135). The type I IFNs in turn induce production of many anti-viral proteins such as myxovirus resistance gene (Mx), the 2'-5' oligoadenylate synthetase (OAS), and ds-RNA activated protein kinase (PKR) which inhibit viral replication (32, 46). PKR is activated by the presence of ds RNA and inhibits protein synthesis in the cell by phosphorylating the α subunit of eukaryotic elongation factor 2 (eIF-2α) hence impeding viral replication (8,63). OAS also recognizes ds RNA and

activates cellular RNAse such as RNAse L which can degrade both cellular and viral RNA (32, 46). Type I IFNs also induce an anti-viral state in uninfected cells (46). C-type lectins, which also function as PPRs in chickens and other species, inhibit hemagglutination (52).

The innate response has been shown to be implicated in the development of the adaptive immune response. The viral NS1 protein antagonizes the innate immunity by sequestering the viral double-stranded RNA intermediates and hence inhibiting the activation of cellular protein kinase R (PKR) and OAS (32, 46, 144). NS1 has also been shown to link directly with PKR and prevent the conformational change required for PKR mediated inhibition of cellular protein synthesis (46). The apoptosis induced by PKR is also inhibited by NS1 by the activation of phosphoinositide 3 kinase (PI3K) (32, 46). Additionally, NS1 has a role in inhibiting host cell protein synthesis by preventing post-transcriptional modification of cellular pre-mRNA (63). The antagonistic role of NS1 on innate immune responses prevents maturation of dendritic cells hence inhibits the development of adaptive immune responses against the virus (46, 63).

Adaptive Immunity: Although the innate immune response is the first line of defense against the virus, the adaptive immune response is ultimately responsible for viral clearance and for protection against subsequent infections (29). While antigen specific in nature, adaptive immunity also has antigenic memory, enabling it to respond more rapidly against subsequent antigen exposure. Adaptive immunity includes humoral (antibody production by B cells) and cell mediated (helper and cytotoxic T cell)

immunity (29). Helper or $CD4^+$ T lymphocytes regulate activation of both B lymphocytes and cytotoxic or $CD8^+$ T lymphocytes (107). B lymphocytes produce antibodies directed against viral glycoproteins HA and NA. While HA houses epitopes that induce neutralizing antibodies which are protective against viral challenge, the antibodies against NA epitopes reduce shedding of the virus by the host (19, 33). Antibodies against conserved internal proteins like NP and M1 are not protective in nature (115). Most commercial vaccines rely on the generation of neutralizing antibodies against HA. However, inability of the neutralizing antibodies to cross-react with heterotypic viruses or even viruses with variants of the same HA subtype limits the efficacy of such vaccines in providing broad spectrum protection (114, 115). The humoral immune response is also generated against the more conserved M2 protein and antibodies against M2 have been shown to be cross- protective in mice (11, 33, 37, 116, 145).

$CD4^+$ T lymphocytes indirectly provide control of viral infection through the regulation of the B lymphocyte and $CD8^+$ T lymphocyte responses. They recognize antigenic peptides derived from exogenously expressed proteins presented in association with MHC-II molecules. MHC-II molecules are expressed only on professional antigen presenting cells (APC), such as dendritic cells, macrophages and B cells (30, 107). The professional APCs can internalize the viral antigens either by phagocytosis or endocytosis. These cells display the exogenously produced antigenic peptides in association with the MHC-II molecules.

$CD8^+$ T or cytotoxic T lymphocytes are responsible for clearance of virus infected cells (49). $CD8^+$ T cell receptors (TCR) recognize the specific antigenic peptide derived from endogenously expressed protein presented by MHC-I molecules expressed on the surface of APCs (14). MHC-I is expressed on the surface of most nucleated cells, including professional APC. The activation of the $CD8^+$ T cells requires the association of TCR with syngenic MHC-I molecule (54). In chickens, the MHC complex is defined within the B genetic locus. There are at least 30 inbred lines of chickens expressing distinct MHC haplotypes (Avian Immunology Research Group, October, 2000) hence chickens provide a rich immunogenetic source of well defined MHC compatible lines.

In addition to the interaction of the T cell receptors and the MHC-I presented antigen peptides, activation of naive $CD8^+$ T lymphocytes also requires the interaction of complementary accessory molecules on APCs, such as members of the B7 family of molecules present on B cells and APCs which engages with CD28 on T lymphocytes (74)(FIG. 1-2). Although the majority of studies investigating the cell mediated immune response against influenza viruses have been conducted in mice and humans, studies with infectious bronchitis virus, also a respiratory viral pathogen of chickens, have demonstrated that chicken immune responses closely follow the paradigms established with mammalian systems (23, 84, 97). Chicken $CD8^+$ T cell responses are also MHC-I restricted as demonstrated by cytolytic studies and adoptive transfer studies (84, 97, 99, 100, 102).

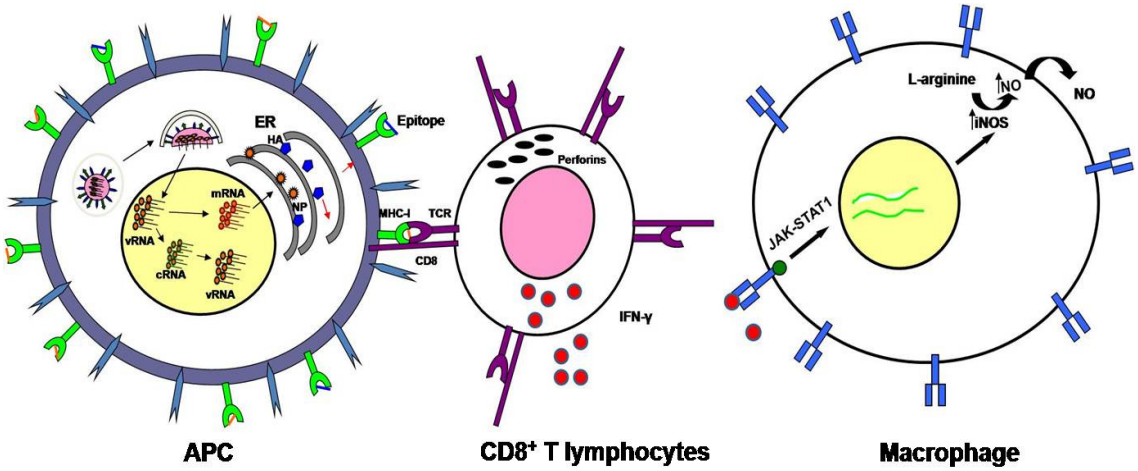

FIG. 1-2. Schematic of APC-CD8^{+} T lymphocyte-macrophage interaction

Following the acute infection of mice with influenza virus, dendritic cells in the lungs interact with $CD4^+$ T cells and by a CD40 dependent mechanism become activated, maturing into APCs expressing higher levels of MHC-I and MHC-II (49, 141, 142). Naive $CD8^+$ T lymphocytes are activated after their T cell receptors recognize the viral peptides presented by MHC-I in the mature dendritic cells (30, 74, 142). The activated T cells undergo extensive proliferation and differentiation leading to generation of functional effector cells (1). It has also been shown in mammals that the effector cells produce cytokines, such as interferon-γ (IFN- γ) and tumor necrosis factor (TNF-α), which activate other cells of the immune system, such as macrophages (1, 30). T cell mediated lysis of infected cells is mediated by perforins, granzymes and the Fas-Fas ligand pathway (1, 30). IBV clearance in chickens has been shown to immediately precede the peak of the effector T cell population, which is followed by the decrease in the population of viral specific T lymphocytes and accompanied by an increase in cell death or apoptosis (23). In chickens, adoptive transfer of effector $CD8^+$ T lymphocytes prepared at 7 to 10 days p.i. with AIV H9N2 protected naïve chickens against a challenge of highly pathogenic AIV H5N1 strain (102).

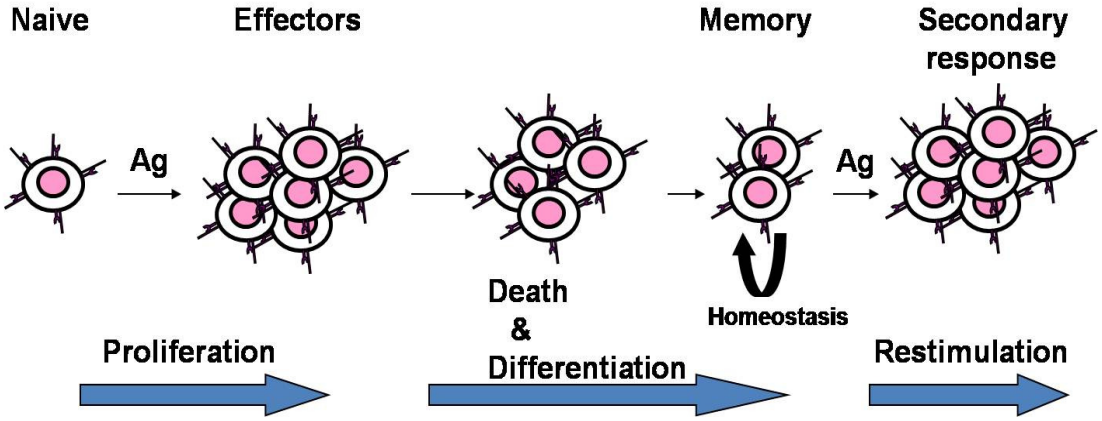

FIG. 1-3. Schematic of effector and memory T lymphocyte development

It has been shown in mammals that a small pool of antigen specific T lymphocytes persists and repopulate as memory T cells since they have the ability to respond efficiently to subsequent encounter with the antigen (1, 20, 64)(FIG. 1-3). In addition to their ability to recall an antigen specific response and the requirement for a lower threshold of activation, memory T cells in mice differ from naïve T cells and effector T cells in their phenotype display (1, 10, 64). Several cell surface markers, including CD44 and CD45, have been defined and investigated as memory cell markers in mammalian cells (1, 9, 10, 48, 133). These are adhesion molecules that can alter the homing pattern of activated T lymphocytes from that of naïve lymphocytes (48, 67). The upregulation of these surface molecules enables the memory $CD8^+$ T cells to infiltrate non-immune tissue when a recall response is required (27, 48, 67). Both CD44 and CD45 are present on chicken lymphocytes (74, 83).

Memory $CD8^+$ T cell responses in mice and humans have been shown to target multiple epitopes on influenza virus proteins. In mice, NP and PA are considered the dominant proteins against which cytotoxic T cell responses are directed (35, 82, 90, 128). However, the studies with humans have revealed that HA, M1 and M2 induce cytotoxic T cell responses in addition to NP (5, 41, 56, 68, 89). This diversity in response correlates with the greater avidity of MHC-I for antigenic peptides (28,122). Mice are not natural hosts of AIV and all the immunological characterization is based only on mouse adapted viruses. It is necessary to define the T lymphocyte mediated immune responses to AIV in chickens since they are pathogen of these animals and can be transmitted directly from chickens to humans. The $CD8^+$ T cell mediated immune

protection, unlike protection resulting from neutralizing antibodies, has the ability to cross protect against heterotypic viruses in mice and chickens (79, 91, 100, 102). It has also been demonstrated in chickens that the protective immunity against a variant AIV is related to the percentage of activated $CD8^+$ T lymphocytes present in the lungs (100).

VACCINES AGAINST AIV

Currently poultry in AIV endemic areas of the world may be vaccinated either with inactivated whole virus vaccine or with fowlpox vectored vaccines (17, 21, 22, 109, 122). These vaccines rely on generating viral antibody responses against the viral glycoprotein, HA and NA (17, 85). Although the use of conventional vaccines reduce the susceptibility of the animal to the infection and shedding of the virus, the major limitation of depending on the induction of antibodies specific for HA and NA is that they are not effective against viral strains with a different or variant HA NA subtype. Therefore, such vaccine strategies, in the absence of effective cross protection, require continuous development of new relevant vaccines that address the threats of new strains (17, 31). Vaccines aimed at generating neutralizing antibodies against viral HA have been helpful in the control of AIV but subsequent immune pressure has been shown to be instrumental in the evolution of evasive virus against which pre-existing antibodies are not effective. In contrast, immune responses mediated by the $CD8^+$ cytotoxic T cells target more conserved epitopes than those required to stimulate humoral immune response and hence have the potential to cross-react with various viral subtypes (121). NP, a highly conserved protein of the influenza A viruses, has been shown to house

epitopes for $CD8^+$ T cells response in mice (147). The $CD8^+$ T cell mediated responses against this protein have been shown to cross protect against heterologous influenza virus in mice (147). Although protective immunity against AIV may rely on neutralizing antibody responses to homologous HA protein, the response of effector and memory $CD8^+$ T lymphocytes can diminish disease preventing mortality, and even morbidity (1).

Another limitation to the efficacy of whole virus vaccine is the inability to distinguish infected from vaccinated animals (DIVA) since both have antibodies against the whole virus (17). In the event of sub-clinical infection the animal may continue to shed the virus despite the presence of antibodies (17). This has implications in trade and export of birds since movement of infected poultry can spread the virus hence it is important to develop vaccines which permit DIVA (17, 21, 22). The advantage of viral vectored vaccines over whole virus vaccines is the ability to DIVA (16). The AIV specific antibodies induced by the viral vector are limited to the encoded recombinant AIV protein. Recombinant TROVAC-H5 fowlpox HA vaccine has been licensed for use in USA (21, 22). In addition to inducing longer lasting immunity this vaccine permits DIVA (17). However pre-exisiting immunity to fowlpox virus in poultry interferes with the ability of this vectored vaccine to induce optimum protective response (17, 114).

A non-replicating human adenovirus vector encoding HA from H5N9 AIV (AdTW68.H5) has been developed and found to elicit an effective humoral immune response that, when given in ovo, protected chickens against highly pathogenic virus challenges (124, 125). Besides the absence of pre-existing immunity to human adenovirus in chickens, another advantage of this non-replicating vector is that it is safer

than a replication competent vector. In mice, recombinant adenovirus vaccine vectors have been shown to generate effective $CD8^+$ T cell immune responses with the potential to cross protect from heterologous viral challenge (38, 50, 51, 53).

CHAPTER II

AVIAN INFLUENZA VIRAL NUCLEOCAPSID AND HEMAGGLUTININ PROTEINS INDUCE CHICKEN CD8$^+$ MEMORY T LYMPHOCYTES

INTRODUCTION

Avian influenza viruses (AIV) belong to *Orthomyxoviridae* family of viruses and have segmented, negative sense RNA genomes. These viruses, natural infectious agents of waterfowl and shorebirds, are classified according to their transmembrane hemagglutinin (HA) and neuraminidase (NA) glycoproteins (3, 60, 81, 138). All 16 hemagglutinin (HA) and 9 neuraminidase (NA) types have been isolated from waterfowl or shore birds (36, 60, 131). Due to their incredibly broad avian host range, AIV strains have been isolated from many different species of birds including ducks, gulls, geese, psittacines and poultry (3, 81). Depending on the virulence of the virus in the poultry, AIV isolates are classified as either low pathogenic (LP) or highly pathogenic (HP) (3, 21 22). LPAIV strains cause asymptomatic to mild respiratory and enteric tract infections while the highly pathogenic strains cause clinical illness and systemic infections. Infections of poultry by the highly pathogenic strains result in especially severe economic losses (17, 27, 47, 122). Human influenza viruses, including those causing high morbidity and significant mortality, such as the H1N1 from 1918 pandemic have been shown to have avian origins (2, 16, 119). Since 1996, highly pathogenic H5N1 AIV strains isolated in Hong Kong have been infecting and subsequently causing deaths in humans, although person-to-person transmission is apparently rare (2, 16, 85,

129).Poultry are the logical intermediate host for adaptation of the viral strains from wild birds to humans and other mammals, such as swine (136, 137). Indeed, human adapted strains have been shown to consist of genome segments of avian, swine and human origin (136, 137, 138).

Vaccination efficacy is traditionally determined by the demonstration of protective humoral immunity, especially targeting AIV HA and putative neutralization of viruses (21, 22, 110, 111, 115). Whereas humoral immunity of chickens to AIV is well characterized, little information is available regarding the more difficult to evaluate, virus specific T cell immune responses (65, 102, 115).

With the availability of a number of poultry lines with defined MHC (located within the chicken B locus), the chicken is one of the few animals for which adaptive T lymphocyte responses can readily be evaluated. T cells have been stimulated ex vivo with known MHC matched chicken kidney cells (CKC) serving as non-professional antigen presenting cells (APCs) and by the adoptive transfer of MHC matched T lymphocytes to naïve chicks prior to viral challenge (23,84, 99, 101). Studies targeting acute infections with a strain of infectious bronchitis virus (IBV), an avian coronavirus, have identified specific $CD8^+$ T cell responses (98, 101). Adoptive transfer of either effector T cells prepared from birds 10 days post-infection (p.i.) or of memory cells prepared from birds 3 weeks after infection with IBV, provided protection against acute disease after viral challenge (84, 94). Following infection with H9N2 AIV, Seo et al (100) described $CD8^+$ T cell responses that correlated with cross-protection to an H5N1 strain. Protection by effector $CD8^+$ T lymphocytes prepared at 7 to 10 days p.i. with

AIV was demonstrated following adoptive transfer one day prior to AIV challenge (102). However, these studies did not identify the viral proteins harboring T lymphocytes epitopes nor described the kinetics of the memory response to AIV.

Whereas sterile immunity may depend on humoral responses to homologous HA and, to a lesser extent, homologous NA, effector and memory $CD8^+$ T cell immunity in mice has been shown to diminish disease preventing mortality, and even morbidity (91, 100, 102). The kinetics of the avian AIV specific memory response has not been defined nor have the avian T cell responses to individual AIV proteins been determined. Although DNA plasmids expressing HA protein from AIVs have been used to generate neutralizing antibody to homologous AIV, the ability of vector-expressed AIV proteins to generate specific T cell responses in chickens has not been examined.

This study describes the AIV HA and/or NP protein memory responses of peripheral blood T lymphocytes from chicks inoculated with plasmids vectors. Responses were evaluated following ex vivo stimulation with MHC-matched or mismatched APCs. Both NP and HA induced AIV specific memory T lymphocyte response between 3 to 9 weeks p.i. Although the T lymphocyte response induced by NP was consistently higher than the response induced by HA until 7 weeks p.i., no differences were detected by 9 weeks p.i.

MATERIALS AND METHODS

Viruses: Low pathogenic AIVs, H5N9 (A/Turkey/Wis/68) and H7N2 (A/Turkey/Virginia/158512/02), were propagated in the allantoic sacs of 10 day-old

embryonated chicken eggs (ECE). The allantoic fluid was harvested 48 hours p.i.and examined for presence of virus by hemagglutination activity (HA) test according to the OIE guidelines (http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf). Viruses were titrated in ECE and titers expressed as embryo infectious dose 50 (EID_{50}) (7).

Experimental Animals: Embryonated eggs of MHC-defined B19/B19 and B2/B2 lines of chickens were obtained from Dr. Briles' laboratory at Northern Illinois University (DeKalb, IL). After hatching, chicks were housed in a specific pathogen free environment at the vivarium facility, Western University of Health Sciences, Pomona, CA. Viral infection studies in chickens were conducted at the biosafety level 2 Lab Animal Research Resource animal facility, Texas A& M University, College Station, TX. All procedures involving the use of chickens were approved by and conducted according to guidelines established by the Institutional Animal Care and Use Committees of Western University of Health Sciences and/or Texas A&M University.

Cloning of NP and HA into a Eukaryotic Expression Plasmid: RNA from H5N9 (A/Turkey/Wis/68) was extracted from allantoic fluid using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. First strand cDNA was synthesized with ImProm-II™ Reverse Transcriptase(Promega, Madison, WI) using AIV specific Universal 12 primer (5'AGCA/GAAAGCAGG 3')(130). PFU polymerase (Stratagene, La Jolla, CA) was used to amplify the open reading frames (ORF) of HA

and NP using specific primer pairs HA-Forward5'-ACCATGGAAAGAATAGTGATT-3'and HA-Reverse 5'-GATGCAAATTCTGCA-3' and NP-Forward 5'-ACCATGGCGTCTCAAGGCACC-3' and NP-Reverse 5'-ATTGTCATACTCCTCTGC-3', respectively. Taq DNA polymerase (New England Biolabs, Ipswich, MA) was then used to add TA overhangs on the Pfu amplified PCR product. The amplified cDNA products were cloned into the eukaryotic expression vector pcDNA3.1/V5-His-TOPO TA (Invitrogen, Carlsbad, CA). Cloned gene segments were sequenced using ABI Big Dye (Applied Biosystems, Foster City, CA) at GenoSeq, UCLA, Los Angeles, CA to confirm the sequence of the ORF. In order to confirm the in vitro expression of NP and HA proteins, an indirect immunofluorescence assay (IFA) was performed following plasmid transfection of CHO-K1 cells. Known chicken serum, positive for AIV (NVSL, Ames, IA) was used at a dilution of 1:100. Mouse anti-chicken IgG FITC at a dilution of 1:500 (Southern Biotech, Birmingham, AL) was used as the secondary antibody.

Generation of APCs: Primary CKC lines were established from 10 day-old chicks of B19/B19 and B2/B2 MHC haplotypes as described previously (98). CKC of the tenth passage were used as non-professional APCs for the stimulation of the $CD8^+$ T lymphocytes. The presence of MHC-I on CKC lines was confirmed by flow cytometric analysis, using anti-chicken MHC-I R-phycoerythrin conjugated monoclonal antibodies (MAbs) (Southern Biotech, Birmingham, AL).

Inoculation of Birds: Three-week old specific, pathogen-free chickens of the B19/B19 MHC haplotype were inoculated intramuscularly (i.m.) with 500 µg of cDNA expressing HA alone, or NP alone, or of 500µg of each HA and NP (HN). Control birds were inoculated with either pcDNA 3.1 vector expressing LacZ (LacZ) or PBS. For viral inoculations, B19/B19 chicks were inoculated at 3 weeks of age, intranasally, with 10^8 ELD_{50} of the low pathogenic H5N9/Tur/Wis/68 AIV strain.

AIV- Specific Antibody Titrations: Serum samples were prepared from blood collected from the jugular vein of chickens at 3 weeks p.i. to evaluate the humoral responses against AIV HA and NP. Hemagglutination inhibition (HI) assays, according to OIE guideline (http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf), were used to identify antibodies specific to H5N9 virus (A/Turkey/Wis/68) HA. HI mediated by the anti-H5 antibodies against H7N2 AIV was also evaluated. Titers were expressed as geometric mean titers (GMT). Titers of ≤ 1 $\log_2$were assigned a titer of 1 $\log_2$.NP specific antibodies were determined using the AIV Plus ELISA kit (Synbiotics, Kansas City, MO) as described by the manufacturer.

Effector Cell Preparation: Effector T lymphocytes used for ex vivo stimulation, were prepared from the peripheral blood mononuclear cells (PBMC) of from 2 to 4 chickens per group (84). Briefly, blood was collected from the jugular vein at 3, 5, 7 and 9 weeks p.i. and diluted 1:2 in Alsever's solution (Sigma-Aldrich, St. Louis, MO). PBMC were prepared by Ficoll-histopaque (Histopaque-1077, Sigma-Aldrich, St. Louis,

MO) density gradient centrifugation (98). Viable cells were collected from the interface and washed twice with phosphate buffered saline (PBS, pH 7.4). Cells were resuspended in 3 ml of RPMI 1640 (Invitrogen, La Jolla, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine, and 0.1mM MEM non-essential amino acids. B lymphocytes were removed by passing the cell suspension through a complete RPMI equilibrated nylon wool column and adherent cells were removed by incubating the cell preparation in 25 cm^2 tissue culture flasks as described previously (98).

Ex vivo Stimulation of T Lymphocytes: T lymphocytes from PBMC were stimulated ex vivo with MHC B19/B19 (matched) and B2/B2 (mismatched) APCs. APCs at a concentration of 1 x 10^5cells/ml were incubated for 8 hours at 39ºC, 5% CO_2 in 96-well tissue culture plates. APCs were infected with 1x10^5 ELD_{50} of H5N9 (A/Turkey/Wis/68) virus for 1 hour followed by removal of the virus and cells were washed 3 times with DMEM supplemented with 10% FBS. One x 10^6 T lymphocytes in complete RPMI were added to each well. Cells were co-cultured for 24 hours, before the media was collected and centrifuged (see FIG. 2-1 on page 28). The clarified supernatants were used to quantify IFNγ production by activated T lymphocytes using a nitric oxide detection assay. At 5 weeks p.i. the pelleted T lymphocytes were collected for FACS analysis to measure the lymphocyte proliferation. Each ex vivo stimulation assay was conducted in duplicate.

Nitric Oxide Induction Assay: In the absence of a commercial ELISA assay and the MHC-I tetramer-peptide based technology the chicken immune system, a modified indirect IFNγ assay based on NO production(24,57, 83) from HD11 cells (a chicken macrophage cell line) was used to demonstrate the ex vivo activation of T lymphocytes by APCs. Briefly, cells were incubated in individual wells of 96-well plates at a concentration of $1x10^5$ cells/well in complete RPMI media for 2 hours at 39ºC, 5% CO_2 prior to the addition of 150µl supernatants from T lymphocyte-APCs cultures. After 24 hours of incubation, the accumulation of nitrite from stimulated HD11 cells was measured using the Griess reagent assay according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO) (FIG. 2-1) The concentration of nitrite produced was determined using sodium nitrite solutions with a concentration of 1-20 µmoles as standards. To ensure that the measured nitric oxide was produced by the IFNγ mediated stimulation of HD11 cells and not due to other soluble inducing factors, nitrite concentration in each sample was normalized by subtracting the nitrite concentration of supernatants from APCs cultured without T lymphocytes from the supernatants of the APCs cultured with T lymphocytes.

FACS Analysis: After ex vivo stimulation with AIV infected APCs, T lymphocytes were collected and dual labeled with phycoerythrin-conjugated MAbs specific for CD44 and fluorescein labeled MAbs specific for either CD8 or CD4 (Southern Biotech Birmingham, AL) as previously described (98). Flow cytometric analysis was used to determine the concentration of T lymphocyte subpopulations. A minimum of 10^4 events were collected for each sample. The percentage of $CD44^+$lymphocytes expressing either CD4 or CD8 surface antigen was determined using FlowJo™ (TreeStar, Inc., Ashland, OR). Cell proliferation was calculated as the percent increase in the population of $CD4^+$ or $CD8^+$T lymphocytes cultured with uninfected APCs after in vitro stimulation by virus-infected APCs for 24 hours.

Statistical Significance of Differences: The nitric oxide concentrations were expressed as average of 4 to 6 birds per group. ANOVA (analysis of variance) with significance of $p < 0.05$ was used to determine statistical differences.

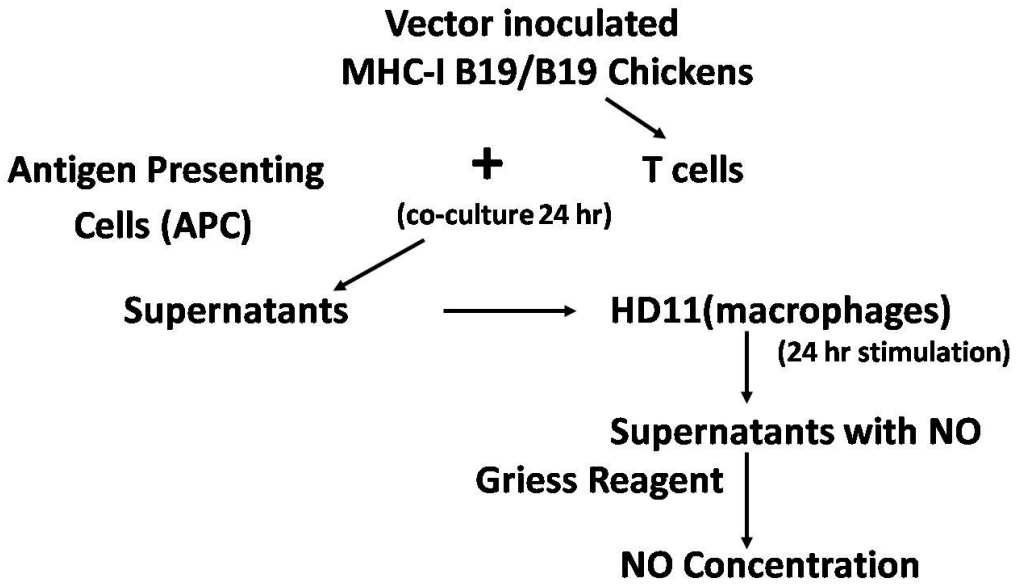

FIG. 2-1. Schematic of $CD8^+$ T lymphocyte ex vivo stimulation assay

RESULTS

In vitro Expression of AIV Proteins: In order to determine the T lymphocyte responses to individual AIV proteins, the NP and HA genes of the low pathogenic H5N9 (Turkey/Wis/68) strain were cloned into the pcDNA3.1/V5-His-TOPO TA vector (Invitrogen, Carlsbad, CA). The eukaryotic expression of the proteins encoded by the plasmids was determined by IFA in CHO-K1 cells 48 hour post-transfection with plasmids expressing either NP or HA (FIG. 2-2). AIV positive chicken polyclonal serum (NVSL, Ames, IA) at a dilution of 1:100 was used as primary antibody and FITC conjugated mouse anti-chicken IgG at a dilution of 1:500 (Southern Biotech, Birmingham, AL) was used as the secondary antibody to detect the presence of protein expression. CHO-K1 cells transfected with either NP or HA expressing plasmids exhibited the presence of fluorescence while no fluorescence was observed in cells transfected with plasmid encoding for LacZ.

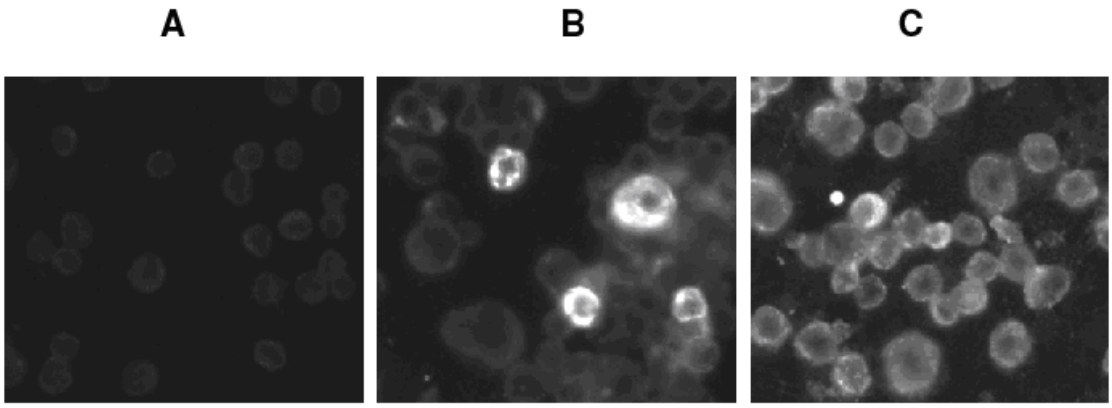

FIG. 2-2. In vitro expression of pcDNA3.1/V5-His-TOPO TA vectored AIV proteins in transfected CHO-K1 cells (magnification, 200x). Expression was detected using IFA using AIV positive reference serum as the source of primary antibodies. Cells were transfected with plasmids expressing (A) LacZ, (B) HA, and (C) NP.

Humoral Immune Response: The in vivo expression of AIV HA and NP and the antibody response to these proteins in the chickens was confirmed by detecting the presence of antibodies specific for HA and NP at 3 weeks p.i. with the plasmids. Serum HI assay was used to confirm the expression of HA. HI titers of sera from 6 chickens inoculated with the HA expression plasmid using the homologous H5N9 AIV strain was found to range from 5 to 6.5 $\log_2$ (GMT) (FIG. 2-3A). No HI activity was detected in sera of the 4 chickens inoculated with either PBS or NP alone (data not shown). Sera from HA inoculated birds failed to inhibit the hemagglutinating activity of a heterologous H7N2 AIV strain. The in vivo expression of NP following inoculation of the chickens with the plasmid was confirmed by a commercial ELISA. The ELISA titers for antibodies against AIV NP in 6 NP expressing plasmid inoculated chickens was found to range between 2.5 to 3.0 $\log_{10}$ (GMT) at 3 weeks p.i. (FIG. 2-3B). The sera from PBS inoculated birds were negative for the presence of anti-NP antibodies. The sera from HA plasmid inoculated chickens were also negative for the presence of anti-NP antibodies (data not shown). Known chicken polyclonal serum positive for AIV was used as a positive control for validation of all titration assays (data not shown).

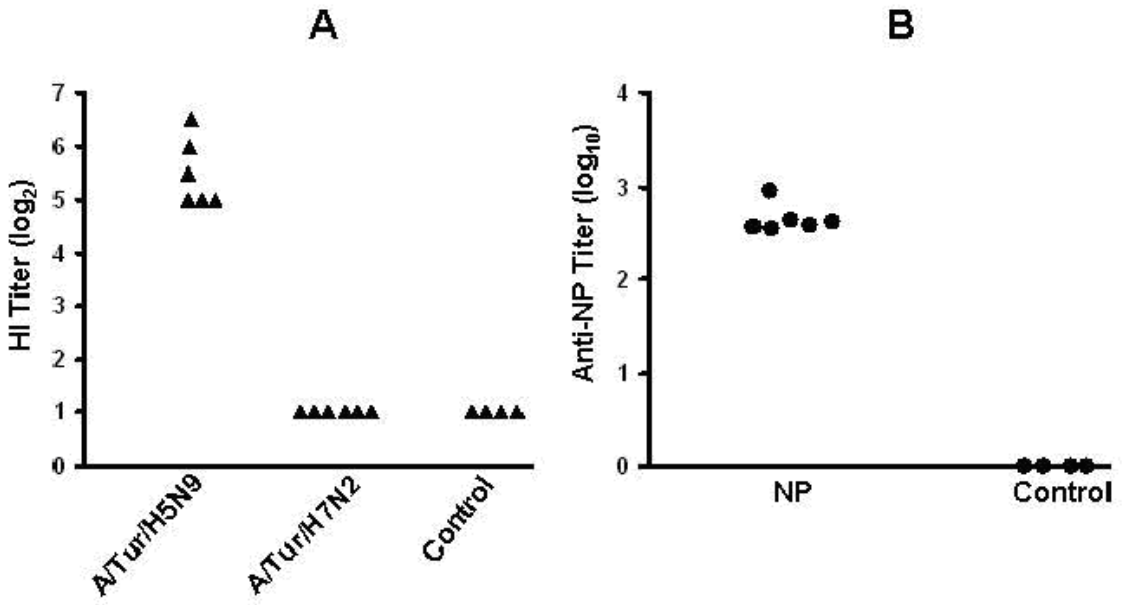

FIG. 2-3. Antibody titers induced in individual birds (n=6) by NP and HA expressing plasmids at 3 weeks p.i. (A) Serum HI antibody titers from chickens inoculated with HA expressing plasmid against the homologous H5N9 AIV strain and heterologous H7N2 AIV strain. (B) Serum anti-NP antibody ELISA titers from chickens inoculated with the NP expressing plasmid. Neither HI nor anti-NP antibodies were detectable in the sera of 4 control birds inoculated with PBS. Each symbol represents the response of an individual chicken.

***Memory T Cell Responses were Detected from Weeks 3 to 9 p.i. with HA and NP Plasmids*:** Because the T cell specificity for individual proteins have not previously been reported, T lymphocyte responses to HA, NP or a combination of both HA and NP (HN) were determined following inoculation of B19/B19 chickens with plasmids expressing the HA or NP ORF. Since the conventional ELISA or intracellular cytokine staining methods to examine T lymphocyte mediated responses are not well established for research with avian immune system an indirect IFNγ assay (NO production from HD11 cells, a chicken macrophage cell line) was standardized for evaluating the ex vivo activation of T lymphocytes by APCs. Considering previous adoptive transfer studies, which identified specific memory T cells to IBV by 3 weeks p.i. with maximal responses occurring between weeks 5 and 6 p.i. (84), the memory AIV response of peripheral blood memory T lymphocytes were evaluated between 3 to 9 weeks p.i. with HA, NP, or HN plasmids.

CKC infected with AIV were used as non-professional APCs for the stimulation of T lymphocytes. Following ex vivo co-culture with MHC matched B19/B19 APCs infected withH5N9 virus, memory responses were detectable in T cell preparations obtained from all chickens receiving plasmids expressing both AIV proteins by 3 weeks p.i. Since neither supernatants from the T cells cultured with uninfected APCs nor T cells from PBS inoculated birds cultured with infected MHC matched B19/B19 birds produced IFNγ (data not shown), the memory T lymphocyte activity was considered AIV specific. Likewise, the memory T lymphocyte responses from each group of chickens receiving AIV plasmids was highly MHC restricted, since the ex vivo

stimulation of the T cells from plasmid inoculated birds with MHC mismatched B2/B2 APCs could only induce basal levels of NO (FIG. 2-4).

The maximum memory T cell response to NP was detected at 5 weeks p.i. However, while still detectable, memory T cells responses at 9 weeks p.i, were dramatically diminished for all birds receiving the AIV expression plasmids (HA, NP or HN). During weeks 3 through 7, the activity of T cells from the HA plasmid inoculated birds was significantly less than that of T cells isolated from birds receiving either NP or NP plus HA plasmids (FIG. 2-4). In addition to weaker APC induced stimulation, the levels of HA specific memory T lymphocyte responses were similar at weeks 3, 5 and 7 p.i. The kinetics and the magnitude of the response mediated by the T lymphocytes derived from chickens inoculated with the combination of both NP and HA (HN) expressing plasmids was similar to the response mediated by the T lymphocytes derived from chickens inoculated with NP alone.

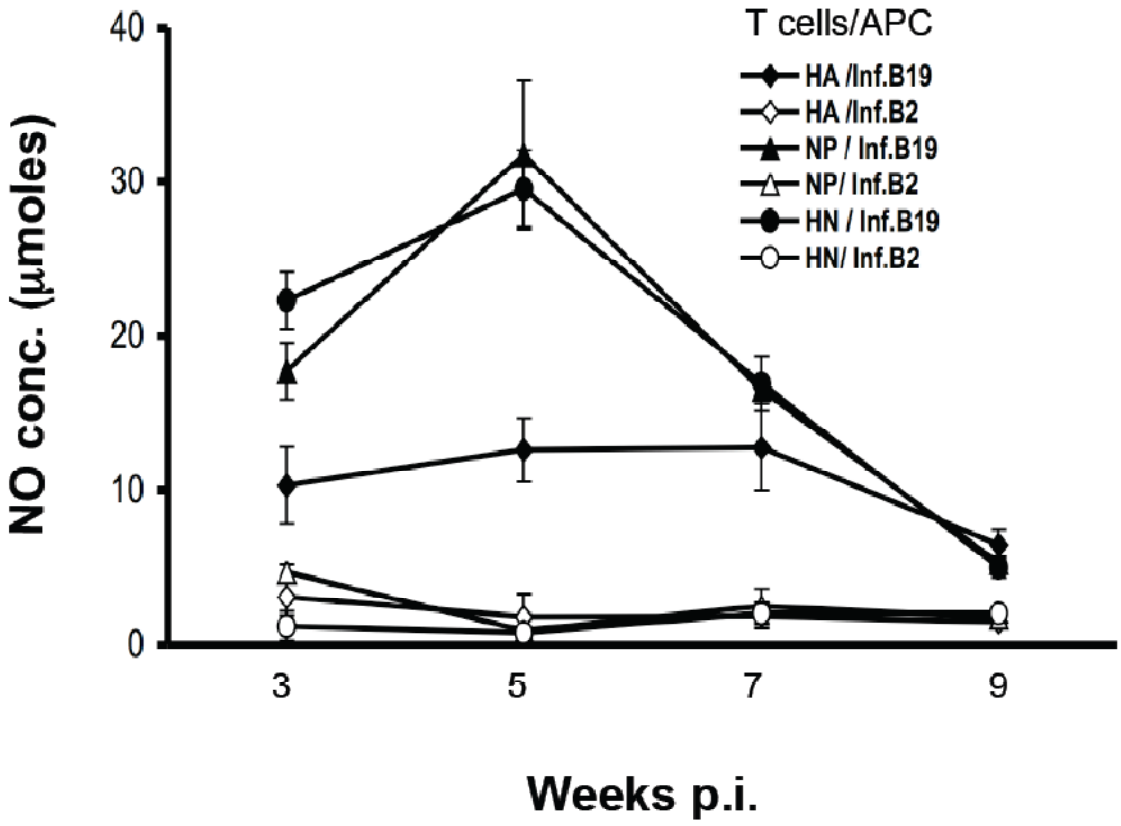

FIG. 2-4. Chicken memory T lymphocyte responses to AIV HA and NP proteins between 3 and 9 weeks p.i. with NP and/or HA expression plasmids. Chickens of the B19/B19 MHC haplotype were inoculated with DNA plasmids expressing AIV HA, NP or both HA and NP (HN). Memory T lymphocytes were stimulated ex vivo with virus infected MHC matched B19/B19 and mismatched B2/B2 APCs. Production of NO by HD11 macrophage cells induced by the the secretion of IFNγ from stimulated T lymphocytes was used to quantify lymphocyte activation. Results, expressed as the average (± S.E.) of two separate experiments. Each ex vivo stimulation assay is denoted by the source of T lymphocyte and virus infected MHC-I APCs. The difference in stimulation by matched and mismatched APCs was significant (p=0.003-0.02) for each inoculated antigen and time point. The responses to HN (p=0.02) at 3 weeks and NP (p=0.02) and HN (p =0.006) at 5 weeks p.i. were significantly greater than the responses to HA at the same time points. The p value for the difference between responses to NP and HA at 3 weeks p.i.was 0.07.

***$CD8^+$ T Lymphocyte Populations Increase with ex vivo Stimulation*:** The phenotype of activated subpopulations of T lymphocytes following co-culture with APCs expressing AIV antigens was determined using flow cytometric analyses (Table 2-1). Since the response of T lymphocytes from HN inoculated chickens would reflect the response to the whole virus more closely than in NP or HA inoculated chickens, the proliferation of the lymphocytes from HN inoculated groups was measured at 5 weeks p.i. Lymphocyte populations were gated using a chicken pan lymphocyte CD44 specific MAb and MAbs specific for either CD4 or CD8 T cell antigens (98). The relative increase in the population of $CD8^+$ T lymphocytes harvested from each HA and NP (HN) plasmid inoculated chicken was between 62 and 91% following ex vivo stimulation with AIV expressing APCs, in contrast to the increase of 31 to 37 % in the $CD4^+$ T lymphocyte population. The increase in the population of T lymphocytes harvested from PBS inoculated birds was 7% and 1% for $CD8^+$ and $CD4^+$ T lymphocyte subpopulations, respectively, following co-culture with AIV infected APCs. The preferential increase in $CD8^+$ cells correlates with their anticipated expansion following exposure to non-professional APCs endogenously expressing AIV and expressing surface MHC-I.

Table 2-1. Proliferation of T lymphocytes from birds 5 weeks p.i. with plasmids using flow cytometry[1].

		$CD4^+$ T lymphocytes (% of T cells)			$CD8^+$ T lymphocytes (% of T cells)		
Bird #	Inocula[2]	Uninfected[3]	Infected[4]	% Increase[5]	Uninfected	Infected	% Increase
1	HA+NP	47.8	65.1	36	20.0	36.4	81
2	HA+NP	44.0	59.5	35	24.7	47.1	91
4	HA+NP	41.3	56.6	37	27.0	43.7	62
5	HA+NP	46.0	60.5	31	22.6	40.4	79
7	PBS	53.8	60.8	1	22.4	24.0	7

[1] Data representative of 2 experiments
[2] HA+NP-Birds inoculated with 500 µg plasmids expressing HA and NP (HN) or PBS only
[3] Population of T lymphocytes after stimulation with uninfected APCs
[4] Population of T lymphocytes after stimulation with infected APCs
[5] Increase in the population of T lymphocytes following stimulation by APCs expressing AIV

***T Cell Responses Cross React with a Heterologous H7N2 AIV Strain*:** A rationale for targeting cellular immunity is the potential for cross-reactivity between vaccine and heterologous viruses. At 8 weeks p.i. the capacity for memory T lymphocytes specific for the NP and HA proteins of the H5N9 strain to be stimulated with an H7N2 (A/Turkey/Virgina/158512/02) strain of AIV was determined following co-culture with MHC matched APCs infected with either AIV strains (FIG. 2-5). Both heterologous H7N2 and homologous H5N9 AIV infected APCs significantly stimulated IFNγ production from memory T lymphocytes isolated from either HA or NP inoculated chickens ($p \leq 0.01$) compared with PBS inoculated chickens. Although the difference between the response to homologous and heterologous AIV to either protein was not significant, the magnitude of the response of T lymphocytes to both HA and NP following stimulation with the homologous H5N9 AIV was observed to be greater. Regardless of the strain used to infect APCs, the observed memory responses generated by T cells obtained from chickens receiving the NP plasmid were again statistically greater ($p=0.007$) than that generated by T cells harvested from HA plasmid inoculated chickens.

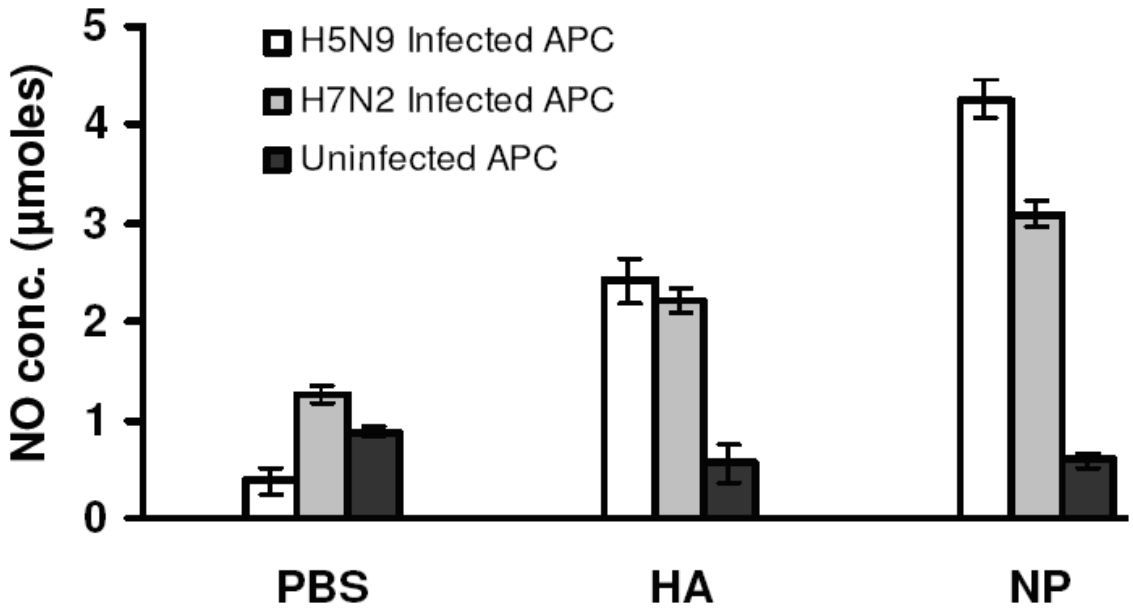

FIG. 2-5. T lymphocytes from B19 birds inoculated with either H5N9 derived HA or NP expression plasmids respond to a heterologous (H7N2) virus. At 8 weeks p.i., T lymphocytes from chickens receiving either HA or NP cloned from the H5N9 strain were cultured with APCs infected with H5N9 or H7N2 viruses. The T cell responses are expressed as the average (± S.E.) of NO production for each treatment group. T lymphocytes from plasmid-inoculated chickens had significantly higher responses to H7N2 AIV strain than those from the PBS control group ($p \leq 0.01$). Three chickens were used for each stimulation assay.

***Similar Memory T Lymphocyte Responses were Observed with Low Pathogenic AIV Infection*:** Memory T cells could be readily detected between 3 and 7 weeks p.i. with plasmids expressing either the NP or the HA proteins. In order to evaluate the memory T lymphocyte responses of chickens inoculated with infectious AIV, chicks with the B19/B19 haplotype were inoculated with the low pathogenic H5N9/Turkey/Wis/68 strain and blood was collected at 5 weeks p.i. The ex vivo activation of T lymphocytes by AIV infected APCs was determined by the indirect IFNγ assay (FIG. 2-6). The mean average NO production induced by the ex vivo stimulation of T lymphocytes from infected birds with B19/B19 APCs was specific compared with the uninfected, MHC matched APCs ($p=0.004$). The responses to AIV infected APCs were MHC restricted as demonstrated by only basal level activation by B2/B2 APCs.

Flow cytometric analysis was used to determine the phenotype of the T lymphocyte subpopulations from the infected chickens responding to ex vivo stimulation. The relative increase in the population of $CD8^+$ T lymphocytes from H5N9 infected chickens was 46 to 95 % while the increase in the $CD4^+$ T lymphocyte population ranged from 6 to 28% following co-culture with MHC-1 matched, H5N9 AIV infected APCs (Table 2-2). The increase in the population of the lymphocytes from uninfected chickens was only 1 to 14% and 10 to 19% for $CD8^+$ and $CD4^+$ T lymphocytes, respectively. Although increases were observed in the CD4 lymphocytes, the greater increased proliferation of $CD8^+$ lymphocytes from birds infected with the low pathogenic virus was consistent with detection of a preferential MHC -I restricted AIV specific, $CD8^+$ memory T cell response.

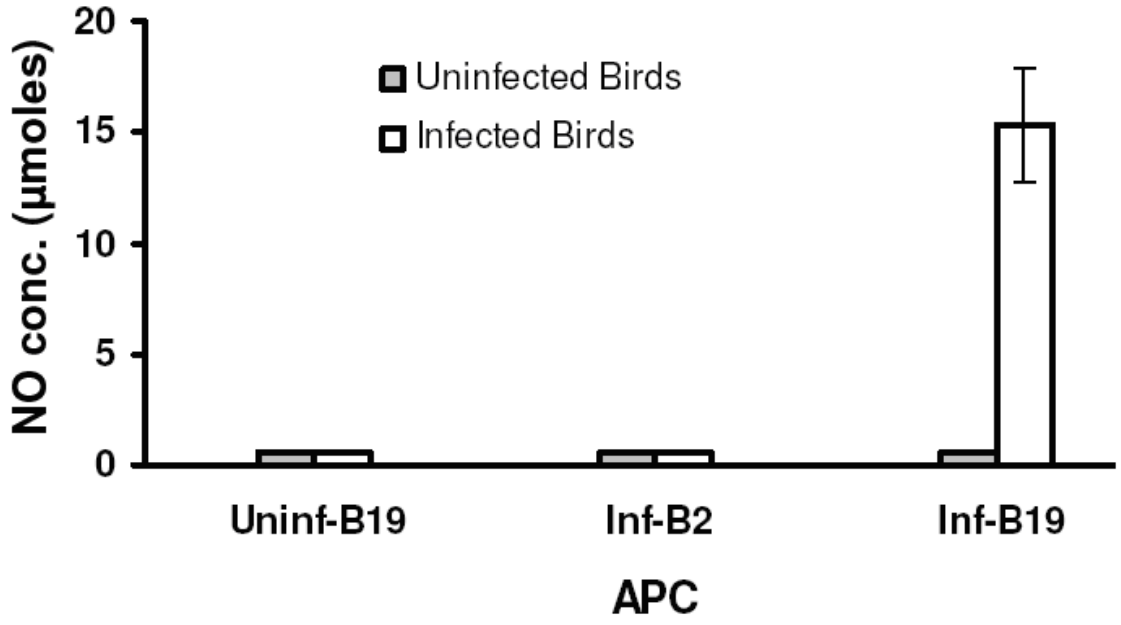

FIG. 2-6. In vivo infection of B19 chickens with the low path H5N9/Tur/Wis/68 AIV generates AIV specific, MHC matched memory T lymphocytes. The potential for infectious AIV to also produce a memory T lymphocyte response was determined 5 weeks p.i. with H5N9. Mean (± S.E.) NO production by each treatment group is represented by the bars. Stimulating APCs were either uninfected (Uninf) or virus-infected (Inf). Mismatched AIV infected APCs derived from the CKC of homozygous B2 chicks indicate MHC restriction. Three birds were used for each ex vivo stimulation assay.

Table 2-2. Proliferation of T lymphocytes from birds 5 weeks p.i. with AIV using flow cytometry.

	$CD4^+$ T lymphocytes (% of T cells)			$CD8^+$ T lymphocytes (% of T cells)		
Bird #	Uninfected[1]	Infected[2]	% Increase[3]	Uninfected	Infected	% Increase
I-1[4]	40.7	52.1	28	16.3	31.7	95
I-2	48.3	58.4	21	12.1	20.5	69
I-3	49.6	60.9	23	17.8	25.9	46
I-4	55.9	59.5	6	18.5	31.7	71
I-5	43.9	53.6	22	9.55	18.5	94
C-1[5]	42.5	50.7	19	27.9	28.1	1
C-2	39.2	43.1	10	10.8	12.3	14

[1] Population of T lymphocytes after stimulation with uninfected APCs
[2] Population of T lymphocytes after stimulation with infected APCs
[3] Increase in the population of T lymphocytes following stimulation with APCs expressing AIV
[4] I-Chickens infected with H5N9/Turkey/Wis/68 AIV
[5] C-Control chickens inoculated with PBS

DISCUSSION

To our knowledge, this is the first study delineating the response of chicken memory $CD8^+$ T lymphocytes to specific AIV proteins. Studies evaluating the $CD8^+$ T lymphocyte response to influenza virus in mice have identified NP as housing the dominant $CD8^+$ T cell epitopes (35,61, 82, 126, 134, 146). In contrast, human cytotoxic T lymphocytes (CTL) have a broader repertoire and the response is directed to multiple influenza viral proteins, including HA (39, 52, 58, 62). Our studies have shown that similar to the responses in humans, memory $CD8^+$ T lymphocytes in chickens are directed against both AIV HA and NP proteins (41, 56, 62, 77). Significantly greater responses were induced by NP than by HA at 3 and 5 weeks p.i. The AIV specific T cell responses were primarily MHC-I restricted as non-professional APCs of B19 and B2 haplotypes were used for ex vivo stimulation of T cells and the APCs of B2 haplotype chickens either failed or could only weakly stimulate the T lymphocytes derived from the B19 line. Therefore, responding T cells were primarily of $CD8^+$phenotype, which also showed significantly greater proliferation than $CD4^+$ T lymphocytes in response to ex vivo APC mediated stimulation. Similar to the MHC-I restricted T lymphocyte responses demonstrated following infection with IBV, $CD8^+$ T cell memory responses to AIV HA and/or NP were detected by 3 weeks p.i. (84).

Furthermore, the current studies quantified the protein specific responses until 9 weeks p.i. The response increased from 3 weeks p.i. until 5 weeks p.i. However, by 9 weeks p.i. with plasmids expressing either NP or HA AIV protein, the memory T cell activity had declined to significantly lower levels. The decline in the more vigorous

$CD8^+$ T lymphocyte response to NP was more rapid after 5 weeks than the $CD8^+$ T cell response stimulated by HA, such that by 9 weeks p.i. the responses to both proteins individually or in combination were similar. A decline in the $CD8^+$ T lymphocyte mediated protection at 10 weeks after challenge of H9N2- infected chicks with H5N1 had been observed by Seo and Webster (102). Similarly, a decline in the memory T lymphocytes response specific to influenza virus infection was also reported in humans (75).

Our study also proved the efficacy of the plasmid delivery approach in providing a mechanism to evaluate the T cell response to AIV HA and NP proteins, either individually or in combination. Protection studies were not included following inoculation of the AIV plasmids because of increased biosafety requirements for AIV. However, hemagglutinating antibodies which can be correlated with protection were demonstrated. The antibodies specific for the HA cloned from a H5N9 virus failed to prevent H7N2 virus-mediated hemagglutination.

The limitation posed by the frequency of bleeding and the volume of blood available precluded our ability to conduct all the assays at the same time points hence the cross-reactivity assay was conducted at 8 weeks p.i. Although the response of the memory T lymphocytes was significantly lower than the response at 5 weeks p.i., it was still detectable and the APCs infected with H7N2 AIV were able to ex vivo stimulate the T lymphocytes from H5 inoculated chickens. These observations indicate that despite the absence of shared HI antibody epitopes, HA does have at least one CD8+ epitope that is shared between both the strains of the virus.

Adoptive transfer studies of $CD8^+$ T lymphocytes specific for IBV and AIV have demonstrated their importance in protection against heterologous viruses (84, 99, 102). In mice, adoptive transfer of memory $CD8^+$ T lymphocytes against influenza virus NP has also been shown to be protective against viral challenge (69, 71, 120). *In vivo* inoculation of chicks with a DNA plasmid expressing the IBV nucleocapsid protein was shown to provide CTL mediated protection against acute respiratory disease (97,101). Consistent with the greater response to NP from the homologous virus, T lymphocytes of chicks inoculated with NP exhibited greater cross-reactivity with the heterologous H7N2 virus than T cells from the birds inoculated with HA. Amino acid sequences in HA from the two AIV strains were 41% identical while the amino acid sequences of NP were 97% identical. The more conserved nature of NP, in compared to HA could be responsible for the immunodominance of NP and a greater cross-reactive response (87). Although the variation could also be attributed to the differences in antigen processing and presentation by the same APCs (14, 25, 28, 29, 127), NP and the HA responses were similar by 9 weeks p.i. using the same standardized assay. Compared with other MHC defined haplotypes, B19 chicken may possess a distinct T cell epitope repertoire for AIV proteins and consequently, respond to AIV proteins differently (15, 76). Hence, it is important to determine the responses to AIV in different MHC lines of chickens. Although the cross-reactivity of memory $CD8^+$ T lymphocytes may not prevent the infection of chickens with a heterotypic strain of AIV, it could contribute to the rapid clearance of the virally infected cells and augment the protection against clinical illness. This study establishes that chickens $CD8^+$ T lymphocytes respond to AIV NP and HA

proteins. The ability of the other AIV proteins besides these in stimulating $CD8^+$ T lymphocytes of chickens needs to be evaluated. Inclusion of such AIV protein targets that can induce cross-reactive $CD8^+$ T lymphocyte responses besides humoral immunity in chickens is critical for the development of efficacious vaccines which can provide protective immunity against a broader range of AIV types (68).

CHAPTER III

MEMORY CD8$^+$ T LYMPHOCYTE RESPONSE TO AVIAN INFLUENZA VIRUS HEMAGGLUTININ AND NUCLEOCAPSID PROTEINS EXPRESSED BY A NON-REPLICATING HUMAN ADENOVIRUS VECTOR

INTRODUCTION

Since 1996, the zoonotic threat posed by avian influenza viruses (AIV) has been realized by the direct transmission of highly pathogenic H5N1 AIV from poultry to humans in many countries throughout the world, including Asian, Africa and Europe (2, 16, 30, 78). These zoonotic infections have resulted in fatality in 60% of the cases reported (3). Although human-to-human transmission of the H5N1 AIV is rare, the emergence of transmissible human virus with genes of avian origin by reassortment is a reality (16, 129, 137). The influenza pandemics of 1918, 1957 and 1968 were caused by human influenza viruses encoding genes of avian, swine and human origins (2, 16, 18).

AIV, influenza A viruses of the Orthmyxoviridae family, have segmented, negative sense RNA genomes. AIV strains are classified according to their transmembrane hemagglutinin (HA) and neuraminidase (NA) glycoproteins (3,18, 21, 22). Although shore birds and waterfowl, such as ducks, swans, geese, waders and terns are considered primary hosts, AIV have also been isolated from pheasants, quails and poultry (3, 18, 20, 21). In poultry, the virulence of AIV determines its classification as either low pathogenic or highly pathogenic virus. Infection with low pathogenic AIV strains produce asymptomatic to mild respiratory and enteric tract disease, while that

with the highly pathogenic strains results in clinical illness and systemic disease (3, 60, 81). Furthermore, poultry are also considered a critical intermediate host for adaptation of the AIV strains from wild birds to mammals, including swine and humans (21, 22, 85).

Both the zoonotic nature of AIV which provides a mechanism for emergence of new human strains and the economic losses sustained by the poultry industry from AIV outbreaks justify efforts to develop more efficacious, safe vaccines (16, 27, 31). While only inactivated whole AIV and fowl pox vectored vaccines are available commercially, AIV vaccines are discouraged or prohibited in many countries, such as the United States (17, 27, 47, 110). The use of inactivated whole virus vaccine as a control strategy for AIV is limited because of the inability to distinguish infected from vaccinated animals (DIVA), and pre-existing immunity against fowl pox virus prevents the development of optimum protective immune response against AIV (17, 110, 112, 114). Incomplete protection allows AIV to survive and circulate in flocks and potentially mutate into highly pathogenic strains (106, 107). The mass slaughter policy applied in the event of highly pathogenic AIV outbreaks in poultry contributes to extensive economic losses (17, 27). Highly pathogenic strains with an increase in infectious viral load within the bird are considered a risk to both birds and mammals.

In both mice and chickens, the efficacy of a non-replicating adenovirus serotype 5 (Ad5) based vector expressing HA of influenza virus to protect against a challenge of H5N1virus, has been demonstrated (38, 50). Toro et al (124) have shown that *in ovo* inoculation of the non-replicating (replication competent (RCA)-free) human

adenovirus vectored vaccine encoding HA transgene of H5N9/Tur/Wis/68 AIV strain induced neutralizing anti-HA antibody in chickens. The vaccine also protected chickens against challenge with highly pathogenic H5N1 (Swan/Mongolia/244L/2005) AIV in 68% cases and H5N2 (Chicken/Queretaro/19/95) AIV in 100 % cases (124). The amino acid sequence identity shared between the HA of the H5N9 AIV (GenBank accession U79456) and the H5N1 (GenBank accession EU723707) or H5N2 (GenBank accession U79448) challenge viruses was 89% and 94%, respectively (119). While the induction of humoral immunity in chickens to H5N9/Tur/Wis/68 AIV is well characterized, the ability of the non replicating adenovirus vectored vaccine to induce HA specific $CD8^+$ T lymphocyte responses in chickens has not been determined.

Although humoral immunity protects against the viral strains expressing homologous HA protein by neutralization of the virus, its efficacy is limited against variant or heterologous viruses (67, 90). The induction of $CD8^+$ T lymphocyte immunity can greatly diminish the clinical disease by clearing viral infected cells (67, 90, 116). Additionally, the $CD8^+$ T lymphocytes target more conserved epitopes than antibodies and thus can confer protection against a broader range of viruses (67, 90, 102). The current studies demonstrate that a viral specific, MHC-I restricted effector and memory $CD8^+$ T lymphocytes are generated by RCA-free human Ad5 vector encoding AIV HA and NP proteins. The T lymphocyte responses do cross-react with heterologous AIV and administration of a booster dose induces a more robust secondary humoral and cellular immune response against AIV.

MATERIALS AND METHODS

Viruses: Viral stocks of low pathogenic AIVs, H5N9 (A/Turkey/Wis/68) and H7N2 (A/Turkey/Virginia/158512/02) types, were propagated in the allantoic sacs of 10 day old embryonated chicken eggs (ECE) for 48 hours and presence of the virus was determined by hemagglutination (HA) assay performed according to OIE guidelines (http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf). Virus titers were determined in eggs and expressed as embryo infectious dose 50 (EID_{50}) (7).

Vector: An RCA-free E1/E3 deleted human adenovirus serotype 5 (Ad5) vector encoding HA gene from H5N9 (A/Turkey/Wis/68) AIV,Ad-HA,) and Ad-NP encoding NP from H1N1/PR8 were provided by Dr. D.C. Tang (Vaxin Inc., Birmingham, AL)(124) .The amino acids of the NP from H1N1 and NP from H5N9 AIV were 93% identical. Virus titers were determined by the Adeno-X rapid titer kit (BD Clontech, Mountain View, CA), according to the manufacturer's protocol, and expressed as infectious units (ifu) per ml.

Experimental Animals: Embryonated eggs of B19/B19 MHC-defined chickens lines were obtained from Dr. Briles' laboratory at Northern Illinois University (DeKalb, IL). Post- hatching,the chicks were housed in a specific, pathogen free environment at the vivarium facility of Western University of Health Sciences, Pomona, CA. All procedures involving the use of chickens were approved by and conducted according to guidelines established by the Institutional Animal Care and Use Committee of Western

University of Health Sciences. At three weeks of age chickens with the B19/B19 MHC haplotype were inoculated intramuscularly (IM) with 0.3 ml (1 X 10^8ifu total) of Ad-HA, or Ad-NP vectors. Control birds were inoculated with either AdE, the empty vector without AIV genes, or PBS only. Nine weeks after the first inoculation, chickens were boosted with the same dose of Ad-HA vector as described for primary inoculation.

Determination of HA-specific Antibodies: Serum samples were prepared from blood collected from the jugular vein of chickens at 10 days, and 3, 5, 7 weeks p.i. and 1 week p.b. to evaluate the humoral responses. Hemagglutination inhibition (HI) assay was carried out to determine titers of anti-HA antibodies specific to H5N9 virus (A/Turkey/Wis/68) HA and expressed as geometric mean titer (GMT) (http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf).

***Generation of Antigen Presenting Cells (APCs)*:** Primary chicken kidney cell CKC lines were established from 10day-old chicks of B19/B19 and B2/B2 MHC haplotypes as described previously (98). Cells from the tenth passage were used as non-professional APCs for the stimulation of the $CD8^+$ T lymphocytes.

T Lymphocyte Preparation: Peripheral Blood mononuclear cells (PBMC) and splenocytes from inoculated chickens were the source of ex vivo stimulated effector T lymphocytes (93). Briefly, blood was collected from the jugular vein at day 10 and, 3, 5, 7 and 8 weeks post inoculation (p.i.) of the primary dose and 1 week post boosting (p.b.).

Splenocytes were isolated at 4 weeks p.b. Ficoll-histopaque (Histopaque-1077, Sigma-Aldrich, St. Louis, MO) density gradient centrifugation method was used to isolated mononuclear cells from whole blood and spleens (98). Viable mononuclear cells were collected from the interface and washed twice with phosphate buffered saline (PBS, pH 7.4). Cells were resuspended in 3 ml of RPMI 1640 (Invitrogen, La Jolla, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine, and 0.1mM MEM non-essential amino acids. B lymphocytes were removed by passing the cell suspension through a complete RPMI equilibrated nylon wool column and adherent cells were removed by incubating the cell preparation in 25 cm^2 tissue culture flasks as described previously (98).

Separation of $CD8^+$ and $CD4^+$ T Lymphocytes: The separation of CD8+ and CD4+ T lymphocytes was done by antibody mediated depletion using Dynabeads (Invitrogen, La Jolla, CA). The purified T lymphocytes were labeled with either mouse anti-chicken CD8 or mouse anti-chicken CD4 monoclonal antibodies (Southern Biotech, Birmingham, AL) at a concentration of 1 $\mu g/10^6$ cells in PBS containing 0.1% bovine serum albumin fraction V (Sigma-Aldrich, St. Louis, MO) and incubated at 4º C for 30 mins. The unattached antibodies were removed by two washes with PBS and the cells were incubated with rat anti-mouse IgG coated Dynabeads according to the manufacturer's protocol. The antibody coated cells were removed using DynaMag-2 (Invitrogen, La Jolla, CA) and the unlabeled cells in the supernatants were collected. The purity of the separation technique was verified by conducting a FACS analysis on T

lymphocyte population labeled with anti-chicken CD8 FITC Mab and anti-chicken CD4 R-PE (Southern Biotech, Birmingham, AL) .

Ex vivo Stimulation of T Lymphocytes: T lymphocytes prepared from PBMC and spleens were stimulated ex vivo with MHC B19/B19 (matched) and B2/B2 (mismatched) APCs (REF). APCs at a concentration of 1 x 10^5cells/ml were incubated for 8 hours at 39ºC, 5% CO_2in 96-well tissue culture plates. Each well of APCs was infected with 1 x 10^5 ELD_{50} ofH5N9 (A/Turkey/Wis/68) virus for 1 hour followed by removal of the unattached virus by three washes with DMEM supplemented with 10% FBS. One x 10^6 T lymphocytes in complete RPMI were then added 4 hours after infection of the APCs. The APCs and T lymphocytes were co-cultured for 24 hours at 39ºC, 5% CO before the media was collected and the supernatant clarified by centrifugation. Each ex vivo stimulation assay was conducted in duplicate.

Determination of IFNγ in Supernatants: Since activated T lymphocytes produce IFNγ, the concentration of IFNγ in the clarified supernatants from T lymphocyte-APC co-culture was determined using both a nitric oxide detection assay and a commercially available ELISA ((Invitrogen, La Jolla, CA). The amount of IFNγ produced by the activated T lymphocytes was determined in the clarified supernatants using an nitric oxide detection assay and an ELISA ((Invitrogen, La Jolla, CA). At 5 weeks p.i., the T lymphocyte pellets were collected for FACS analysis. Each ex vivo stimulation assay was conducted in duplicate.

Nitric oxide secretion by IFNγ stimulated HD11 cells, a chicken macrophage cell line, was evaluated using a modification of the assay described by Karaca et al (57), Crippen (24) and Pei et al (84). Cells were incubated in individual wells of 96-well plates at a concentration of 10^5 cells/ well in complete RPMI for 2 hours at 39ºC, 5% CO_2, prior to the addition of 100µl of supernatants from T lymphocyte-APCs cultures. After 24 hours of incubation, the accumulation of nitrite from stimulated HD11 cells was measured using the Griess reagent assay according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). The concentration of nitrite produced was determined using sodium nitrite solutions with a concentration of 1-20 µmoles as standards. The concentration of any non-specific production of nitric oxide by any soluble factors was removed by subtracting the nitrite concentration of supernatants from APCs cultured without T lymphocytes from the supernatants of the APCs cultured with T lymphocytes. The calculated sample nitrite concentration was induced by IFNγ mediated stimulation.

The concentration of chicken IFN γ (ChIFN-γ)in 100µl of clarified supernatants collected after ex vivo stimulation was also determined at 10 days, 5 and 7 weeks p.i. and 1 week p.b. using a recently marketed, commercial ELISA kit according to the manufacturer's protocol (Invitrogen, La Jolla, CA) (4).

Statistical Significance of Differences: The nitric oxide and the ChIFN-γ concentrations were expressed as average of three to four birds per group. ANOVA (analysis of variance) with significance of $p < 0.05$ was used to determine statistical differences.

RESULTS

Humoral Immune Response to HA Expressed by the Non-Replicating Adenovirus: Chicks were inoculated IM with $1x10^8$ i.f.u. of adenovirus vector expressing H5 protein of H5N9 (A/Turkey/Wis/68) (Ad-HA) or with an equivalent amount of the empty vector (AdE) (124). Between 10 days p.i. to 7 weeks p.i., the HI titers of sera to the homologous H5N9 AIV strain from chickens inoculated with the Ad-HA vector ranged from 4 $\log_2$ to 8 $\log_2$ (GMT) (FIG. 3-1). The HI titers from 10 days p.i. to 7 weeks p.i. continued to maintain steady levels. However, there was an increase of over 16 fold in the HI titers at 1 week p.b. which ranged from 9 $\log_2$ to 12 $\log_2$ (GMT). The sera from Ad-HA inoculated birds failed to inhibit the hemagglutinating activity of a heterologous H7N2 AIV. The sera from AdE and PBS inoculated chickens were also negative for any HI activity.

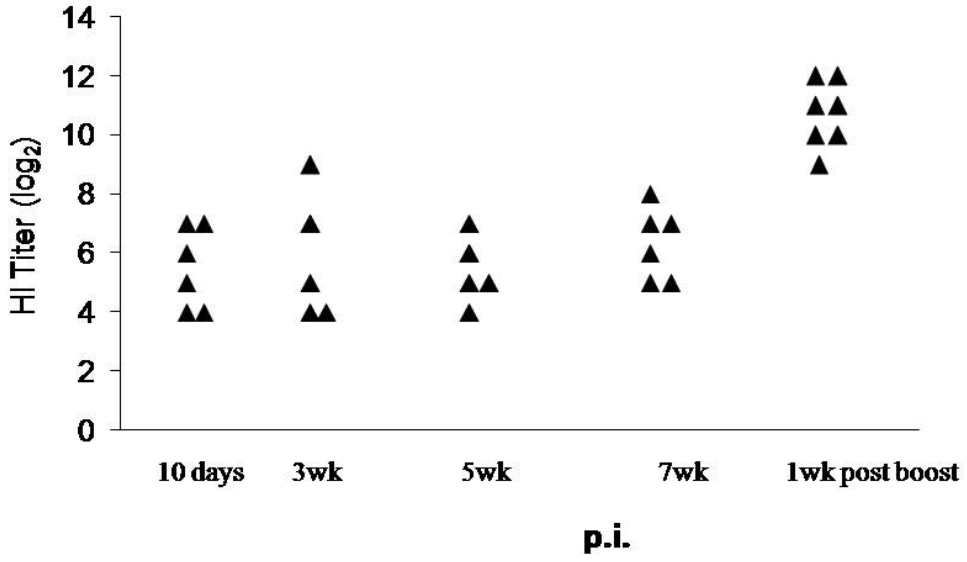

FIG. 3-1. Kinetics of the humoral immune response induced by Ad-HA inoculation at 10 days, 3, 5, and 7 weeks post prime and 1 week post secondary administration. Serum HI antibody titers of individual chickens were evaluated and expressed as $\log_2$ of the reciprocal of the greatest dilution of serum inhibiting agglutination of 1% chicken RBCs by 4 HA units of H5N9/Turkey/Wis/68 AIV. Results are from two separate experiments.

AIV Specific Effector and Memory T Lymphocyte Responses: To determine whether Ad-HA could induce antigen specific T lymphocyte responses, 3 week old chickens of B19/B19 MHC-I haplotype were inoculated IM with Ad-HA at a dose of 1 x 10^8 ifu. Since adoptive transfer studies with infectious IBV and AIV had detected effector T lymphocytes at 10 days p.i, effector cells were evaluated at 10 days p.i. (84). An indirect IFN-γ assay relying on the production of NO by the HD-11 chicken macrophage cell line was used to demonstrate AIV specific ex vivo activation of T lymphocyte response by infected APCs.

An effector T cell response to the HA, detected at 10 days after administration of the Ad-HA, declined to basal levels by 16 days p.i. Memory T lymphocyte responses were detected by 3 weeks p.i. and maximum stimulation of viral specific T lymphocytes derived from Ad-HA inoculated chickens were detected at 5 weeks p.i. By 8 weeks p.i., T cell responses had declined to undetectable levels. Following the decline of the memory response of peripheral blood T lymphocytes, 4 birds were boosted with a second, 1 X 10^8 ifu dose of Ad-HA given IM at 8 weeks after the primary inoculation. The T lymphocyte responses were evaluated at one and two weeks post boosting (p.b.).

At 1 week p.b. with the Ad-HA, significantly higher memory effector T cell responses from Ad-HA inoculated chickens were detected after ex vivo stimulation of T lymphocytes than by T lymphocytes from AdE or PBS inoculated chickens. This secondary effector response markedly declined at 2 weeks post boosting (p.b.) similar to the decline observed following the primary effector response.

The activation of the B19/B19 derived T lymphocytes by AIV infected B19/B19 APCs was considered MHC-I restricted since B2/B2 CKC infected with AIV did not activate these T lymphocytes (data not shown). While at all time points T lymphocytes from Ad-HA inoculated chickens cultured with AIV infected APCs could stimulate NO production by the macrophage cell line, the response was considered primarily antigen specific (FIG. 3-2). Lymphocytes from chickens inoculated with empty Adeno vector (AdE) stimulated only basal levels of NO. T lymphocytes derived from PBS inoculated chickens were also minimally stimulated by infected B19/B19 APCs. T lymphocytes derived from AdE inoculated chickens were non specifically stimulated by infected APCs one week p.b. but this response was significantly lower than the response mediated by T lymphocytes derived from Ad-HA inoculated chickens.

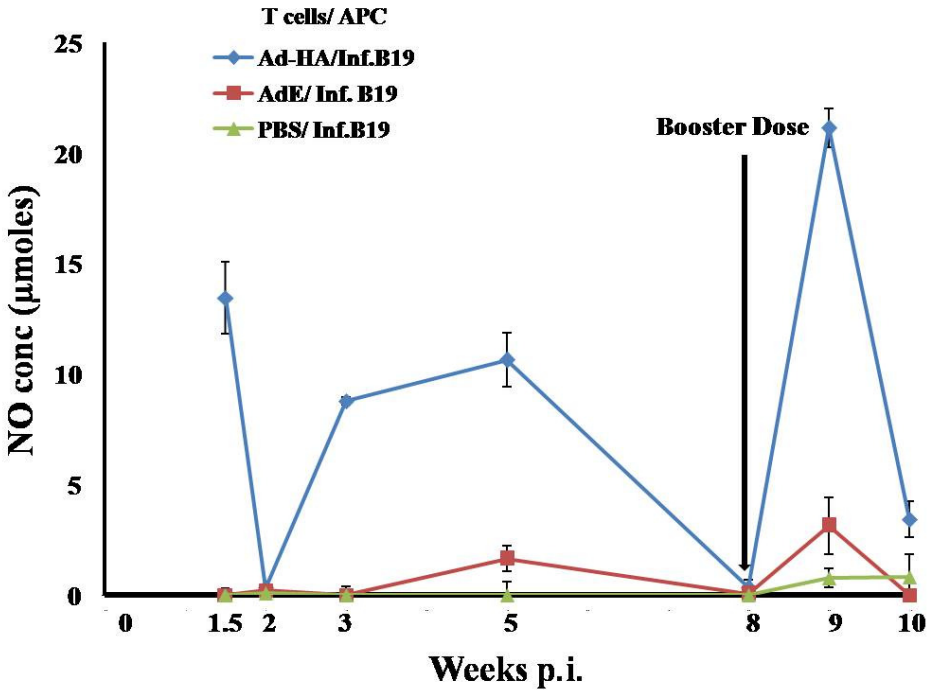

FIG. 3-2. Kinetics of the AIV specific T lymphocyte responses from B19/B19 MHC-I haplotype chickens following primary and booster inoculations with 1X 10^8 ifu of RCA-free human Ad5 adenovirus vector expressing AIV HA (Ad-HA). T lymphocytes were ex vivo *stimulated* with H5N9 AIV infected MHC matched B19/B19 APCs before evaluating activation of the T lymphocytes. Activation was quantified as IFNγ secretion from T cells determined through production of NO by an HD11 macrophage cell line. Results are expressed at average (± S.E.) of two experiments. Each ex vivo stimulation assay is denoted bythe source of T lymphocyte and virus infected MHC-I APCs. The responses of T lymphocytes from Ad-HA inoculated birds and AdE inoculated control birds at 10 days p.i. and at 3 and 5 weeks were significant ($p \leq 0.001$).

Direct IFN ELISA Confirmed HA Specific T Lymphocyte Responses: Using a recently developed commercial kit, ELISA directly evaluating the IFNγ production by activated T lymphocytes confirmed the AIV specific response of T lymphocytes at 10 days, 5 weeks and 9 weeks (one week p.b) p.i. from chickens receiving Ad-HA. The mean amount of IFNγ produced by the primary effector T lymphocytes from Ad-HA inoculated chickens was 49.5 pgm which was nearly 24 folds of that secreted from lymphocytes of chickens inoculated with AdE (FIG. 3-3). The memory T lymphocytes collected from chickens 5 weeks p.i. with HA secreted an average of 44 pgm of IFNγ which was more than 20 folds greater than that produced by the T lymphocytes derived from chickens receiving the empty AdE control. The HA specific response was highly MHC restricted at 10 days, as well as 5 weeks. At 5 weeks p.i., IFNγ secreted was more than 44 fold by the T lymphocytes of Ad-HA inoculated birds stimulated with the B19/B19 match compared to those stimulated by mismatched B2/B2 infected APCs (data not shown). The IFNγ production from the memory effector cells as detected by the ELISA 1 week p.b. was also greater than the primary effector response and was also confirmed to be highly AIV specific and MHC-I restricted.

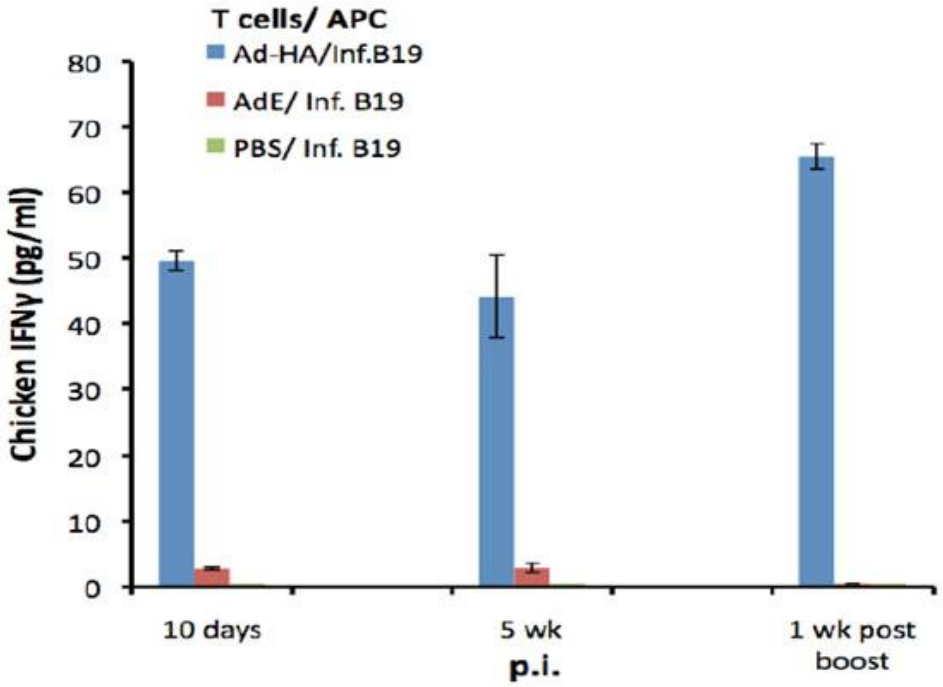

FIG. 3-3. ELISA results measuring IFNγ secretion by T lymphocytes following primary and booster administration of Ad-HA. Concentration of IFNγ secreted in the supernatant of ex vivo stimulated T lymphocytes was determined using a commercial sandwich ELISA (Invitrogen, La Jolla, CA). Results are expressed as the average (± S.E.) of 3 birds. Each ex vivo stimulation assay is denoted by the source of T lymphocyte and virus infected MHC-I APCs. The difference between IFNγ secretion by activation of T lymphocytes derived from Ad-HA and AdE inoculated chickens was significant ($p \leq 0.001$).

AIV Specific Memory T Lymphocytes Are Mostly CD8$^+$: To determine the phenotype of the responding memory T lymphocytes reacting to stimulation by AIV infected APCs, at 6 weeks p.i. the T lymphocyte subpopulations expressing either CD8 or CD4 were enriched by negative selection using specific mouse anti-chicken CD4 or mouse anti-chicken CD8 antibodies and magnetic beads coated with rat anti-mouse IgG antibodies (97). Following ex vivo stimulation with the H5N9 AIV infected B19/B19 haplotype APCs, the purified CD4$^+$ T lymphocytes were activated but at a much lower level compared to the T lymphocyte enriched for the CD8 phenotype ($p \leq 0.06$) (FIG. 3-4). The activation of the CD8$^+$ T lymphocytes derived from Ad-HA inoculated chickens was specifically stimulated by AIV infected APCs. Uninfected B19/B19 APCs had a significantly lower stimulatory effect on CD8$^+$ T lymphocytes derived from Ad-HA inoculated chickens. Although in this assay, CD4$^+$ lymphocytes from chickens given the AdE without HA were also stimulated by AIV infected APCs, these observations were not reproducible. The T lymphocytes derived from PBS inoculated chickens of either CD4 or CD8 subtype were stimulated only at basal levels.

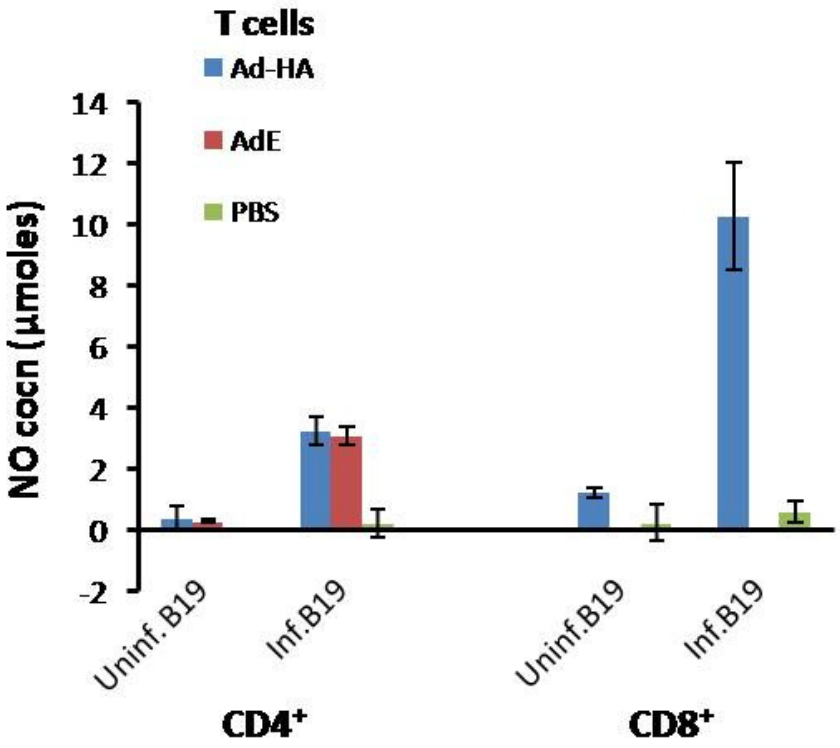

FIG. 3-4. Response of memory $CD4^+$ and $CD8^+$ T lymphocytes from Ad-HA inoculated chickens. At 6 weeks p.i. the T lymphocytes from the peripheral blood mononuclear cells were separated into $CD4^+$ and $CD8^+$ T lymphocyte subpopulations and stimulated with AIV infected and uninfected B19 APCs. The activation of the T lymphocytes was measured by indirect stimulation of NO production. Results are expressed as average NO production (±S.E) of an N of 3 birds.

H5 Specific CD8$^+$ T Lymphocytes also Recognize H7 Infected APCs: The potential for T lymphocytes from H5 Ad-HA to cross react with H7 was determined at 7 weeks p.i., while the responses remained detectable. Using H7N2 infected APCs for ex vivo stimulation of T lymphocytes, activation was detected by secretion of IFN□ using the indirect assay and a newly available commercial direct ELISA (Invitrogen, La Jolla, CA). The responses of T cells from Ad-HA inoculated birds were not significantly different whether the APCs were infected with the H5 or the H7 virus although the response to H5 virus infected APCs was slightly greater (FIG. 3-5)

Using either the ELISA or NO production of macrophages to detect T cell secretion of IFNγ, the responses were again found to be primarily mediated by the activation of CD8$^+$ T lymphocytes rather than CD4$^+$ lymphocytes ($p \leq 0.001$-0.0001). Stimulation of either CD8$^+$ or CD4$^+$ T lymphocyte populations from AdE and PBS inoculated chickens was either weak or absent.

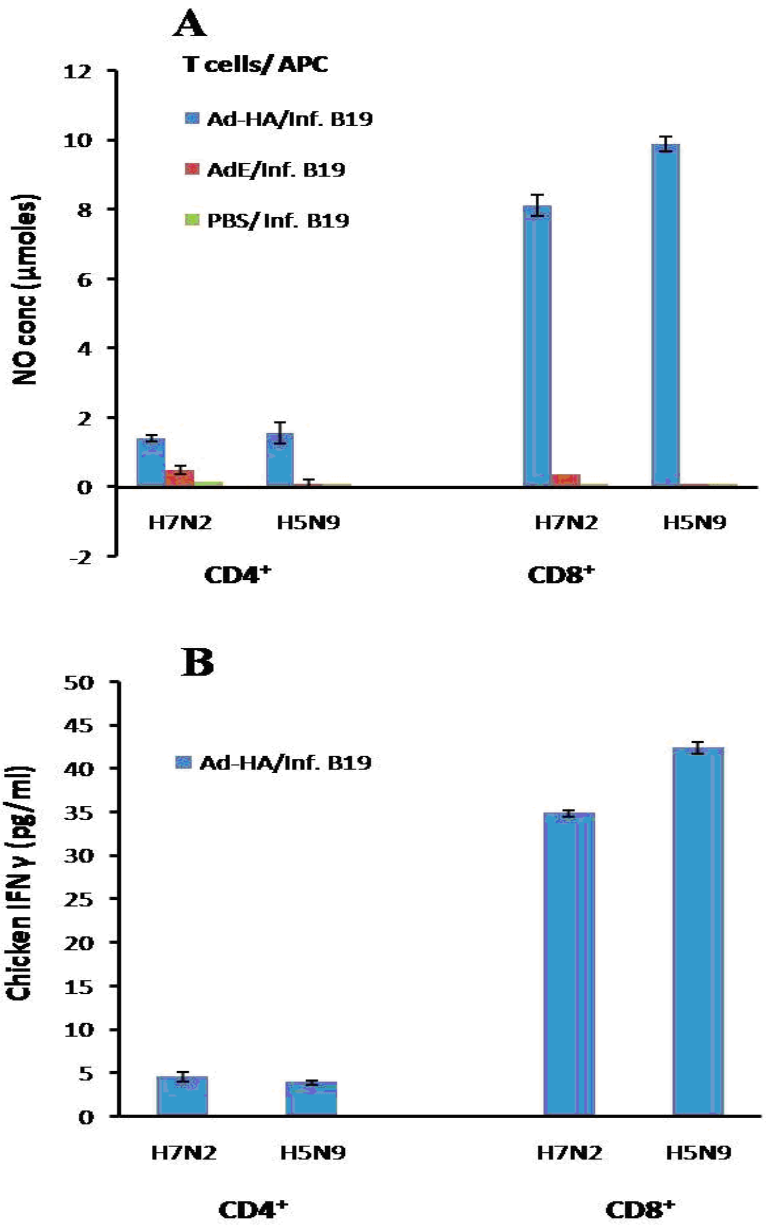

FIG. 3-5. $CD8^+$ T lymphocytes from B19 birds inoculated with Ad-HA respond to APCs infected with a heterologous (H7N2) virus. At 7 weeks p.i., purified $CD4^+$ and $CD8^+$ T lymphocytes from chickens (n=3) inoculated with Ad-HA were stimulated by APCs infected with either homologous virus H5N9 or a heterologous H7N2 strain. Assays represent the mean (± S.E.) of T lymphocytes of 3 birds. (A) The T lymphocyte responses as determined by the indirect IFNγ assay. (B) The T lymphocyte responses of the same birds as determined by a direct IFNγ ELISA. Responses of $CD8^+$ T lymphocytes were significantly greater than those of the $CD4^+$ T lymphocytes ($p \leq 0.01$ and $p \leq 0.001$, respectively).

Memory Response of Spleen Derived T Lymphocytes: Memory T lymphocyte subpopulations in the spleens were evaluated 4 weeks p.b. when the study was terminated. Whole T lymphocyte populations and the purified $CD4^+$ and $CD8^+$ T lymphocyte subpopulations were stimulated ex vivo with AIV infected and uninfected B19/B19 APCs. The stimulation of the T lymphocytes from the spleens of the Ad-HA inoculated chickens was AIV specific and MHC-I restricted. These memory splenic T lymphocytes responses were mediated primarily by the activation of $CD8^+$lymphocytes (FIG. 3-6). The magnitude of the response of whole T lymphocyte population was similar to the response of $CD8^+$ T lymphocytes. In contrast, the activation of the $CD4^+$T lymphocytes was significantly lower ($p \leq 0.003$) and non-specific as suggested by stimulation with uninfected APCs. The significantly lower response of the T lymphocyte subpopulations derived from either AdE or PBS inoculated birds was non specific since they were stimulated by both uninfected and AIV infected APCs.

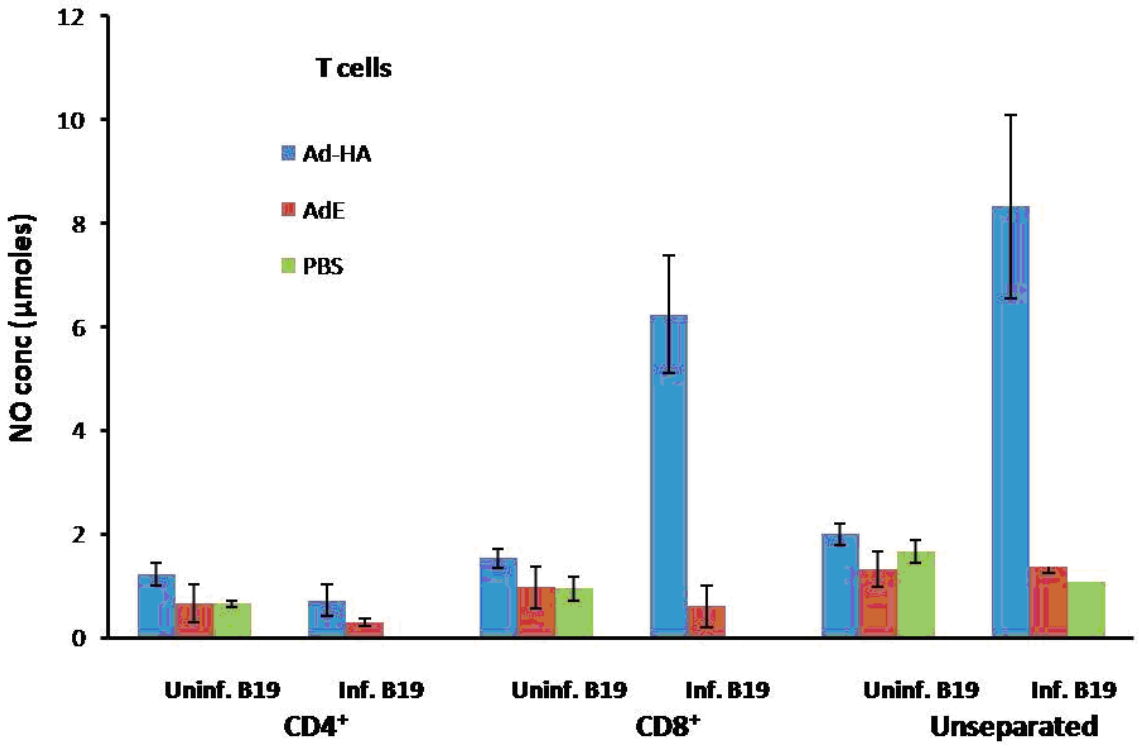

FIG. 3-6. Response of T lymphocytes derived from spleens of Ad-HA inoculated chickens 4 weeks post boosting. Splenic T lymphocytes were separated into $CD4^+$ and $CD8^+$ subpopulations and were stimulated ex vivo along with un-separated T lymphocytes using MHC matched B19/B19 APCs infected with homologous H5N9 virus. Production of NO by HD11 macrophage cells induced by secretion of IFNγ from stimulated T lymphocytes was used to quantify the activation of the lymphocytes. Results were expressed as the average (± S.E.) of 4 birds. Each ex vivo stimulation assay is denoted by the source of T lymphocyte and virus infected MHC-I APCs. AIV specific memory $CD8^+$ T lymphocyte responses in the spleens were demonstrated by the significantly greater production of IFN following co-culture with AIV infected APCs as compared to CD4+ T lymphocytes ($p \leq 0.003$).

Non-Replicating Adenovirus Expressing NP Stimulates Greater T Lymphocyte Responses: Previous studies using plasmid expression vectors had indicated that in chickens the $CD8^+$ T lymphocyte response directed against AIV NP may be more vigorous than that against HA. Therefore, the T lymphocyte response following inoculation of the RCA-free Ad5 human adenovirus vector expressing NP (Ad-NP) was evaluated and compared with the Ad-HA (FIG. 3-7). Although the origin of the NP was H1N1, the NP gene has 93 % identity with the H5N9 AIV NP. Six 3 week old B19 haplotype chickens were inoculated with $1X\ 10^8$ ifu Ad-NP or Ad-HA. The AIV specific responses of T lymphocytes prepared from PBMCs collected at 10 days, 5 weeks and 7 weeks p.i. were evaluated by ex vivo activation using H5N9 infected APCs or the uninfected APCs. The activation of lymphocytes was determined with the direct ELISA. The effector and 5 week p.i. memory responses of T lymphocytes collected from the Ad-NP inoculated chickens were significantly greater than that from HA inoculated chickens at the same time p.i. ($p \leq 0.02$ and $p \leq 0.09$, respectively). Moreover the stimulation of the T lymphocytes collected from Ad-NP inoculated chickens was induced by the cross reaction to a heterologous virus, H5N9/Turkey/ Wis/68 used infected APCs. At 7 weeks p.i., the separation of T lymphocytes into $CD4^+$ and $CD8^+$ subpopulations indicated a significantly greater activation of $CD8^+$ T lymphocytes by 48 folds in comparison to $CD4^+$ T lymphocytes. However, as the responses declined at 7 weeks and the differences between the stimulation of cells from NP and HA inoculated birds decreased it was still significant ($p \leq 0.01$).

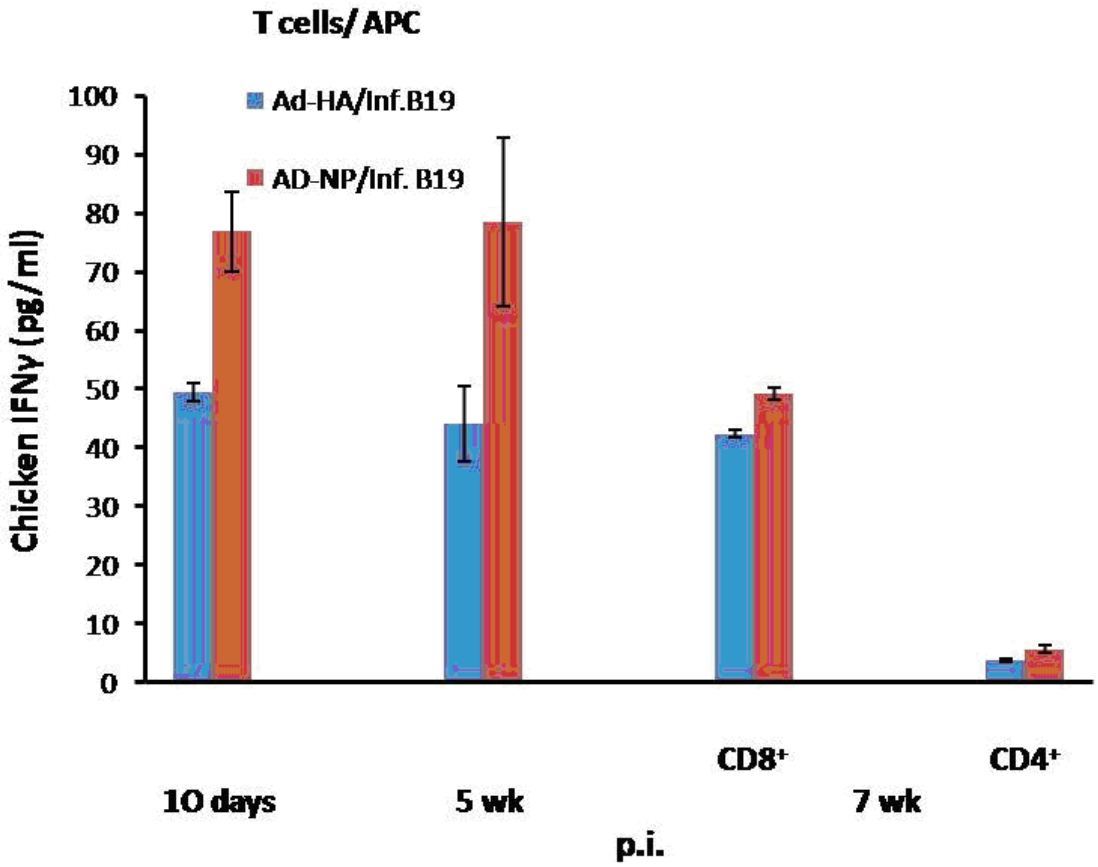

FIG. 3-7. IFNγ secretion by T lymphocytes from birds (n=3) inoculated with Ad-NP or Ad-HA. Concentration of IFNγ produced by T lymphocytes following ex vivo stimulation with H5N9 AIV infected B19 APCs at 10 days, 5 weeks and 7 weeks p.i. Results are expressed as the average (± S.E.) of 3 birds. Each ex vivo stimulation assay is denoted by the source of T lymphocytes and virus infected MHC-I APCs.

DISCUSSION

The increased incidence of the outbreaks of highly pathogenic AIV infections in poultry throughout the world and the threat of emergence of a zoonotic pandemic are the compelling reasons which make the development of an efficacious vaccine against these viruses imperative (17, 22, 31, 116). Although humoral immunity provides protective immunity against a specific AIV strain, their ability to protect against serologically distinct variants is limited (68, 91). In contrast, the immune response mediated by $CD8^+$ T lymphocytes protect against a broader range of antigenically distinct strains (68, 100, 102). In this study, we have examined the ability of an RCA-free human Ad5 vector expressing AIV HA and NP to elicit $CD8^+$T lymphocyte responses to AIV in chickens.

The capacity of adenovirus vectored vaccine expressing influenza virus proteins to stimulate $CD8^+$ T lymphocytes has been demonstrated in mice and swine (38, 50, 51, 53, 103, 117, 133). Gao et al (38) have also established the effectiveness of adenovirus vectored vaccine in protecting chickens from a challenge of homologous virus (38). Toro et al (124) have developed an RCA- free human Ad5 vector expressing H5N9 AIV HA that ability protects chickens against challenge of highly pathogenic AIV variants, H5N1 and H5N2 strains (124).

Although the Ad5 vectored adenovirus infection of chickens resulted in the production of anti-HA antibodies and HI of homologous virus, these antibodies failed to inhibit hemagglutination mediated by the heterologous, H7N2, AIV strain. The ability of this vaccine to protect against a challenge of variant virus in the absence of HI could be explained by a contribution of T lymphocyte mediated immunity. In the current study we

demonstrated that IM inoculation of HA expressing Ad-vectored AI vaccine induces $CD8^+$ T lymphocyte response specifically against AIV. Previous studies in our lab have shown that plasmid DNA expressing HA stimulates $CD8^+$ T lymphocytes in chickens (Chapter II). This response was primarily MHC-I restricted as only AIV infected APCs of B19 MHC-I haplotype could activate the T lymphocytes. The stimulatory effect of infected APCs of B2 MHC-I mismatched APCs was either absent or very weak. The Ad-HA response was mediated primarily by $CD8^+$ T lymphocytes since the NO production and IFNγ secretion by purified T lymphocyte subpopulation bearing $CD8^+$ phenotype was significantly greater than subpopulation with $CD4^+$ phenotype following ex vivo activation by infected APCs at 7 weeks p.i. Although at 6 weeks p.i. the $CD4^+$ T lymphocytes from Ad-HA inoculated chickens induced NO production following stimulation with both AIV infected and uninfected APCs, these results were not observed at other time points. The T lymphocytes induced by RCA-free Ad5 vector also reacted with a heterologous H7N2 AIV, which shared only 41% amino acid sequence identity with the HA expressed by the adenovirus vector.

The current studies further established the kinetics the $CD8^+$ T lymphocyte response following primary and booster administration of Ad-HA. Similar to the MHC-I restricted T lymphocyte responses stimulated by infection with whole IBV or AIV infection, the primary effector $CD8^+$ T lymphocyte response was observed at 10 days p.i. (99 , 102). However at 16 days p.i., the response of the T lymphocytes was undetectable. The AIV specific memory T lymphocyte response induced by Ad-HA could be observed between 3 weeks and 7 weeks p.i. Although the magnitude of the memory response was

greatest at 5 weeks p.i., it remained lower than the primary effector response. The response of the memory T lymphocytes declined sharply 7 weeks after the primary inoculation of the vector and was reduced to undetectable levels at 8 weeks p.i. These observations of the memory T lymphocyte response induced by Ad-HA are consistent with our previous findings with T lymphocyte response to HA expressing plasmid (Chapter II). Seo and Webster also observed that in chickens inoculated with H9N2 AIV, the protection against a variant viral challenge had greatly declined by 10 weeks p.i. (97). Similarly, human memory T lymphocyte responses have also been reported to decline with time (75).

The current study also evaluated the impact of boosting on the chicken T lymphocyte response. Studies with mice and non human primates have indicated the induction of immune responses to vaccine vectors following primary inoculation as an impediment to their use for further homologous boosting with the same vector (65, 90, 113). However, our study demonstrated the induction of a robust T lymphocyte response with a 16 fold increase in anti-HA antibody titers 1 week post boosting with the same Ad-HA vector, indicates the contrary. Toro et al (125) have also established that *in ovo* vaccination of chickens with H5 Ad-vectored vaccine did not impair the development of protective immune response induced by the post hatch vaccination with the same Ad-vector expressing different HA (125). The presence of pre-existing immunity against the Ad- vector did not inhibit the efficacy of this vaccine to stimulate a secondary immune response against HA. This could be attributed to the non replicating

nature of the vector which induces the development of a weaker immune response against its self (125).

Additionally, the present study also established that the non-replicating Ad-vector encoding an NP originating from a human H1N1/PR8 influenza virus has the ability to induce a cross reactive chicken T lymphocyte mediated responses specific for an avian derived NP. Studies with mice have demonstrated the immunodominance of NP in induction of $CD8^+$ T lymphocytes (68, 120, 134, 146). The observations from this study also concur with our previous findings that NP from influenza virus is a better stimulator of T lymphocytes than HA, however the presence of the memory T lymphocyte in the peripheral blood against both the proteins is detectable only for 7 weeks p.i. of the primary inoculation. The presence of responding memory T lymphocytes in the spleen 4 weeks post boosting suggest that following clearance of the antigens the circulating memory lymphocytes localize to central immune organs in chickens.

The results of this study demonstrate that RCA-free Ad5 human adenovirus vectored vaccine expressing HA has the potential to induce both cell mediated and humoral immunity against AIV HA. This vaccine can cross react at the $CD8^+$ T cell level with variant and heterologous AIV strains hence has a better ability to provide immunity against broader range of AIV. This vaccine can also be used effectively in a homologous prime-boost vaccination program.

CHAPTER IV

CHICKEN CD8$^+$ MEMORY T LYMPHOCYTES HAVE GREATER EXPRESSION OF CD44 AND CD45 THAN NAÏVE T LYMPHOCYTES

INTRODUCTION

Although water fowl and shore birds are natural reservoirs of avian influenza viruses (AIV), these viruses have also been isolated from domestic poultry, humans, pigs, tiger and seals (16, 43). Depending on the virulence of the virus, poultry may suffer from mild respiratory and enteric tract infection due to low pathogenic viruses or systemic infections, which may result in mortality due to highly pathogenic viruses (3, 21, 60). The direct transmission of the highly pathogenic H5N1 AIV from chickens to humans with 60% fatalities of reported cases has made these viruses of grave zoonotic concern (3).

Genetic versatility of the HA and NA genes makes both humoral and cell mediated immunity critical in establishing effective immunity. Although the antibodies against the viral hemagglutinin (HA) protein may be viral neutralizing in nature and prevent infection, their efficacy is limited to homologous viral HA (3, 5). The more conserved nature of T lymphocyte epitopes in comparison to B lymphocyte epitopes allows them to respond to a broader range of serotypes (5). More cross reactive, T lymphocyte responses against viral infections have been shown to reduce disease by clearing infection (1, 29, 30). Vaccines that can induce T lymphocyte mediated immune

responses are more likely to be effective in preventing and limiting the disease caused by variant viruses (5).

Following viral infection, the receptors of naïve T lymphocytes recognize the antigenic epitopes presented by dendritic cells and other antigen presenting cells (APC), responding by proliferating and differentiating into effector T lymphocytes that can clear the virus one to three weeks post infection (1, 29). The elimination of infection is followed by the contraction in the population of the effector T lymphocytes where the majority of the cells die due to apoptosis while a small population either persists or arises as memory T lymphocytes (1). The memory T lymphocytes have the ability to respond to the specific antigen more rapidly than naïve T lymphocytes (1). Memory T lymphocytes in mice and humans have been shown to express surface molecules that serve as markers to distinguish them from naïve lymphocytes. Adhesion molecules, CD44 and CD45, are two such phenotypic markers (26). These molecules have been shown to influence the migration, localization and activation of the T lymphocytes (29, 30, 34).

In mice and humans, T lymphocytes express different isoforms of CD45, which differentiate memory from naïve cells (8). In other species, such as rats, quantitative differences in the levels of CD45 on the surface of T lymphocytes are used to distinguish memory and naïve T lymphocytes (8). CD45 is a membrane protein tyrosine phosphatase and has a role in the signaling cascades that influence the differentiation and proliferation of T lymphocytes (8, 83).

Similarly, the level of expression of CD44 distinguishes memory T lymphocytes from naïve T lymphocytes (26). CD44 is a primary receptor of hyaluronate present in extracellular matrix and its interaction with the hyaluronic acid regulates cell adhesion, migration and homing (40). Naïve T cells in mice and humans have been shown to express low levels of CD44 and their activation leads to higher expression of CD44 which continue to remain elevated on both effector and memory T lymphocytes (1, 26). The CD44 antigen also can have a role in signal transduction which is mediated by its coupling with tyrosine kinases p185 and s-SRC and regulates cell growth, activation and differentiation (44, 49, 54).

Since the development of immunological memory is the fundamental basis of vaccination, it is essential to recognize and differentiate the key components of the T lymphocyte mediated immune response. Although avian T lymphocyte progenitors have been shown to express adhesion proteins like CD44, BEN, HEMCAM and CD45 on their surface, the phenotype of avian memory and naïve T lymphocytes remain to be described (79, 83).

Previous studies in our lab have demonstrated the induction of an AIV HA specific, memory $CD8^{+}$T lymphocyte response in chickens inoculated with either a plasmid or a replication competent (RCA)-free human adenovirus vector encoding HA of H5N9/Tur/Wis/68 AIV strain (Chapter I, Chapter II). This memory response is detected at 3 weeks p.i. and a peak response is observed at 5 weeks p.i. The current study evaluates the expression of CD44 and CD45 surface molecules on the memory T lymphocytes at 5 weeks p.i. with replication competent (RCA)-free human adenovirus

vector encoding HA. The study demonstrates that the median fluorescence intensities of both CD44 and CD45 on the surface of memory chicken $CD8^+$ and $CD4^+$ T lymphocytes specific for AIV HA were greater than that of naïve T lymphocytes. Moreover, the differential expression of CD44 and CD45 on mature T lymphocytes could be used to distinguish memory T lymphocytes from naïve T lymphocytes.

MATERIALS AND METHODS

Viruses: Virus stock of low pathogenic H5N9 (A/Turkey/Wis/68) AIV was propagated in the allantoic sacs of 10 day old embryonated chicken eggs (ECE) for 48 hours. Presence of the virus was determined by hemagglutination (HA) assays performed according to OIE guidelines (http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf).Virus titers were determined in eggs and expressed as embryo infectious dose 50 (EID_{50}) (7).

Vector: An RCA-free E1/E3 deleted human adenovirus serotype 5 (Ad5) vector encoding the HA gene from H5N9 (A/Turkey/Wis/68) AIV (Ad-HA) was provided by Dr. D.C. Tang (Vaxin Inc., Birmingham, AL)(119) . Virus titers were determined by the Adeno-X rapid titer kit (BD Clontech, Mountain View, CA), according to the manufacturer's protocol, and expressed as infectious units (ifu) per ml.

Experimental Animals: Embryonated eggs of B19/B19 MHC-defined chickens lines were obtained from Dr. Briles' laboratory at Northern Illinois University (DeKalb,

IL). Post-hatching, the chicks were housed in a specific, pathogen free environment at the vivarium facility of Western University of Health Sciences, Pomona, CA. All procedures involving the use of chickens were approved by and conducted according to guidelines established by the Institutional Animal Care and Use Committee of Western University of Health Sciences. At three weeks of age, chickens with the B19/B19 MHC haplotype were inoculated intramuscularly (i.m.) with 0.3 ml (1 X 10^8 ifu total) of Ad-HA. Control birds were inoculated with either AdE empty vector or PBS.

Determination of HA-specific Antibodies: Serum samples were prepared from blood collected from the jugular vein of chickens at 5 weeks p.i. to evaluate the humoral responses. Hemagglutination inhibition (HI) assays (http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf) were carried out to determine titers of anti-HA antibodies specific to H5N9 (A/Turkey/Wis/68) HA and expressed as geometric mean titer (GMT).

Generation of Antigen Presenting Cells (APCs): Primary chicken kidney cell CKC lines were established from 10 day-old chicks of B19/B19 and B2/B2 MHC haplotypes as described previously (98). Cells from the tenth passage were used as non-professional APCs for the stimulation of the $CD8^+$ T lymphocytes.

T Lymphocyte Preparation: Peripheral blood mononuclear cells (PBMC) and splenocytes from vaccinated chickens were the source of ex vivo stimulated effector T

lymphocytes (92). Briefly, blood was collected from the jugular vein at varying times post-inoculation (p.i.). Viable cells were collected from the interface and washed twice with phosphate buffered saline (PBS, pH 7.4). Cells were resuspended in 3 ml of RPMI 1640 (Invitrogen, La Jolla, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine, and 0.1mM MEM non-essential amino acids. B lymphocytes were removed by passing the cell suspension through an RPMI 1640 equilibrated nylon wool column and adherent cells were removed by incubating the cell preparation in 25 cm^2 tissue culture flasks as described previously (98).

Ex vivo stimulation of T Lymphocytes: T lymphocytes prepared from PBMC and spleens were stimulated ex vivo with MHC B19/B19 (matched) and B2/B2 (mismatched) APCs. APCs at a concentration of 1 x 10^5 cells/ml were incubated for 8 hours at 39ºC, 5% CO_2 in 96-well tissue culture plates. Each well of APCs was infected with 1 x 10^5 ELD_{50} of H5N9 (A/Turkey/Wis/68) virus for 1 hour followed by removal of the unattached virus by three washes with DMEM supplemented with 10% FBS. One x 10^6 T lymphocytes in complete RPMI were then added 4 hours after infection of the APCs. The APCs and T lymphocytes were co-cultured for 24 hours at 39ºC, 5% CO before the media was collected and the supernatant clarified by centrifugation. Each ex vivo stimulation assay was conducted in duplicate.

Determination of IFNγ in Supernatants: Since activated T lymphocytes produce IFNγ, the concentration of chicken IFNγ (ChIFN-γ) in 100µl of clarified supernatants collected after ex vivo stimulation was also determined at 5 weeks p.i. using a recently marketed, commercial ELISA kit (Invitrogen, La Jolla, CA) according to the manufacturer's protocol (4).

***FACS Analysis*:** After ex vivo stimulation with AIV infected APCs, T lymphocytes were collected and labeled with antibodies for flow cytometric analysis as described by Bohls et al (12). One hundred µl of normal goat IgG (Sigma Aldrich, St. Louis, MO) at a concentration of 2 mg/ml was used to block non-specific binding by cellular Fc receptors on 10^6 cells for 20 min. Following blocking, the cells were washed twice with PBS and then labeled with phycoerythrin-conjugated monoclonal antibodies (MAbs) specific for either CD44 or CD45 at a concentration of 4 µg / 10^6 cells and fluorescein labeled MAbs specific for either CD8 or CD4 at a concentration of 1 µg / 10^6 cells (Southern Biotech Birmingham, AL) as previously described (98). Biotinylated K55 Mab, labeled with streptavidin conjugated to R-phycoerythrin Cy5, was used for gating the lymphocytes as described by Bohls et al (12). Controls included unstained cells.

Flow cytometric analyses were performed on Beckman Coulter Cytomics FC 500 Flow Cytometer (Beckman Coulter, Fullerton, CA). A minimum of 10^4 events were collected for each sample. FlowJo (TreeStar, Inc., Ashland, OR) was used to perform compensation and analyses of the data.

Statistical Significance of Differences: The ChIFN-γ concentrations and median fluorescence intensities of CD44 and CD45 expression were expressed as average of three to six birds per group. ANOVA (analysis of variance) with significance of $p < 0.05$ was used to determine statistical differences.

RESULTS

RCA Ad-HA Vector Induces Both $CD4^+$ and $CD8^+$ T Lymphocyte Mediated Responses: Since activation of the B lymphocytes is regulated by the $CD4^+$ T helper lymphocytes, the presence of viral antibodies reflects on the induction of a $CD4^+$ T lymphocyte response. At 5 weeks p.i., the anti-HA antibodies against the homologous H5N9 AIV were detected by HI serum assays following Ad-HA inoculation of chickens. The HI titers of these anti-HA antibodies ranged from 4 $\log_2$ to 7$\log_2$. No HI activity was detected in the sera collected from the birds inoculated with PBS (FIG. 4-1).

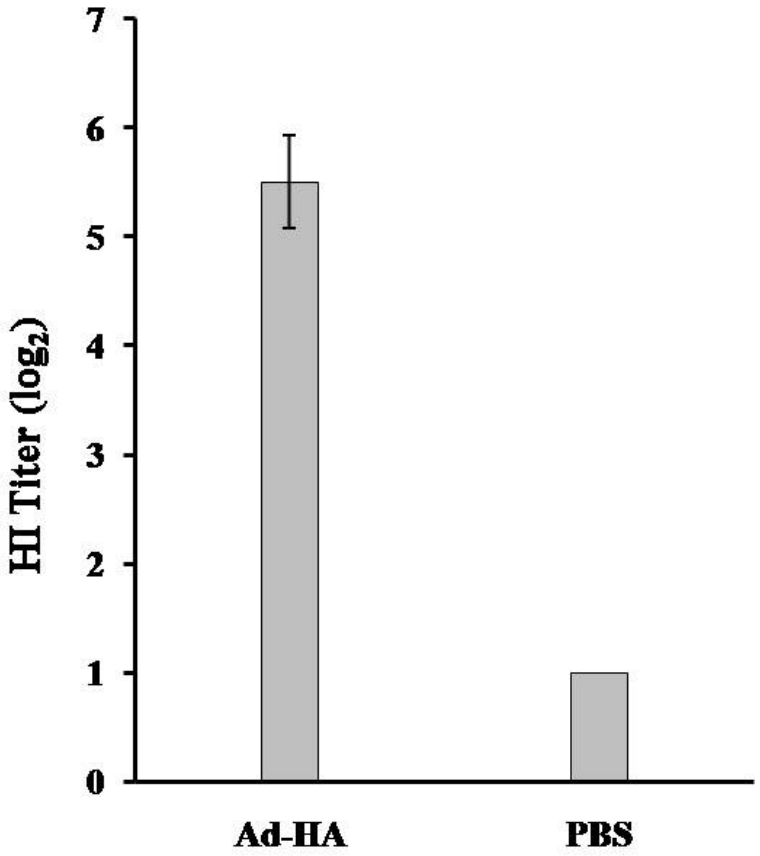

FIG. 4-1. Average (±S.E.) HI antibody titers against H5N9 AIV in the sera of chickens inoculated with Ad-HA (n=6) or PBS (n=4) at 5 weeks p.i.

The induction of memory $CD8^+$ T lymphocyte mediated response was analyzed by a capture ELISA evaluating the IFNγ production by activation of T lymphocytes by MHC-I matched AIV infected APC . Since the APCs used for stimulation of T lymphocytes were non-professional in nature and FACS analysis confirmed only expression of MHC-I and not MHC-II molecule on their surface, the stimulated T lymphocytes were of $CD8^+$ phenotype. At 5 weeks p.i., the T lymphocytes from the chickens inoculated with either Ad-HA or PBS were ex vivo stimulated by co-culturing with AIV infected or uninfected APCs of the B19 MHC-I haplotype. After 24 hours of stimulation, the concentration of the IFNγ secreted in the supernatants of co-culture was evaluated using an ELISA. The stimulation mediated by AIV infected APCs resulted in an average of 44 pgm/ml of IFNγ secretion by the memory T lymphocytes derived from Ad-HA inoculated chickens while no detectable IFNγ was secreted by the T lymphocytes derived from PBS inoculated chickens. The memory T lymphocyte stimulation was AIV specific since neither T lymphocytes derived from Ad-HA nor from PBS inoculated chickens secreted any detectable levels of IFNγ following co-culture with uninfected APCs (FIG. 4-2).

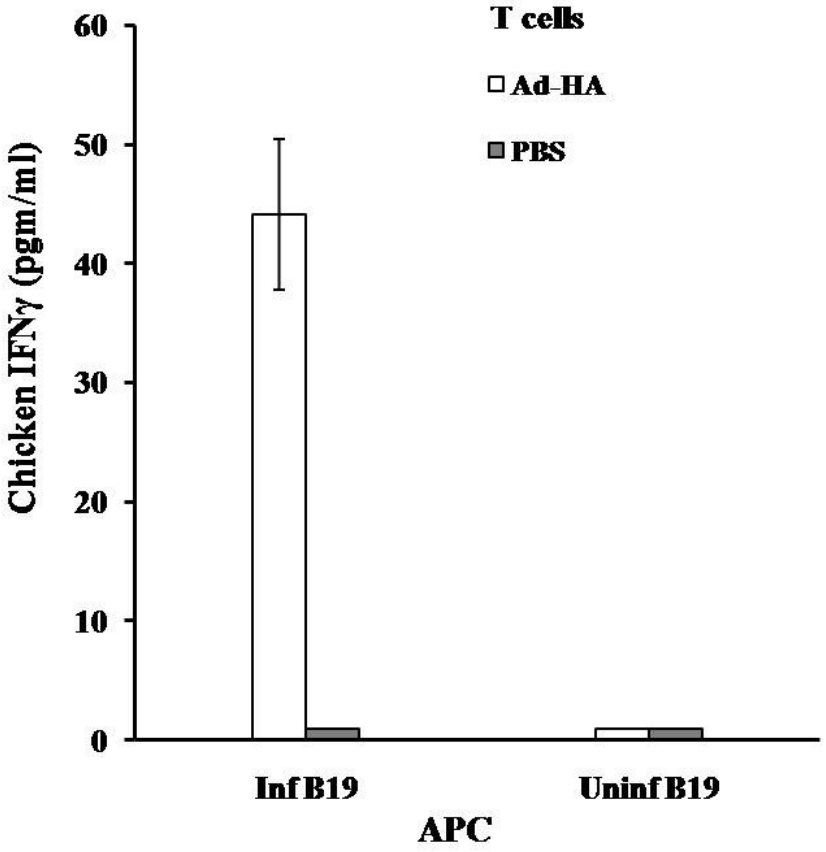

FIG. 4-2. Concentration of IFNγ secretion by T lymphocytes isolated from chickens inoculated with either Ad-HA or PBS at 5 weeks p.i. following ex vivo stimulation with uninfected and AIV infected APCs of the B19 MHC-I haplotype. Results are expressed as the average (± S.E.) of 3 birds. The difference between IFNγ secreted by T lymphocytes derived from Ad-HA and PBS inoculated chickens stimulated with AIV infected APCs was significant ($p \leq 0.001$).

Analysis of CD44 Expression on T Lymphocytes: The expression of the pan leukocyte marker CD44 on T lymphocytes from Ad-HA or PBS inoculated chickens at 5 weeks p.i. was evaluated after ex vivo stimulation with uninfected and AIV infected B19 APCs. Each sample of the T lymphocytes was divided into two aliquots and dual labeled with either anti-chicken $CD4^+$ Mab or anti-chicken $CD8^+$ Mab conjugated to FITC and anti-chicken CD44 Mab conjugated to PE. Biotinylated K55 Mab labeled with streptavidin conjugated to R-phycoerythrin Cy5 was used for gating the lymphocytes as described by Bohls et al. (11). All T lymphocytes of either CD4 or CD8 phenotype were positive for the expression of CD44. However, the median fluorescence intensity of CD44 expression on the $CD8^+$ T lymphocytes was greater than the intensity of expression on $CD4^+$ T lymphocytes (FIG. 4-3(a) and FIG. 4-3 (b)). The T lymphocytes of both CD4 and CD8 phenotype derived from Ad-HA inoculated chickens had significantly higher median fluorescence intensity ($p \leq 0.01$) of CD44 expression than the lymphocytes derived from PBS inoculated chickens. Although the average median fluorescence of the CD44 expression on $CD8^+$ T lymphocytes from Ad-HA inoculated chickens was greater following co-culture with infected APC than with uninfected APCs, the difference was not significant (Table 4-1). In contrast, the difference in the median fluorescence intensity of the CD44 expression on $CD4^+$ T lymphocytes co-cultured with infected APCs was significantly ($p=0.03$) greater than that of $CD4^+$ T lymphocytes co-cultured with uninfected APCs.

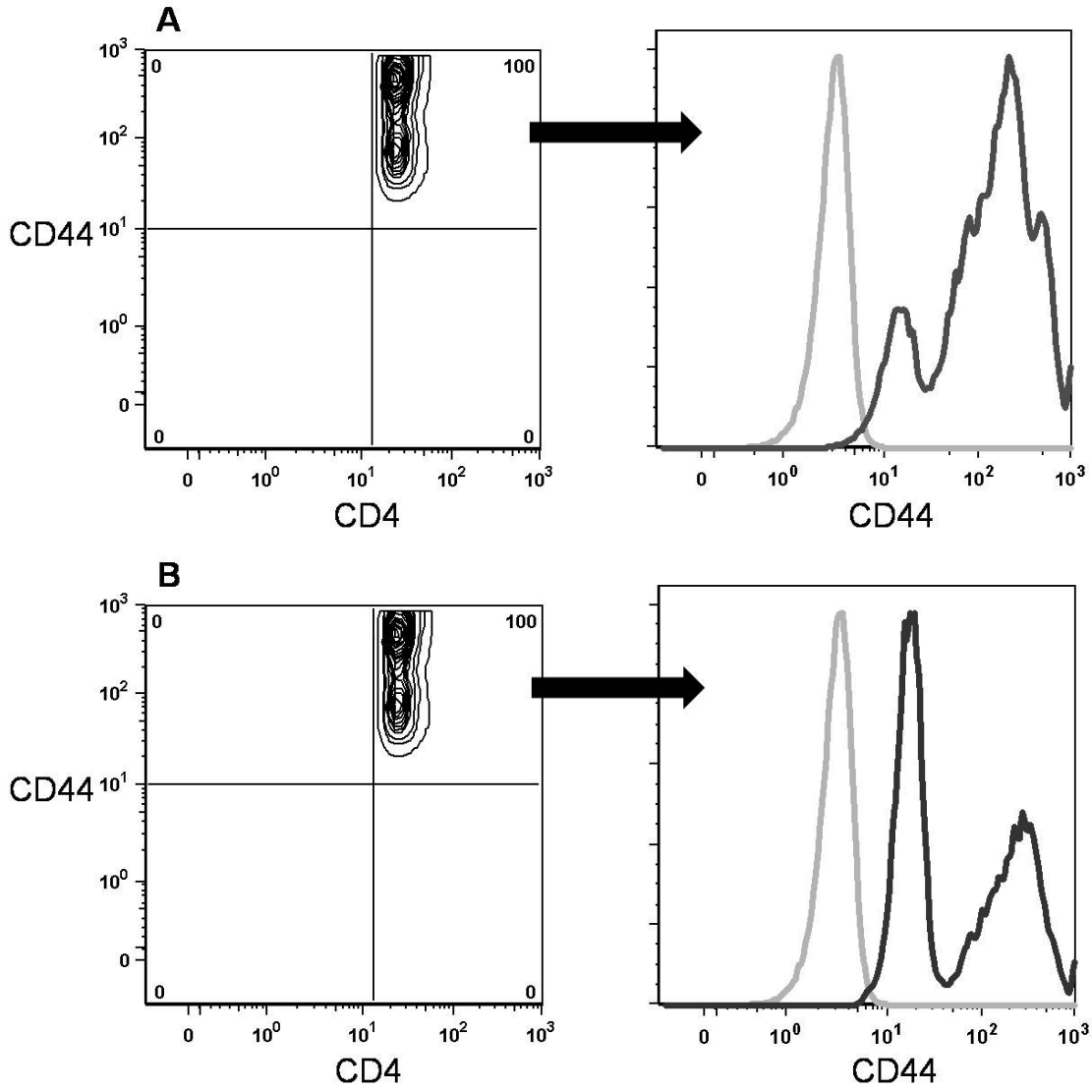

FIG. 4-3(a). Analysis of the CD44 expression on the surface of T lymphocytes after ex vivo stimulation with H5N9 AIV infected APCs. Contours of the T lymphocyte populations stained with anti-chicken CD44 PE and anti-chicken CD4 or CD8 FITC. Histogram of the log fluorescence (Fl2) of CD44 expression (black) on T lymphocyte populations and the unlabeled control cells (gray). (A) $CD4^+$ T lymphocytes from Ad-HA, (B) $CD4^+$ T lymphocytes from PBS.

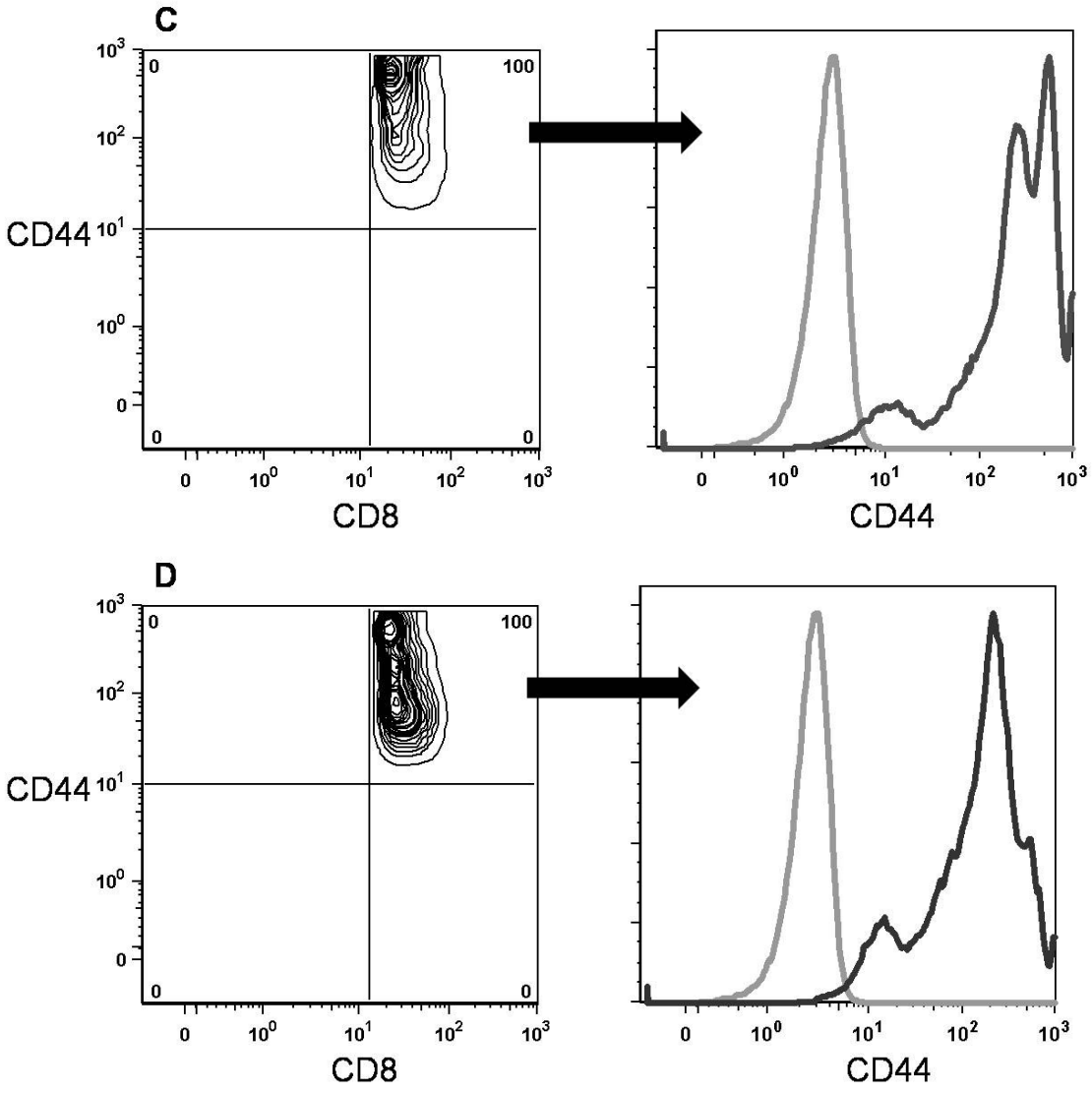

FIG. 4-3(b). Analysis of the CD44 expression on the surface of T lymphocytes after ex vivo stimulation with H5N9 AIV infected APCs. Contours of the T lymphocyte populations stained with anti-chicken CD44 PE and anti-chicken CD4 or CD8 FITC. Histogram of the log fluorescence (Fl2) of CD44 expression (black) on T lymphocyte populations and the unlabeled control cells (gray). (C) $CD8^+$ T lymphocytes from Ad-HA and (D) $CD8^+$ T lymphocytes from PBS inoculated chickens co-cultured with AIV infected APC.

Table 4-1. Median fluorescence intensity of CD44 expression on ex vivo stimulated T lymphocytes from chicken 5 weeks p.i. with Ad-HA and PBS.

Source of T lymphocytes	APC	$CD8^+/CD44^+$	$CD4^+/CD44^+$
Ad-HA inoculated chickens [a]	AIV infected	464 (12)	406 (9)
PBS inoculated chickens [b]	AIV infected	288(2)	257(13)
Ad-HA inoculated chickens [a]	Uninfected	433 (10)	376(9)

[a] values represent the mean (SE) from 6 chickens
[b] values represent the mean (SE) from 4 chickens

Analysis of CD45 Expression on T Lymphocytes: CD45 is also a pan leukocyte marker and the level of its expression has been used to define the phenotype of memory and naïve T lymphocytes in rats (8). Flow cytometric analysis of the T lymphocytes labeled with anti-CD45 MAbs conjugated with PE revealed the presence of this molecule on both $CD4^+$ and $CD8^+$ T lymphocytes derived from either Ad-HA or PBS inoculated chickens. Similar to the CD44 expression, following ex vivo stimulation with AIV infected APCs , the median fluorescence intensity of CD45 expression was significantly greater on $CD8^+$ T lymphocytes than $CD4^+$ T lymphocytes. T lymphocytes of both the CD4 and CD8 phenotype derived from Ad-HA inoculated chickens had higher expressions of CD45 than those derived from PBS inoculated chickens as indicated by the levels of the fluorescence intensities (FIG. 4-4(a) and FIG. 4-4(b)). The activation of the T lymphocytes by the infected APCs had an impact on the expression of the CD45 on $CD8^+$ T lymphocytes since the fluorescence intensities of the CD45 expression on $CD8^+$ T lymphocytes co-cultured with uninfected APCs was significantly less than those co-cultured with infected APCs ($p \leq 0.001$). There was no difference in the levels of fluorescence intensity of CD45 expression on the $CD4^+$ T lymphocytes from Ad-HA chickens cultured with either infected or uninfected APCs (Table 4-2).

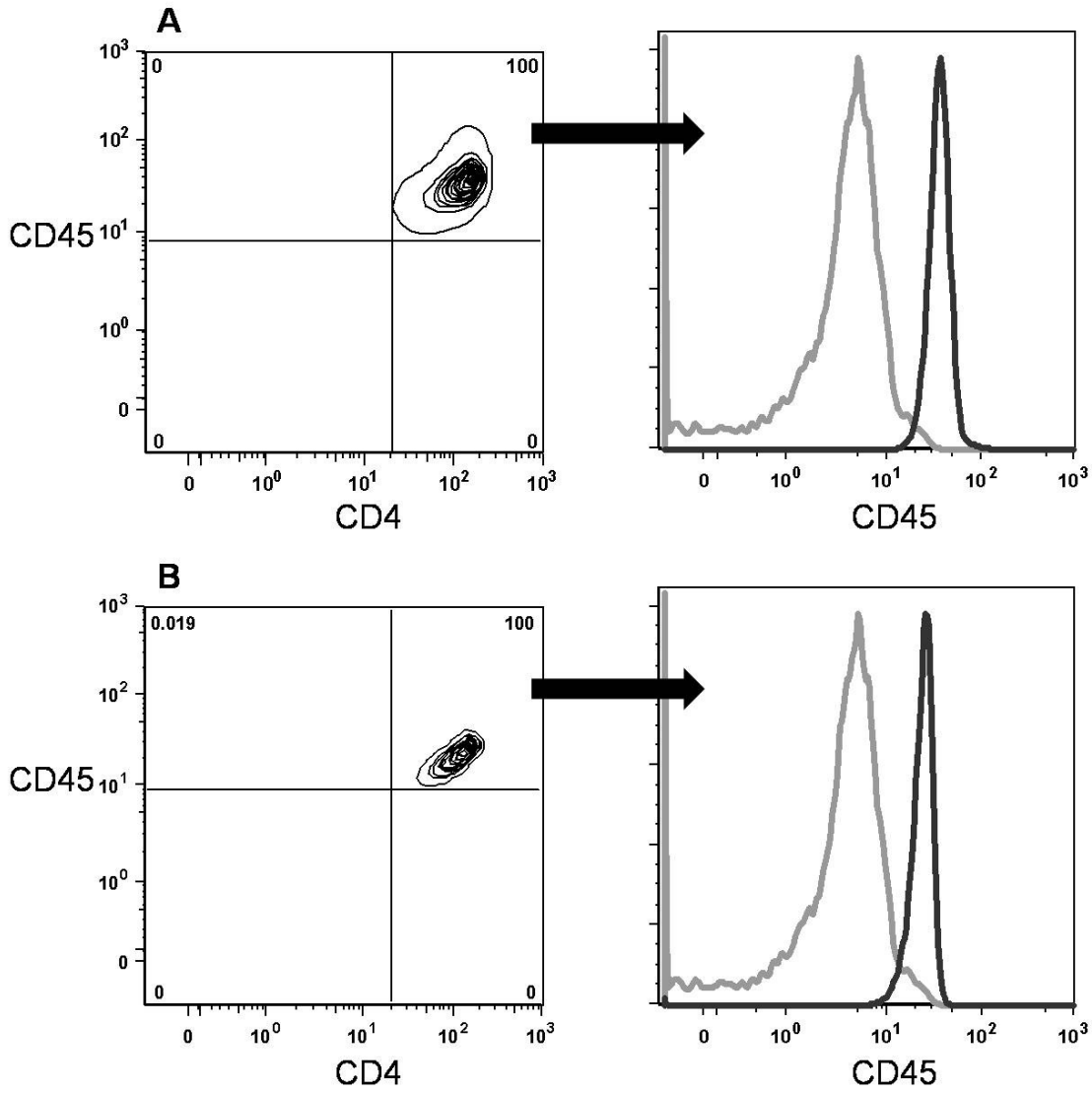

FIG. 4-4(a). Analysis of the CD45 expression on the surface of T lymphocytes after ex vivo stimulation with H5N9 AIV infected APCs. Contour plots of the T lymphocyte populations stained with anti-chicken CD45 PE and anti-chicken CD4 or CD8 FITC. Histograms of the log fluorescence (Fl2) of CD45 expression (black) on T lymphocyte populations and the unlabeled control T cells (gray). (A) $CD4^+$ T lymphocytes from Ad-HA ,(B) $CD4^+$ T lymphocytes from PBS.

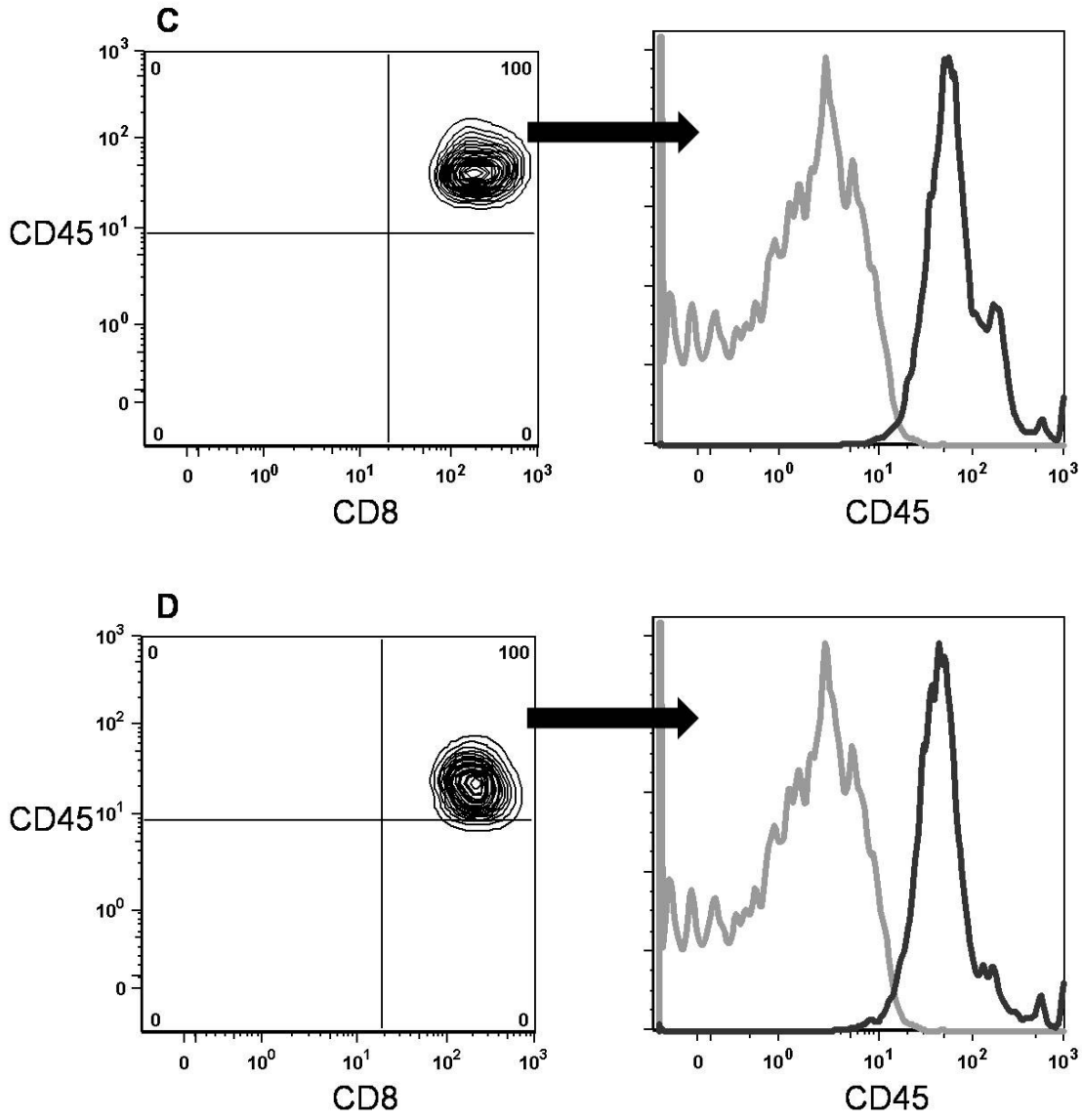

FIG. 4-4(b). Analysis of the CD45 expression on the surface of T lymphocytes after ex vivo stimulation with H5N9 AIV infected APCs. Contour plots of the T lymphocyte populations stained with anti-chicken CD45 PE and anti-chicken CD4 or CD8 FITC. Histograms of the log fluorescence (Fl2) of CD45 expression (black) on T lymphocyte populations and the unlabeled control T cells (gray). (C) $CD8^+$ T lymphocytes from Ad-HA and (D) $CD8^+$ T lymphocytes from PBS inoculated chickens co-cultured with AIV infected APC.

Table 4-2. Median fluorescence intensity of CD45 expression on ex vivo stimulated T lymphocytes from chicken 5 weeks p.i. with Ad-HA and PBS.

Source of T lymphocytes	APC	$CD8^+/CD45^+$	$CD4^+/CD45^+$
Ad-HA inoculated chickens [a]	AIV infected	47 (1)	34 (1)
PBS inoculated chickens [b]	AIV infected	28 (0.3)	22(1)
Ad-HA inoculated chickens [a]	Uninfected	43 (0.3)	34(0.3)

[a] values represent the mean (SE) from 6 chickens
[b] values represent the mean (SE) from 4 chickens

DISCUSSION

This is the first study that describes the phenotype of the memory and naïve T lymphocytes of the chicken. The study examined differential expression of pan leukocyte markers CD44 and CD45 on the surface of T lymphocytes. Studies with human and mice T lymphocytes have described differences in the expression of CD44 and CD45 on the memory and naïve T lymphocytes, implicating differences in the quality of the response mediated by them (1, 9, 10, 29, 30).

The CD44 high expression of the memory T lymphocytes has been shownto facilitate the activation by a lower antigenic stimulation as compared with activation needed for naïve T cells, probably by allowing stronger interactions with dendritic cells (1). Studies using purified MHC-I and antigenic peptide complexes immobilized on latex microspheres lacking any other adhesion or co-receptor molecules have demonstrated the ability of CD44 high memory T lymphocytes to respond to antigen without the requirement for co-stimulation (26). The ability of the memory T lymphocytes to extravasate and localize to non lymphoid tissue like skin, lungs and intestinal tract is also due to CD44 mediated adhesion with hyaluronic acid present in these tissues (29, 30 , 34).

In the current study, the T lymphocytes from Ad-HA inoculated chickens were shown to express higher levels of CD44 on their surface than T lymphocytes from PBS inoculated chickens. Moreover the expression of CD44 was greater on the $CD8^+$ T lymphocytes than on $CD4^+$ T lymphocytes from both Ad-HA and PBS inoculated chickens. Although the expression of CD44 on AIV stimulated memory $CD8^+$T

lymphocytes was greater than the unstimulated memory T lymphocytes, the difference was not significant enough to allow distinguishing of effector and memory $CD8^+$T lymphocytes on the basis of CD44 expression.

CD45 by virtue of its tyrosine phosphatase activity functions in the signaling during activation of the T lymphocytes (72). The different isoforms of CD45 allow distinction between naive and memory T lymphocytes of humans and mice (9, 30). Although Paramithioitis et al (83) have shown the presence of various isoforms of CD45 in chicken thymocytes, the expression of these isoforms in chicken memory and naïve T lymphocytes have not been established (83). However, the current study does clearly indicate greater expression of CD45 on either $CD4^+$ or $CD8^+$ chicken T lymphocytes derived from Ad-HA inoculated chickens than on T lymphocytes derived from PBS inoculated chickens. Additionally, the activation of the memory $CD8^+$ T lymphocytes with infected APCs resulted in a greater CD45 expression on the cells as compared to those with uninfected APCs.

Since the ex vivo stimulation of the T lymphocytes was mediated by non-professional APCs with MHC 1 expression, the responding T lymphocytes were primarily of $CD8^+$ phenotype. Hence, the current studies clearly establish that the chicken memory and naïve $CD8^+$T lymphocytes can be phenotypically distinguished on the basis of CD44 and CD45 expression. Although the expression of both CD44 and CD45 was found elevated on the $CD4^+$ T lymphocytes from Ad-HA inoculated chickens compared with those of PBS inoculated chickens, the distinction between memory and

naïve $CD4^+$ T lymphocytes would be established more definitely by assays evaluating $CD4^+$ T lymphocyte function.

In addition to CD44 and CD45, other surface adhesion and signaling molecules, such as CD62L and CD27, have been show to be important for distinguishing the phenotype of memory and naïve T lymphocytes in humans and mice (1, 9). Identification of the chicken homologues of these molecules and the subsequent development of antibodies that can recognize these molecules would be of value in determining the function and development of T lymphocyte mediated responses in chickens.

CHAPTER V

CONCLUSIONS AND DISCUSSION

Infections with the avian influenza viruses (AIV) are concerns for veterinary and public health. Although shore birds and waterfowl are reservoirs for subclinical infections, the incidental infection of and adaptation to domestic poultry with these viruses cause mild respiratory and enteric disease to severe systemic disease that can even result in 100% fatality (2, 3, 17, 18).Since 1996, the increased incidence of the highly pathogenic AIV infection of humans in many Asian, African and European countries by direct transmission from poultry has made AIV a zoontoic threat (2, 78) . Although human-to-human transmission of the H5N1 AIV is rare, there are risks of emergence of a reassortant transmissible human virus with genes of avian origin viruses (16, 129, 136). The viruses of the influenza pandemics of 1918, 1957 and 1968 and the currently circulating swine origin H1N1 influenza virus encode genes of viruses of avian, swine and human origins (2, 6, 16, 18, 39). Although pre-emptive mass slaughter policies practiced in the event of highly pathogenic AIV outbreaks in poultry curtails the spread of the virus, extensive economic losses are inevitably sustained (16, 27). Highly pathogenic strains with an increase in infectious viral loads within the bird are considered a risk to both birds and mammals (27, 115). Therefore, it is of great importance for both veterinary and public health to develop vaccines that limit viral proliferation and spread in chickens.

Currently, the available vaccines used in chickens rely on generating antibody mediated immunity primarily against the viral hemagglutinin (HA) and to a lesser extent, neuraminidase (NA) glycoproteins (3, 21, 22). Such humoral immunity protects against the viral strains expressing homologous HA protein and reduces shedding of viruses expressing homologous NA protein (3, 21, 22). However, with the presence of strains with any one of 16 different HA subtypes and any one of 9 different NA subtypes, the efficacies of the vaccines are limited for variant or heterologous viruses (3,5). Therefore, such vaccine strategies, in the absence of effective cross protection, require continuous development of new relevant vaccines that address the threats of a variety of serologically distinct strains (3, 9).

In contrast, the induction of $CD8^+$ T lymphocyte immunity can greatly diminish the clinical disease by clearing viral infected cells. Additionally, the $CD8^+$T lymphocytes target more conserved epitopes and hence have the potential to cross react with various viral subtypes (67, 68). Adoptive transfer studies have shown viral specific $CD8^+$ T cells to be protective against a serologically heterologous avian influenza virus (100, 102). The responses of T cells to antigen by the initial activation of naïve cells can be described as the primary effector response, followed by the eventual appearance of memory cells which after activation by boosting or restimulation mediate the secondary effector response (1, 140).

The use of commercially available inactivated whole AIV and fowl poxvirus vectored vaccines against AIV is discouraged or prohibited in many countries, such as the United States (16, 26, 47, 110). While the inability to distinguish infected from

vaccinated animals (DIVA) has limited the use of inactivated whole virus vaccine as a control strategy for AIV, pre-existing immunity to fowl poxvirus precludes the development of optimum protective immune response against AIV by this vectored vaccine (110, 114, 115, 122). Incomplete protection allows low pathogenic AIV to survive and circulate in flocks and potentially mutate into highly pathogenic strains (112, 113).

The adenovirus vectored vaccine expressing influenza virus proteins have been shown to stimulate $CD8^+$ T lymphocytes in mice and swine (38, 50, 51, 53, 103, 117, 133). The non-replicating (RCA- free) human Ad5 vector expressing H5N9 AIV HA (Ad-HA) developed by Toro et al provided partial to complete protection in chickens challenged with highly pathogenic H5N1 and H5N2 AIV strains (124).

Development of safe and efficacious vaccines that have the ability to cross protect is critical for the prophylactic control of AIV. Vaccines that activate $CD8^+$ T cell mediated immune responses, in addition to neutralizing antibody response, provide a rational approach for achieving cross protection. While the antibody mediated immune response to individual AIV proteins is well studied, the kinetics of the AIV specific T lymphocyte responses to these proteins have not been described in chickens. **Ultimate protection against AIV challenge infection requires immunologic memory that is relevant for the virus.** Memory responses are thus, the essence of an efficacious vaccine. The rationale of this study was to identify the AIV proteins that can activate $CD8^+$ memory T lymphocyte responses providing tools to develop more efficacious

vaccines against broader spectrum of AIV infections in chickens, which unlike mice, can be naturally infected by the field strains of these viruses.

The study describes the modulation of chicken T lymphocyte responses specific to AIV HA and NP proteins. This study focused on delineating the responses of chicken memory $CD8^+$ T lymphocytes to specific AIV hemagglutinin (HA) and nucleocapsid (NP) proteins expressed by plasmids or non replicating human adeno Ad5 virus vector and describing the kinetics of both primary and secondary responses of T cells. Additionally, this study identifies markers that differentiate the phenotype of the memory and naïve chicken T lymphocytes based on the expression of the pan leukocyte CD44 and CD45 markers on the surface of T lymphocytes.

Studies evaluating the $CD8^+$ T lymphocyte responses to influenza virus in mice have identified epitopes in the NP as being dominant in induction of specific $CD8^+$ T cells (35, 61, 82, 126, 134, 146). In contrast, the repertoire of human $CD8^+$ T lymphocytes has been found to be broader and the responses are directed against multiple influenza viral proteins, including NP and HA (42,56, 62, 66). This study also demonstrated that similar to the responses in humans, the memory $CD8^+$ T lymphocytes of chickens respond to both AIV HA and NP proteins expressed by either plasmid or a non replicating (replication competent (RCA)-free) human Ad5 vector (41, 56, 62, 77). This, primarily MHC-I restricted, response ex vivo stimulated specifically by the AIV infected non-professional APCs resulted in the activation and proliferation of $CD8^+$ T lymphocytes. Similar to the MHC-I restricted T lymphocyte responses stimulated by whole IBV or AIV, the primary effector $CD8^+$ T lymphocytes resulting from inoculation

of adenovirus vector expressing either HA or NP was observed at 10 days p.i. (99, 102). The decline in effector T lymphocyte responses at 16 days p.i. was followed by emergence of AIV specific memory T lymphocyte responses. AIV protein specific memory $CD8^+$ T lymphocyte responses induced by either plasmid or adenovirus vector expressing AIV proteins were detected by 3 weeks p.i. and increased until 5 weeks p.i. The response of the memory T lymphocytes to either protein declined after 5 weeks p.i. to nearly undetectable or undetectable levels by 8 to 9 weeks p.i. (83). Consistent with our studies, a decline in the protection of chickens against a challenge of H5N1AIV, 10 weeks after infection with an H9N2 AIV has been observed by Seo and Webster (100, 102).

The magnitude of the responses of viral specific memory T lymphocytes was consistently lower than that by the primary effector T lymphocytes. However, $CD8^+$ T lymphocytes were the primary inducers of this response because the MHC matched CKC used for ex vivo stimulation were non-professional APCs. The non-professional APCs express MHC-I, in the absence of MHC-II and thus stimulate $CD8^+$ T lymphocytes rather than $CD4^+$ T lymphocytes. In order to further substantiate that the stimulated T cells expressed the CD8 rather than the CD4 antigen, populations enriched by negative selection using antibody coated Dynabeads (Invitrogen, La Jolla, CA) were stimulated and the response of $CD8^+$ T lymphocytes were observed to be significantly greater than the response of $CD4^+$ T lymphocytes.

While comparing the responses of individual AIV proteins NP expressed by either vector was observed to be a significantly more effective at inducing $CD8^+$ T

lymphocytes than HA at 3 and 5 weeks p.i. when memory responses were most evident. Consistent with this greater response to NP than HA stimulated by the homologous virus, the T lymphocytes from the chickens inoculated with NP had a greater cross-reactive response to the heterologous H7N2 virus than T cells from the birds inoculated with HA. It is possible that NP has more common T lymphocyte epitopes since, the amino acid identity between NP from H5N9 AIV and H7N2 AIV was 98% while that of HA was 41%.

After reactivation by a secondary or booster inoculation, the secondary effector response appeared and declined more rapidly than the primary effector response (Chapter III). Whereas the initial effector response is greater than the memory response, the booster effector was even greater than the primary effector activity. Administration of a booster dose of Ad-HA stimulates a 16 fold increase in the anti-HA antibody titer and a secondary effector T lymphocyte response which is induced more rapidly and has a higher magnitude than the primary effector T lymphocyte response. The ability of Ad-HA to induce a secondary immune response in chickens is not inhibited by the immunity against this non-replicating adeno virus vector.

In the absence of an IFNγ ELISA, the initial studies evaluated IFNγ produced by activated T cells using NO production from stimulated macrophage cells. Although not commonly used to evaluate T cell responses, the latter assay was in our hands highly reproducible with strict standardization of cell conditions, such as the concentration, passage number and incubation. However, with the availability of a recently marketed commercial chicken IFNγ assay, an ELISA was used to evaluate the secretion of IFNγ

by the T lymphocytes ex vivo stimulated with AIV infected APCs. The results observed by this assay were corroborated with the simultaneously executed indirect NO production assay. However, the background as determined by PBS and AdE controls was actually lower using the ELISA as compared with the macrophage NO secretion to detect IFNγ. Therefore, the ELISA is not only more efficient and less expensive, but also is likely more specific at detecting T lymphocyte activation.

The phenotypic difference between memory and naïve T lymphocytes of the chickens 5 weeks p.i. with Ad-HA, when the memory responses were greatest, was also described in these studies. The pan leukocyte markers for chicken CD44 and CD45 were available to quantify their expression on the surface of T lymphocytes from birds inoculated with either Ad-HA or PBS. Studies with human and mice T lymphocytes have implicated that the differences in the expression of CD44 and CD45 on the memory and naïve T lymphocytes impact the quality of the response mediated by them (1, 9, 10, 29, 30). The mice CD44 high expressing T lymphocytes were activated by lower levels of antigenic stimulation than CD44 low expressing T lymphocytes (26). The homing and migration pattern of the T lymphocytes expressing higher or lower levels of CD44 were also shown to differ (1, 9). CD45 is a tyrosine phosphatase and functions in signaling during activation of the T lymphocytes (72). Naive and memory T lymphocytes of humans and mice have been shown to express different isoforms of CD45 and in rats the differential level of CD45 is the basis to distinguish naïve from memory T lymphocytes (9, 30).

In the current study, it was observed that both $CD4^+$ and $CD8^+$ T lymphocytes from Ad-HA inoculated chickens expressed higher levels of CD44 and CD45 than the T lymphocytes derived from PBS inoculated chickens. The surface expression of both CD44 and CD45 was greater on T lymphocytes with $CD8^+$ than $CD4^+$ phenotype. Although ex vivo stimulation of memory $CD8^+$T lymphocytes with APCs expressing AIV proteins had no effect on the CD44 expression, the expression of CD45 on stimulated memory cells was observed to be greater than in the absence of AIV specific APC stimulation.

In conclusion both AIV HA and NP delivered by plasmid DNA or a non replicating (RCA-free) Ad5 human adenovirus vectored vaccine have the ability to stimulate memory $CD8^+$ T lymphocytes that can be activated by both homologous and heterologous AIV viruses. This non-replicating Ad5 human adenovirus vectored vaccine can be used effectively in a homologous prime-boost vaccination program in chickens AIV. The phenotype of the chicken memory $CD8^+$ and naïve $CD8^+$ T lymphocytes can be distinguished by the levels of the expression of CD44 and CD45 molecules on their surface.

Future studies should be aimed at determining the response of $CD8^+$ T lymphocytes to other AIV proteins besides HA and NP. Our preliminary studies have indicated that the M1 and to a lesser extent M2 may have T cell epitopes. Since there are estimated to be at least 30 distinct MHC chicken haplotypes, additional studies should also evaluate the $CD8^+$T lymphocyte repertoire to AIV in different MHC-I defined lines of chickens. The identification of the chicken homologues to other surface

adhesion molecules on mice and human T lymphocytes such as CD62L and CD27 would be relevant in defining the phenotype of and mechanisms of action for the response T lymphocytes in chickens. Development of reagents to detect cytokine expression by the T cells following activation would be helpful in understanding and manipulating the avian immunology. Only vaccine strategies that maximize induction of both memory cross-reactive $CD8^+$ T lymphocyte responses and humoral immunity in chickens can address the challenge of providing protective immunity against a broader range of AIV types.

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