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THE SUBTYPE SPECIFIC AND CROSS-REACTIVE T CELL RESPONSES TO INFLUENZA VIRUSES IN HUMANS

A Dissertation Presented

By

JENNY AURIELLE BALILI BABON

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

03 APRIL 2012

IMMUNOLOGY AND VIROLOGY

THE SUBTYPE SPECIFIC AND CROSS-REACTIVE T CELL RESPONSES TO INFLUENZA VIRUSES IN HUMANS

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And I have come to the end of tunnel. That was one long tunnel!

As I reflect on my training as a scientist and the time I spent in graduate school, I have come to realize that I am truly blessed! I am surrounded by a bunch of wonderful people who have helped me throughout this journey and I appreciate everything that they have contributed to who I am today.

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ABSTRACT

Human influenza is a contagious respiratory disease resulting in substantial morbidity and mortality worldwide. With the recent cases of avian influenza infections in humans and the heightened concern for an influenza pandemic arising from these infections, it is essential to understand host responses that would confer protective immunity to influenza. The cell-mediated immune responses to influenza virus play an important role during influenza infection.

To analyze the specificity and diversity of memory T-cell responses, we performed a genome-wide screening of T cell epitopes to influenza A virus in healthy adult donors. We identified a total of 83 peptides, 54 of them novel, to which specific T cells were detectable in interferon-(IFN-γ) enzyme-linked immunosorbent spot assays (ELISPOT) using peripheral blood mononuclear cells (PBMCs) from four healthy adult donors. We found that among 11 influenza viral proteins, hemagglutinin (HA) and matrix protein 1 (M1) had more T-cell epitopes than other viral proteins. The donors were not previously exposed to H5N1 subtype, but we detected H5 HA T cell responses in two of the four donors. To confirm that HA is a major target of T cell responses we also analyzed H1 and H3 HA-specific T-cell responses using PBMC of additional 30 adult donors. Fifteen out of thirty donors gave a positive response to H3 HA peptides, whereas five of thirty donors gave a positive response to H1 HA peptides.

Because we detected T cell responses to the H5 HA peptides in donors without prior exposure to H5N1 subtype, we asked if cross-reactive T cells to H5 HA peptides

can be attributed to a prior exposure to H2N2 subtype, the closest HA to the H5 based on their phylogeny. We compared younger donors who have no prior exposure to H2N2 subtype and older donors who were likely to be exposed to H2N2 subtype, and both groups responded H2N2 peptides at similar level, suggesting that memory T cells cross-reactive to H5 HA peptides can be generated by prior exposure to the H1N1 and H3N2 subtypes, and the exposure to H2N2 subtype is not necessary. We subsequently identified a $CD4^+$ T cell epitope that lies in the fusion peptide of the HA. This epitope is well conserved in all 16 subtypes of HA of influenza A and the HA of the influenza B virus. A $CD4^+$ T cell line specific to this epitope recognizes target cells infected with various influenza A viruses including seasonal H1N1 and H3N2, a reassortant H2N1, the 2009 pandemic H1N1, H5N1 and influenza B virus in cytotoxicity assays and intracellular cytokine staining assays. Individuals who have the HLA-DRB1*09 allele have *ex vivo* IFN-γ responses to this epitope peptide in ELISPOT. Although natural infection or standard vaccination may not induce strong T and B cell responses to this very conserved epitope in the fusion peptide, it may be possible to develop a vaccination strategy to induce these $CD4^+$ T cells which are cross-reactive to both influenza A and B viruses.

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ABBREVIATIONS

^{51}Cr	radiolabeled chromium
a	avian
A/PR8	A/Puerto Rico/8/34 (H1N1)
AIDS	acquired immunodeficiency syndrome
AIM-V	Adoptive Immunotherapy Media V
APC	allophycocyanin
APC	antigen presenting cell
ASC	antibody secreting cell
BEI	Biodefense and Emerging Infections
BLAST	Basic Local Alignment Search Tool
BLCL	B lymphoblastoid cell line
BSA	bovine serum albumin
BSL	biosafety level
CD	cluster of differentiation

CDC	Centers for Disease Control and Prevention
CEF	cytomegalovirus, epstein-barr virus, influenza virus
CFR	case fatality rate
CMV	cytomegalovirus
cRNA	complementary ribonucleic acid
CTL	cytotoxic T lymphocyte
DMSO	dimethyl sulfoxide
EBV	epstein-barr virus
ELISPOT	enzyme-linked immunosorbent spot
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein
FSC	forward scatter
h	human
HA	hemagglutinin
HAI	hemagglutination-inhibition

HEF	hemagglutinin esterase fusion protein
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSI	heterosubtypic immunity
IAV	influenza A virus
IBV	influenza B virus
IC_{50}	half maximal inhibitory concentration
ICS	intracellular cytokine staining
IEDB	Immune Epitope Database
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LAIV	live attenuated influenza vaccine
LDA	live/dead aqua
M1	matrix 1 protein
M2	matrix 2 protein

MACS	magnetic-activated cell sorting
MHC	major histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MxA	myxovirus resistant A
NA	neuraminidase
NCBI	National Center for Biotechnology Information
ND	not determined
NEP	nuclear export protein
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NK	natural killer
NLR	NOD-like receptor
NP	nucleoprotein
NS1	non-structural protein 1
NS2	non-structural protein 2

OAS	2',5'-oligoadenylate synthetase
ORF	open reading frame
PA	polymerase acid
PAMP	pattern associated molecular pattern
PB1	polymerase basic 1
PB2	polymerase basic 2
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PE	phycoerythrin
Per-CP	peridinin-chlorophyll protein
PHA	phytohemagglutinin
PKR	protein kinase R
PMA	phorbol 12-myristate 13-acetate
pMHC	peptide-MHC complex
preHA	precursor form of hemagglutinin
PRR	pattern recognition receptor

rh	recombinant human
RIG-I	retinoic acid inducible gene-I
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPMI	Rosewell Park Memorial Institute cell culture medium
SCID	severe combined immunodeficiency
SD	standard deviation
SFC	spot forming cells
SIL	specific immune lysis
SSC	side scatter
TCR	T cell receptor
TIV	trivalent inactivated vaccine
TNF	tumor necrosis factor
vRNA	viral ribonucleic acid
WHO	World Health Organization

PREFACE

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Chapter III:

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Chapter IV:

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Terajima, M., J. Cruz, A. M. Leporati, L. Orphin, J. A. Babon, M. D. Co, P. Pazoles, J. Jameson, and F. A. Ennis. 2008. Influenza A virus matrix protein 1-specific human CD8+ T-cell response induced in trivalent inactivated vaccine recipients. J Virol 82:9283-9287.

CHAPTER I

INTRODUCTION

Influenza is a contagious respiratory illness in humans ranging from mild to severe, and at times can lead to death. It causes significant morbidity and mortality worldwide. There are, on average, more than 200,000 hospitalizations associated with influenza illness each year (220). In a survey of influenza-related mortality in the United States between 1976 and 2007, the number of deaths can range from 3,000 to 48, 000 (32). The elderly and children younger than five years are more susceptible to influenza, as shown by the rates of influenza-associated primary respiratory and circulatory hospitalizations (220). Influenza A viruses (IAV) are the major type of influenza virus that causes disease in humans, and while influenza B viruses (IBV) can also infect humans, they do so to a less severe extent (151). These viruses cause acute illness and do not result into persistent infections in humans, but they are maintained in circulation in the population and are detectable all year round by direct person-to-person spread during acute infections (240).

A. The nature of influenza viruses

The influenza viruses are segmented negative stranded RNA viruses that belong to the family *Orthomyxoviridae*. There are three genera or types of influenza virus - *influenzavirus A*, *influenzavirus B*, and *influenzavirus C* - based on the antigenic

differences in the matrix protein (M) and the nucleoprotein (NP) (124). Influenza A, B, and C viruses have a common evolutionary precursor (151). Based on comparative sequencing studies using the hemagglutinin (HA) protein component, it is estimated the IAV HA gene diverged from the IBV HA gene more recently than from the hemagglutinin-esterase-fusion (HEF) gene, the HA equivalent in influenza C virus (212). The divergences between the different subtypes of IAV HA genes are estimated to have occurred from several thousand to several hundred years based on the rate of amino acid substitution in HAs isolated from aquatic birds (212). Influenza B and C viruses seem to be near or at an evolutionary equilibrium in humans, while the genes of type A viruses were introduced into the human population less than 150 years ago and were most likely derived from birds (240).

Influenza A viruses are classified into subtypes, which are determined by differences in the nucleic acid sequences of the hemagglutinin (HA) and the neuraminidase (NA) viral proteins (151). At present, there are 17 known HA subtypes and nine NA subtypes (67, 222, 231). Except for the most recently identified H17 HA which was isolated from a bat species (222), all 16 HAs and nine NAs have been isolated and identified from wild aquatic birds, which are considered to be the natural reservoir for IAV (240). IAV can also naturally infect swine, horses, seals, whales and mink (151). Virus strains are named accordingly and they include the host of origin (if the host organism is not human), geographic location of the first isolation, strain number and year of isolation, with the particular subtype in parenthesis (4). Thus, an IAV that was the 8th virus isolated from a person in Puerto Rico in 1934 was given the name A/PuertoRico/8/1934 (H1N1), while

an IAV isolated from a duck in Vietnam is named A/Dk/Vietnam/568/2005 (H5N1). Influenza B viruses are not classified into subtypes, although strains are usually identified by the lineage that they belong to. IBV strains that have been described in the last century or so started out as a homogenous group that eventually diverged into two distinct lineages – the B/Victoria and the B/Yamagata lineages (179). IBV strains are also named following the guidelines for IAV, except that there is no subtype designation (4).

i. Virus structure and protein components

The IAV particles are usually spherical in shape with a diameter of about 100 nm, although filamentous particles that can be as large as 300 nm have been observed in certain conditions (151). The virus structure is quite complex and is depicted in Figure 1.1. It is characterized by distinctive spikes comprising of the HA and NA proteins that are jutting out of the viral lipid envelope, which is derived from the host cell membrane where the virus has previously replicated (151). The matrix 2 protein (M2) also comprises the viral envelope (125) and serves as an ion-channel, playing an important role in the release of the viral genome into the cytoplasm (37, 157). Underlying the viral envelope is a shell of matrix 1 protein (M1) (69) that encapsulates the ribonucleoprotein (RNP) complex. The RNP complex is made up of an RNA segment in close association with the nucleoprotein (NP) and the viral polymerase proteins PB1, PB2 and PA

Figure 1.1. A schematic representation of the structure of influenza A virus. Reprinted with permission from Nelson and Holmes *Nature Reviews Genetics* 2007: **8**, 196–205. (License No. 2845511024333).

(113, 146). IAV and IBV are made up of the eight RNA segments, while influenza C viruses only have seven segments. These RNA segments have a negative sense, are single-stranded, and comprise the influenza viral genome (151). The eight RNA segments and the corresponding viral protein they encode for are listed in Table 1.1.

More recently, an alternative open reading frame in the PB1 gene was identified encoding for a novel influenza protein called PB1-F2 (36). It localizes in the mitochondria and promotes apoptosis in cells that are exposed to synthetic PB1-F2 (36). A third major polypeptide from the PB1 segment called N40 has also been recently described which is derived from a differential AUG codon usage (237). Not all influenza isolates express the PB1-F2 and N40 proteins. The non-structural proteins 1 and 2 (NS1 and NS2) are only found in infected cells and are not part of the virion.

Influenza B viruses are very similar to IAV by electron microscopy (181). The IAV and IBV may have the same number of RNA segments, the IBV genome only encodes for ten proteins, as the PB1-F2 protein or the N40 has not yet been identified in IBV (151). Table 1.1 highlights the main differences between IAV and IBV in terms of their genome and sequence similarities between related proteins.

Table 1.1. Comparison of IAV[a] and IBV[b] viral proteins.[d]

Segment	Name	aa length in IAV	aa length in IBV	aa similarity (%)
1	PB2	759	770	37
2	PB1	757	750	61
3	PA	716	726	36
4	HA	566	585	28
5	NP	498	566	37
6	NA	469	466	30
7	M1	252	248	31
7[c]	M2/BM2	97	109	26
8	NS1	230	281	very low
8[c]	NS2/NEP	121	122	24

[a] Prototype strain used is A/Sydney/5/1997 (H3N2).

[b] Prototype strain used is B/Florida/02/2006.

[c] The alternate ORF of this protein is encoded by a spliced mRNA within the vRNA segment.

[d] Table is modified from (98).

ii. Influenza A virus life cycle

The influenza virus infects the host cell through the binding of the viral HA to sialic-acid containing receptors on the cell membrane (233, 235). The human influenza A viruses preferentially bind to sialic acid residues attached to galactose by an α-2,6 linkage, while the avian influenza A viruses prefer the α-2,3 linkage (43) . These α-2,6 sialic acid receptors are preferentially expressed on human airway epithelial cells (70). The virus is internalized via the endocytic pathway, where the low pH in the endosomes allows for the acidification of the virus interior by pumping H^+ through the M2 ion channels leading to the dissociation of M1 from the RNP (92). The low pH also causes a structural change in the HA, promoting the fusion of the viral membrane with the endosomal membrane (51, 198) and releasing the dissociated RNPs into the cytoplasm.

The RNPs, which contain the genetic material needed for the replication of the virus, are then transported into the nucleus via interactions with NP (161), where the viral RNA segments are transcribed into mRNA through the aid of the viral polymerase PB1 (95). The polymerase PB2 cleaves 5'-capped fragments from newly synthesized host cell mRNA that will serve as primers for viral mRNA synthesis, a phenomenon called cap-snatching (22, 117, 159). A poly-A tail is also added to the newly transcribed viral mRNA (160). This mRNA is transported back into the cytoplasm for protein translation (193), where newly synthesized HA, NA and M2 proteins are shuttled to the cell membrane through the ER and the Golgi apparatus (144, 201) while the polymerase proteins PB1, PB2, PA and NP are brought back to the nucleus to form new RNP

complexes (201). On the other hand, NS1 sequesters host mRNA in the nucleus and prevents pre-mRNA splicing and polyadenylation, providing an ample supply of cap structures for viral transcription while inhibiting host gene expression (166).

The PA is required for both transcription of viral proteins and replication of the viral genome (87, 143). M1 and NS2 mediate the nuclear export of the newly formed RNP (131, 147). M1 associates with the RNP and both are brought , by yet unknown mechanisms, to the apical side of the plasma membrane where the HA, NA and M2 proteins are assembled, eventually packaging the M1 and RNP complex into new virus particles (144). The virus particles are released from the cell membrane by the action of NA, where it cleaves off the sialic acid from the virion and the cellular glycoproteins (144).

iii. The Influenza Hemagglutinin

The HA is one of two surface viral glycoproteins, making up about 25% of the total viral protein (142). As described briefly above, HA is indispensable in the viral life cycle because it is necessary for binding the viral receptor on target cells and mediating the fusion of viral and cellular membranes (48, 199). The active form of HA consists of trimer of identical subunits that are anchored in the viral membrane due to the hydrophobic transmembrane sequences in the C-terminal region of the protein (199). The two subunits that consists a monomer of HA, HA1 and HA2, are linked by a disulfide bond, and they are products of the enzymatic cleavage of the precursor protein

HA0. This cleavage step occurs extracellularly by a trypsin-like protease that is restricted in the respiratory tract epithelia (73) and renders the virus infectious. For some of the HAs of the H5 and H7 subtypes, polybasic sequences are inserted at the cleavage site, allowing these HAs to be cleaved intracellularly by furin-like enzymes and is thought to be related to the widespread systemic and virulent infections by these subtypes in birds (199).

The homology among the different IAV HAs is variable. H2 and H5 HA are the most closely related HA (80% homology), while H3 and H1 HA are the most divergent (25% homology) (7, 67) . Most of the homology is located in the HA2 subunit, including the highly conserved fusion peptide sequence (48). In fact, analysis of evolutionarily conserved sequences in the different influenza A viral components reveal that the FGAIAGFIE sequence of the fusion peptide is the only region in the HA protein that is 98-100% conserved in influenza viral strains of the different human and avian influenza subtypes that circulated between 1997 to 2006 (91). Comparisons between the HAs of A/Puerto Rico/8/34 (A/PR/8) and B/Lee/40 also show significant conservation in the first 12 amino acids of the fusion peptide sequence (118). This conservancy is probably due to the critical role of this domain in triggering fusion and destabilizing target membranes during the fusion process (48).

B. Influenza genetics and epidemiology

Influenza viruses undergo constant antigenic variation to escape the host immune response. This characteristic largely defines the epidemiology of the viruses. These variations are brought about by two distinct mechanisms that are influenced by the nature and design of the viral genome as well as by selective immune pressure. Antigenic drift is a consequence of the error-prone RNA polymerase-dependent replication, which introduces point mutations leading to gradual antigenic changes in the HA or NA proteins (231). Some drift variants can amplify and survive because of escape from neutralizing antibodies (240). These gradual changes may also affect host species range and influence disease severity. Drift variants can occasionally cause epidemics and can typically prevail for two to five years before it is replaced by another variant (240). The HA is the major antigenic component of influenza and all neutralizing antibodies that confer sterilizing immunity are targeted to the HA. Five antigenic domains have been defined by studying the structure of H3 HA, all of which are located in the HA ectodomain (240). Antibodies to NA have also been detected and they help to prevent cell-to-cell spread of the virus, but do not neutralize it (112). Antigenic drift in the NA has also been reported (240).

A second mechanism in which viral diversity is achieved is through antigenic shift. A shift is brought about by the reassortment of the viral RNA segments from one virus subtype with the genes of a different subtype. This typically occurs between human and avian viruses, where *in vivo* reassortment has been seen among human and avian strains

as well as between human and avian strains (240). Antigenic shift can also occur by direct transmission of an avian or swine virus into the human population (29). This "mixing" of genes can generate viral proteins that are now immunologically distinct from previously circulating strains. When a virus subtype resulting from an antigenic shift is able to successfully establish transmission within a species, in particular in humans, higher infection rates and increased morbidity and mortality are observed, which can ultimately lead to a pandemic (240).

C. Influenza pandemics: a historical perspective

Influenza pandemics occur when there are major changes brought about by antigen shift, introducing a novel influenza virus to which human population has not been previously exposed. In theory, novel influenza viruses that are encountered by our immune system have the capacity to initiate an influenza pandemic if they accrue enough mutations that would allow them to infect human cells and be efficiently transmitted from one person to the next (241). In the last 400 years, about 31 pandemics have been recorded (127), four of which occurred in the recent century (Figure 1.2). The mortality impact of these pandemics can range from devastating to mild (127), as exemplified by the 1918 "Spanish" influenza pandemic and the 2009 swine-origin H1N1 pandemic respectively.

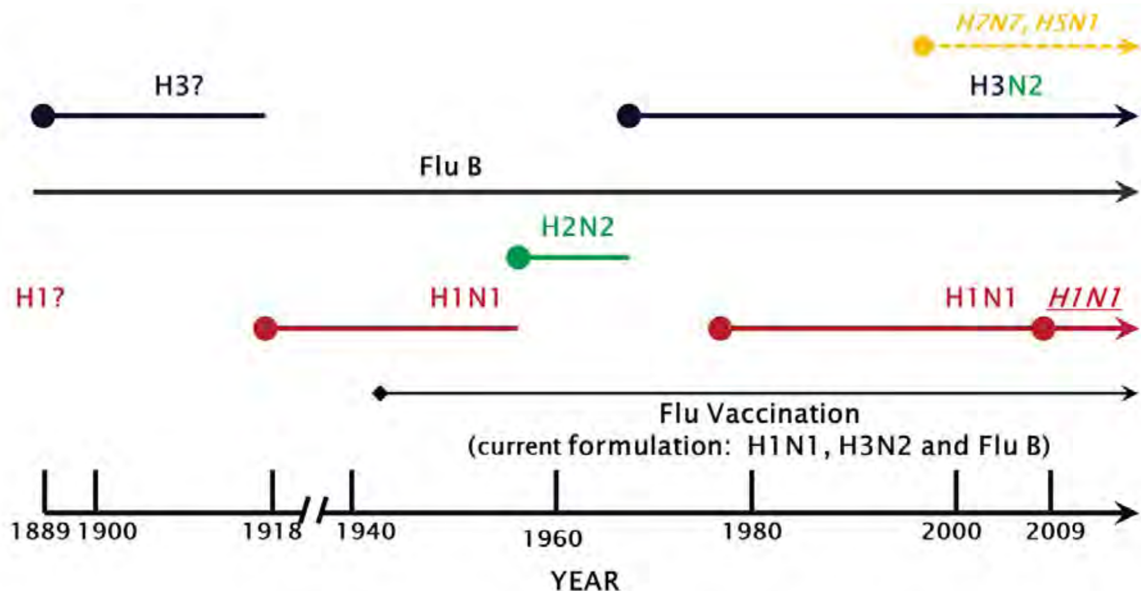


Figure 1.2. Influenza through the years. We are constantly exposed to influenza antigens in our lifetime. Influenza viruses have been circulating in the human population in the last hundred years or so. As of late, only three IAV subtypes have successfully established human-to-human transmission: H1N1 emerged in 1918 and was replaced by H2N2 in 1957. H3N2 replaced H2N2 in 1968 and has been circulating since then, amidst constant antigen drift. The H1N1 subtype re-emerged in 1977 and is believed to be related to the H1N1 of the 1940's. It co-circulated with H3N2 until the emergence of a new H1N1 subtype in 2009, which has effectively replaced the previous H1N1. In the past fifteen years, some avian influenza subtypes (e.g. H5N1, H7N7) have directly infected humans, although transmission within the human population has not been established. IBV co-circulates with IAV. In the 1940's, influenza vaccination was introduced and since then has been available annually.

A usual hallmark of pandemic influenza is the distinct shift in the mortality rate, where younger individuals are more affected. During the three pandemics of the 20th century, a significant proportion of influenza-related mortality included individuals less than 65 years old (196).

The first influenza pandemic of the 20th century came in three distinct waves in 1918 and spread throughout Europe, Asia and North America (14, 214). Called Spanish flu, it is deemed to be the deadliest pandemic in history, where an estimated 50 million people died from influenza, more than the number of people killed during World War I, which was happening simultaneously (106). The young and healthy (between 15 to 35 years old) were disproportionally affected and 99% of the deaths were in people younger than 65 years old. Taubenberger and colleagues were able to recover the genomic RNA of the 1918 virus from archived formalin-fixed lung autopsy material (215) and from frozen, unfixed tissue from an influenza victim who was buried in permafrost in November 1918 (170) and performed initial genetic characterization of the 1918 flu virus, identifying it as subtype H1N1virus that may have arose from an avian-like predecessor that was able to adapt to humans (170).

The succeeding pandemics that came after 1918 were milder and had fewer excess deaths. These happened in 1957 (Asian flu, H2N2), 1968 (Hong Kong flu, H3N2) and 1977 (Russian flu, H1N1) (96) (Figure 1.2). The 1957 pandemic was caused by an H2N2 subtype, effectively replacing the H1N1 that had been in circulation. This new subtype had acquired novel HA, NA and PB1 genes from an avian H2N2 virus (185,

240). H2N2 circulated for about ten years and has not been detected in the human population since 1968. The H3N2 subtype is also thought to have originated from China and caused an epidemic in Hong Kong in 1968, eventually spreading to other countries (41). The HA of the previously circulating H2N2 was replaced with an avian-derived H3 HA gene segment, retaining the N2 NA gene segment (185, 232). The PB1 gene segment was also replaced, and is speculated to be derived from an avian source as well (110). In 1977, an H1N1 subtype caused epidemics in Russia and China that quickly spread globally and primarily affected younger people with relatively mild presentation (111). It has been determined that the 1977 H1N1 is closely related to H1N1 strains that were isolated between 1947 and 1957 (186). The 1977 H1N1 subtype did not replace the H3N2 in circulation; instead it co-circulated with H3N2 and maintained its presence in seasonal epidemics (251), alongside IBV (Figure 1.2).

More recently, a novel H1N1 subtype emerged in 2009 (6). The 2009 H1N1 caused large epidemics in Mexico and the United States that eventually spread across the globe (2, 33, 99). The 2009 H1N1 was eventually determined to be of swine origin, where majority of the gene segments are closely related to common swine influenza viruses (1, 227). This 2009 H1N1 subtype effectively replaced the seasonal H1N1 strains that circulated previously.

D. Avian influenza in humans

The zoonosis of avian influenza is usually only limited to birds and these subtypes have caused major outbreaks in livestock chicken populations intermittently, leading to significant economic and social impact (reviewed in (31)). However, the Centers for Disease Control and Prevention (CDC) has documented avian influenza of the subtypes H5, H7, and H9 to have infected humans (http://www.cdc.gov/flu/avian/gen-info/avian-flu-humans.htm). Highly pathogenic strains of influenza A have only been identified and restricted to the H5 and H7 subtypes (reviewed in (8)).

In the last two decades, there were documented bird-to-human transmissions of H5N1 in 1997 (3) and H7N7 in 2003 (66), causing outbreaks in Hong Kong and the Netherlands respectively. In 2005, avian H5N1 infections in humans were documented in Southeast Asia, which eventually spread out to the rest of the continent (218). This avian influenza subtype was determined to be of the highly pathogenic strain and brought about elevated concern with regards to its ability to be transmitted from one person to another. Since then, global surveillance of avian influenza species have become a norm because of the possibility that these viruses may acquire mutations that would allow for human-to-human transmission that can potentially result in a pandemic. As of December 30, 2011, there have been 574 recorded cases of human avian influenza infections globally, 337 of which turned out to be fatal, a case fatality rate (CFR) of 58.7% (http://www.wpro.who.int/NR/rdonlyres/B44D349B-3DF1-4573-8286-8F81C5FC9B02/0/AIWeekly313WPRO30Dec2011.pdf). From the same report, 23.2%

of the cases occurred in individuals between the age range of 20-29 and the highest CFR was seen in the 10-19 age range (73%). However, these numbers do not include those who may have been infected by H5N1 but were asymptomatic or did not present febrile or respiratory illness. The stringent criteria that the World Health Organization (WHO) utilize to confirm an H5N1 infection prompted a recent meta-analysis of data from different studies that determined the seroprevalence of avian H5N1 infection in humans (230). They found that about 1 to 2% of the 12,500 study participants from 20 studies have sero-evidence for prior H5N1 infection (230).

E. Influenza vaccination

One of the more effective ways to reduce disease burden by a viral infection is through vaccination. The goal of any vaccination regimen is to produce a long-lasting, (and if possible, a lifetime) protective immune memory. However, due to the nature of influenza viruses, this has become an elusive goal. Influenza viruses are constantly undergoing antigenic drifts and can also undergo occasional shifts. Additionally, there are two subtypes of IAV and a strain of IBV that have been are co-circulating every flu season since 1977. Thus, the current vaccination strategy of influenza requires accurate prediction of the influenza strains that may circulate in the coming flu season and this relies on viral surveillance of circulating strains causing human disease in the past season worldwide (65). The WHO recommends the composition of the seasonal influenza vaccine based on this surveillance data. Although vaccination is not mandatory, most

countries have special recommendations and guidelines that are in place for vaccination of high-risk individuals for influenza (149). According to the CDC, this group includes children under five years old and adults over 65 years old, pregnant women, those with medical conditions that may leave them immunocompromised, and health care workers (http://www.cdc.gov/flu/keyfacts.htm).

In the United States, there are two types of licensed influenza vaccines being administered. The more common one is the trivalent, inactivated vaccine (TIV) composed of H1N1 and H3N2 seasonal flu strains and an IBV strain. Since the emergence of the novel H1N1 in 2009, the TIV composition for the 2011-2012 influenza season replaced the seasonal H1N1 with the 2009 H1N1. TIV is introduced intramuscularly and given to individuals 6 months and older (CDC recommendations). They are commercially available and are regulated by the amount of HA (15ug per flu strain). Alternatively, a live attenuated influenza vaccine (LAIV) is also available and is administered intranasally to individuals age 2 to 49 years old (CDC recommendations). In Europe, both formulations are available, as well as adjuvanted influenza vaccines (149). Both the LAIV and the TIV can induce HA-specific antibody responses (60), which are determined by the hemagglutination-inhibition (HAI) antibody titers.

The level of serum anti-HA antibodies has been used extensively as a correlate of protection and is indicative of vaccine efficacy (47). Susceptibility to infection is inversely correlated with the anti-HA titers, usually determined by HAI, wherein a post-vaccination titer of 1:40 indicates the level of antibody that is able to protect 50% of the

population (163). With recent advances in the field of viral immunology, there is a need to include other immune correlates of protection to evaluate vaccine efficacies. In elderly populations, serum antibody levels may be limited as a measure of vaccine efficacy, while T cell responses correlated with protection (135). There are also other antibodies generated to other components of the influenza virus, as well as other immune mechanisms, as discussed below, that may also serve as correlates of protection.

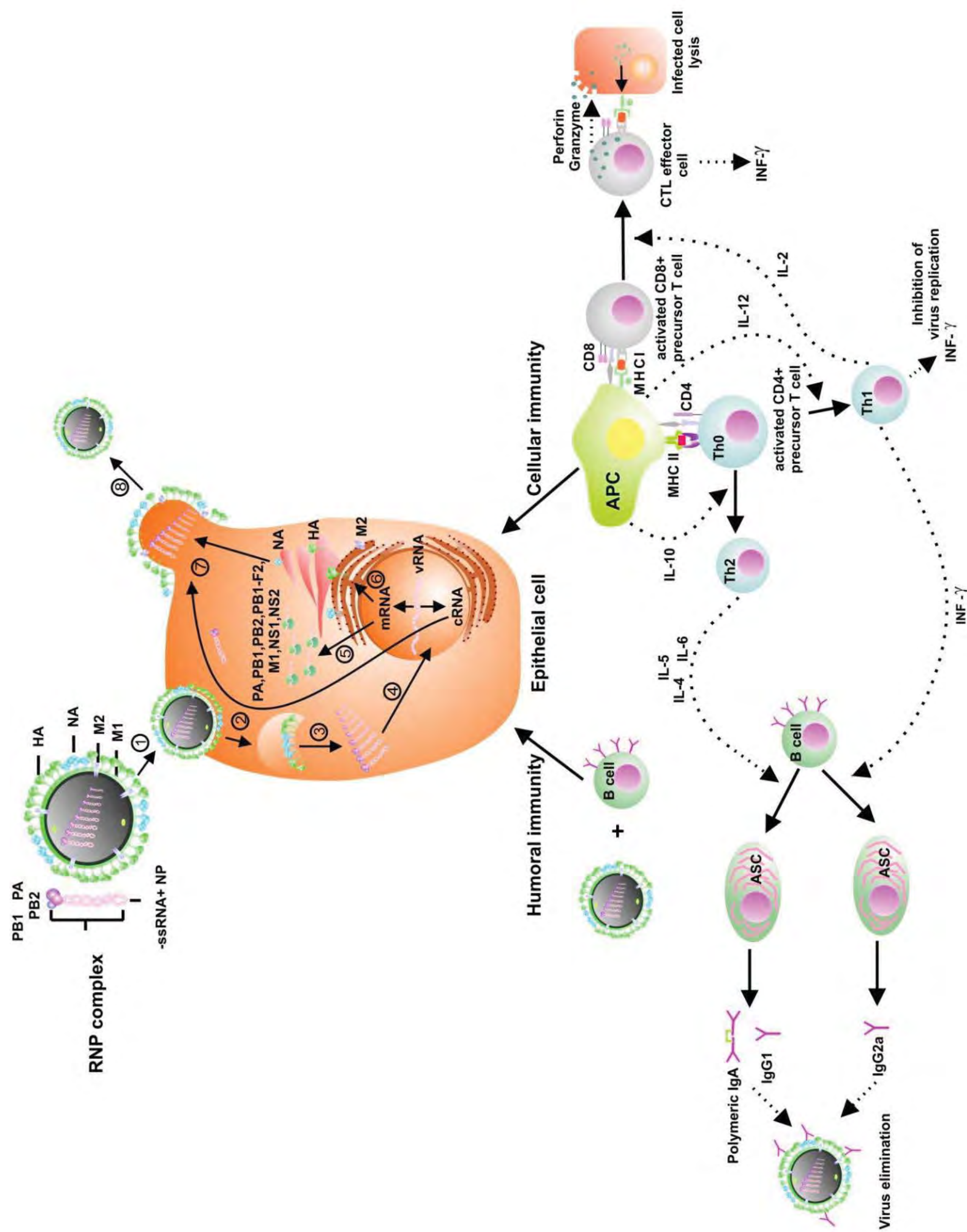
F. The innate immune response to influenza

The innate immune system serves as our first line of defense against an invading pathogen. It is characterized by a non-specific response to a pathogen that can be readily mobilized upon infection. On a cellular level, innate immunity is comprised of intracellular signaling cascades that trigger the production of cytokines that contribute to modulating and containing the infection. These signaling cascades are initiated by the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Among these PRRs are the membrane-associated toll-like receptors (TLRs), which can recognize a variety of PAMPs, including viral RNA. TLR-3 recognizes double-stranded RNA, while TLR-7 recognizes single stranded RNA in the endosomal compartment (54, 85). The influenza viral life cycle allows for both mRNA species to trigger the innate immune response via these TLRs. Influenza is also recognized by a cytosolic sensor of PAMPs called retinoic acid inducible gene – I (RIG-I) (109). Recognition of these influenza components by the PRRs and cytosolic sensors

leads to the induction of the type I interferon (IFN) pathway, a known potent antiviral mechanism (reviewed in (206)) that triggers a global antiviral state by the production of IFN-inducible effectors such as PKR, OAS and MxA (86). The antiviral effects of type I IFN activation in influenza is antagonized by the NS1 protein, where a deletion of this gene in a reverse genetic system increased interferon induction and the virus generated following NS1 deletion were attenuated (72). A third player in the innate immune recognition of influenza includes the intracellular NOD-like receptors (NLRs). These NLRs have been shown to activate the inflammasome, a molecular platform involving several protein complexes that can activate caspase 1, an important enzyme that cleaves precursor forms of inflammatory cytokines such as interleukin (IL)-1 (140). More recently, activation of the inflammasome by NLR recognition of influenza can influence the outcome of the subsequent adaptive immune response (97).

G. The adaptive immune response to influenza

The adaptive immune response plays an important role in protecting the host and eliminating pathogen. It is characterized by high degree of specificity to distinct portions of the pathogen and the ability to establish memory to that pathogen. This allows for a more rapid response to repeated exposures of the same pathogen. There are two arms to this response – the humoral, antibody-mediated response and the cellular T cell-mediated response. Both arms of the adaptive immune response are involved during influenza infection (Fig. 1.3).



RNP complex
PB1
PA
PB2
-ssRNA+ NP
HA
NA
M2
M1
Epithelial cell
PA,PB1,PB2,PB1-F2,
M1,NS1,NS2
mRNA
cRNA
vRNA
NA
HA
M2
Humoral immunity
Cellular immunity
B cell
APC
CD8
MHC I
MHC II
CD4
activated CD8+
precursor T cell
CTL effector
cell
Perforin
Granzyme
Infected cell
lysis
INF-γ
IL-2
IL-12
IL-10
Th0
Th1
Th2
activated CD4+
precursor T cell
Inhibition of
virus replication
INF-γ
IL-5
IL-4
IL-6
ASC
Polymeric IgA
IgG1
IgG2a
Virus elimination

Figure 1.3. Humoral and cellular immunity induced by influenza virus infection. (1) Influenza virus binds to the receptor on the host cell and entry the cell by receptor-mediated endocytosis. (2) The endosomal acidification permits fusion of the host and viral membranes by altering the conformation of hemagglutinin. (3) Upon the fusion, viral RNP complexes are released into the cytoplasm and (4) transported to the nucleus, where the viral RNAs (vRNA) are transcribed into messenger RNAs (mRNA) and replicated by the viral RNA-dependent RNA polymerase complex into complementary RNA (cRNA). (5) mRNA are exported to the cytoplasm for translation of structural proteins. (6) Synthesis of envelope proteins takes place on ribosomes of endoplasmic reticulum. (7) The newly synthesized viral RNPs are exported from the nucleus to the assembly site at the apical plasma membrane, where (8) new virus particles are budding and release out of host cells. Influenza virus infection triggers innate (not shown) and adaptive immune response where the effector cells and molecules are involved in restriction of viral spread, as follows: The cellular immune response *(right)* is initiated after recognition of viral antigens presented via MHCI and MHC II molecules by antigen presenting cells (APC), which then leads to activation, proliferation and differentiation of antigen-specific $CD8^+$ T or $CD4^+$ cells. These cells gain effector cell function and either they help directly (Th1 or Th2 cell) to produce antibodies or, CTL effector cells recognize antigen peptides presented by MHCI on APC and kill the virus infected cells by exocytosis of cytolytic granules. The humoral immune response *(left)*is mediated by specific antibodies (e.g IgG, IgA) produced by antibody secreting plasma cells (ASC) which are the final stage of B cell development. This process is aided by $CD4^+$ T helper and T cell-derived cytokines essential for the activation and differentiation of both B-cell responses and $CD8^+$ T cell responses. (Stanekova and Vareckova. 2010. Virology Journal. 7:351. Reprinted with permission under the terms of the Creative Commons Attribu tion License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.)

i. Antibody-mediated responses to influenza

The humoral response is contributed by B cells and the antibodies that they secrete. These antibodies recognize distinct structural components of the intact viral proteins that are present on the surface of the virus particle or on virus-infected cells. Neutralizing antibodies are particularly important because they can provide sterilizing immunity. They can directly bind to virus particles, preventing virus entry into host cells. The antibody isotype IgA is locally secreted in the respiratory epithelia in response to IAV infection and persists for at least three to five months post infection in mice (34). Virus-infected cells that are coated with IgG antibody are recognized and lysed by natural killer (NK) cells via antibody-dependent cell- mediated cytotoxicity (88). These antibodies may also bind infected cells and lyse them by activating the complement system (167, 228). It has been established that the levels of HA- and NA-specific antibodies in the serum correlate with protection from illness following natural (46) or experimental infection (38).

Antibodies to HA, NA, NP and M are produced during influenza infections in humans (163). In both mice and men, antibodies to HA and NA correlate with resistance to infection, while antibodies to M1 and NP do not (240). The neutralizing antibodies to influenza are directed to HA and antibodies to NA modify severity. Passive transfer of anti-HA antibodies can resolve influenza infection in T and B cell deficient SCID mice (184). Monoclonal antibodies to M2 were previously generated and characterized from *in vitro* cell lines (248) and passive transfer of these antibodies conferred protection in

mice by reducing the level of viral replication in the lungs (226). However, protection by antibody becomes progressively less efficient through time. There is a gradual decrease of IgG and IgA antibodies as well as antibody secreting cells specific to influenza within the first year after initial infection (107, 239). High mutation rates of HA and NA genes also result to altered antibody binding sites leading to virus escape from antibody recognition, as described above.

ii. T cell-mediated immune responses to influenza

The T cell-mediated immune response to viral infections is also widely studied because of its importance in controlling pathogens. Unlike antibodies that recognize intact viral proteins, T cells recognize short peptide fragments presented by professional antigen presenting cells in the context of an MHC molecule through the T cell receptor (TCR). This MHC restriction is influenced by the expression of either the CD8 or the CD4 co-receptor on the T cell surface (103).

A detailed description of the functions of $CD8^+$ and $CD4^+$ T cells has been reviewed by Janeway (103, 104). In summary, $CD8^+$ T cells recognize peptide bound to MHC class I molecules which are expressed on virtually all cell types. The MHC class I molecules typically present peptides that 8-10 amino acids in length and are derived from endogenously synthesized proteins, including viral proteins that are actively produced during infection. On the other hand, $CD4^+$ T cells recognize peptide bound to MHC class II molecules. The expression of MHC class II is limited to professional antigen

presenting cells and B cells. Structural differences in the peptide binding groove of MHC class I and MHC class II allow for longer peptides (between 12 to 17 amino acids in length) to bind to MHC class II molecules. In addition, MHC class II molecules present antigens derived extracellularly. Peptide fragments generated from the uptake of exogenous antigen into the acidified endosomes are then loaded onto the MHC class II complexes. The recognition of a particular TCR and co-receptor with its cognate peptide-MHC complex stimulates a cascade of signaling events in the T cells, leading to their proliferation and differentiation into effector T cells. These effector T cells can either directly or indirectly promote the lysis of infected cells. Following resolution of infection, the effector T cell populations are down-regulated and a memory T cell pool is established and maintained, with the potential to rapidly respond against subsequent viral exposures.

Much of what is known about the cell-mediated immunity to influenza has been gleaned using mouse models. The availability of reagents and genetically-modified mouse models has allowed for an extensive analysis of the T cell response to influenza, although influenza in mice does not necessarily replicate what is seen in natural infection in humans, birds and other vertebrate species (219). Mice that lack B cells were more susceptible to a lethal challenge of influenza, but priming with sub-lethal doses of influenza promoted resistance to a subsequent lethal infection and adoptive transfer of influenza specific $CD8^+$ and $CD4^+$ T cells prior to lethal challenge conferred protection in mice (80). These results suggest a contribution by both $CD8^+$ and $CD4^+$ T cells to immunity to IAV.

The $CD8^+$ T cell response is characterized by its ability to rapidly proliferate during the initial phase of a viral infection and its cytotoxic capacity. Influenza-specific $CD8^+$ T cells can contribute to protective immunity based on studies done in $CD8^+$ T cell-deficient mice, where there is delayed viral clearance (18). Early on, $CD8^+$ cytotoxic T cells (CTL) have been shown in murine studies to limit influenza A virus replication and to protect against lethal influenza A virus challenge (119, 121, 130). Passive transfer of NP-specific $CD8^+$ CTL can also protect against a lethal influenza infection in mice (216). They are also able to mediate clearance of virus in mice that were depleted of $CD4^+$ T cells (56). Several of these CTL responses are cross-reactive, recognizing conserved components of the viral proteins, thus allowing them to lyse target cells that are infected with different influenza subtypes (240). Indeed, several of these influenza-specific CTLs target the viral internal proteins NP, M1 and viral polymerases (15, 19, 20, 78, 171, 223, 224, 247). The CTL memory response to influenza has also been studied in humans. $CD8^+$ CTL were detected in the peripheral blood lymphocytes of infected or vaccinated individuals by day 6 until day 14 and undergo contraction by day 21 (59). A live influenza infection in human volunteers demonstrated that CTL can clear virus as seen by the reduction in viral titers and recovery in donors that had a robust T cell responses (137).

$CD4^+$ T cells are not essential in providing protective immunity in mouse models of influenza infection when both $CD8^+$ T cells and B cells are present (reviewed in (27)). Optimal humoral and cellular immunity to influenza require the activation of $CD4^+$ T helper cells (reviewed in (27)). Influenza specific $CD4^+$ T cells can provide help to B

cells and cross-reactive CD8^{+} T cells during infection and they may also regulate early innate immune responses by indirectly upregulating inflammatory cytokines and chemokines during the early stage of infection (209). Mice that lack CD4^{+} T cells had compromised CD8^{+} T cell memory response after influenza challenge, suggesting that CD4^{+} T cells play a role in maintaining the CD8^{+} T cell cytotoxic responses and the transition to memory phase (17).

CD4^{+} T cells can also act as antiviral effectors themselves upon sequential infection of two different influenza subtypes (136). This cytotoxic effector function is carried out by a perforin-mediated mechanism, mediating protection against a lethal influenza infection in mice and inducing higher levels of neutralizing antibody titers (26). Teijaro and colleagues have shown that memory CD4^{+} T cells specific to the H1N1 influenza virus provide a protective immune response in the lungs after a lethal challenge in mice by enhancing T cell recruitment to the lungs (217). Moreover, B cell deficient mice with HA-specific or polyclonal memory CD4^{+} T cells were protected from influenza virus challenge in the presence of CD8-depleting antibodies, thus demonstrating an intrinsic effector function for influenza-specific CD4^{+} T cells (217).

In humans, the role of CD4^{+} T cells is not fully understood. A recent study performed challenge experiments where they inoculated healthy human volunteers intranasally with influenza virus to demonstrate the contribution of the CD4^{+} T cell response to protection (236). The challenge virus given to these individuals was determined by the absence of neutralizing antibody titers to that particular IAV in the sera

prior to infection. They found significant correlation between influenza-specific $CD4^+$ T cell responses and disease protection, suggesting a role for memory $CD4^+$ T cells in influenza infection in humans.

iii. Identification of T cell epitopes to influenza

The identification of T cell epitopes is important for understanding and analyzing cell-mediated immune responses in general, and is relevant in our understanding of disease pathogenesis, monitoring disease progression and developing vaccines (5). T cell epitopes to various viral pathogens have been identified, including influenza. Defining the T cell epitopes to influenza will enable us to determine which responses are specific for a given virus strain or subtype or which ones are cross-reactive, among others (28). Bui and colleagues compiled all influenza T cell epitopes described in literature in 2007 (28). Although several T cell epitopes have been determined from human samples (76, 101, 102), they are biased because they utilized the sequences of the A/Puerto Rico/8/34 (H1N1) strain (A/PR/8), a prototype strain that was isolated more than 70 years ago and is not a circulating strain. Moreover, only a few epitopes have been determined using current isolates of human pathogenic strains (~1.2%, on average, for a given strain) compared to A/PR/8 (~24%) or A/X-31 (H3N2), a reassortant virus with internal genes from A/PR/8 strain and external genes of the A/Hong Kong/68, which is the H3N2 prototype strain (~32%) (28). There are also limited epitopes determined for influenza strains of pandemic potential, including the avian influenza subtype H5N1 strains (102).

H. Cytokines produced during influenza infection

Cytokines are secreted signaling protein molecules that are rapidly produced by a variety of cell types during an immune response. In the course of an influenza infection, cytokines are secreted by both innate and adaptive immune cells. A subset of cytokines, called chemokines, is also produced. These chemokines act as chemoattractants that bring cells of the immune system to the site of infection. Human plasmacytoid and myeloid dendritic cells have been shown to produce distinct waves of chemokines that allows for a coordinated recruitment of immune effectors, including neutrophils, natural killer (NK) cells, cytotoxic T cells and effector memory T cells (158). Cytokines involved during the course of an influenza infection include IL-1α/β, TNF-α/β, IL-6, IL-8, IFN-α/γ and MIP1-α/β (16).

IFN- γ is a pleiotropic cytokine that is secreted mainly by T lymphocytes, including T-helper 1 $CD4^+$ T cells and cytolytic $CD8^+$ T cells, and by NK cells (reviewed in (13)). During viral infection, IFN- γ promotes an antiviral state by inducing the synthesis of host proteins that are able to inhibit viral replication (reviewed in (154)). It also contributes to enhanced antigen presentation by inducing the expression of MHC molecules on antigen presenting cells (64) and by augmenting peptide degradation during antigen processing in the proteasome (245). In a heterologous secondary influenza infection, IFN- γ deficient mice have impaired ability to clear the challenge virus, even though they have similar number of T cells and antibody (HAI) titers to wild-type mice (25). This indicates a protective role for IFN-γ during secondary influenza infection.

TNF-α is another pleiotropic cytokine that is produced mainly by macrophages, but can also be secreted by T lymphocytes. Although TNF-α is associated with systemic inflammation that may lead to septic shock (reviewed in (225)), it has been shown to mediate inflammatory responses to several autoimmune diseases (116), as well as microbial (53) and viral infections (93), including influenza . An influenza-specific $CD8^+$ cytotoxic lymphocyte line producing both TNF- α and IFN-γ was shown to lyse mediated by both cytokines during the course of influenza infection (120). Another cytokine produced during influenza infection is MIP1-β. It is a chemokine secreted mainly by monocytes, $CD4^+$ T cells and NK cells, and preferentially recruits $CD4^+$ and $CD8^+$ T cells and promotes neutrophil infiltration (132). The outcome of the immune response to infection can lead to either protection or immunopathology depending on the interplay, timing, magnitude and location of these cytokines.

I. Heterosubtypic Immunity to Influenza

Heterosubtypic immunity (HSI) is the immunity generated by a given IAV subtype or its antigens that protects against challenge with a virus of another IAV subtype (e.g. immunity to H1N1 protecting against an infection with H3N2) (81). It is hypothesized that T cells are the major contributors to HSI, especially T cells that target the internal proteins of influenza (81). Because the internal viral proteins are conserved, T cells generated against them are potentially cross-reactive, allowing them to participate in a more rapid response upon challenge, thus enhancing virus clearance and reducing

immunopathology (81). However, pre-existing T cells to influenza cannot prevent infection (sterilizing immunity) as this is the function of neutralizing antibodies. These antibodies are usually specific only to the strain that they were raised against, and because of the propensity of influenza to antigenic drift, antibodies generated to a previous influenza infection may not be efficient in neutralizing a future influenza infection. However, antibodies should not be totally discounted for a possible contribution to HSI. Cross-reactive monoclonal antibodies directed to the stem region of the HA have been described and characterized recently (45, 57, 210). These recent developments also point to the contribution of cross reactive antibodies to HSI.

i. HSI in mice and other animal models

HSI was first demonstrated by Shulman and Kilbourne wherein mice that were previously infected or immunized with an H1 subtype of influenza virus had partial immunity against challenge with an H2 subtype of influenza as seen by reduced pulmonary viral titers, reduced mortality and less severe lung lesions (188). This partial protection is specific only to influenza A viruses, since infection with an influenza B virus strain did not provide the same protection. It is now well-established that a mild influenza infection in animals can provide protection against a subsequent and more severe challenge with a heterosubtypic virus containing a different HA and NA (reviewed in (63)). This protection due to HSI has also been demonstrated in other vertebrate animals. In ferrets, HSI was shown by a decrease in viral titers after a heterosubtypic

challenge and the immunity generated to the initial IAV infection lasted for 18 months (246). Chickens that were primed with H9N2 survived a lethal challenge with H5N1 and this protection was mediated by T cells (191). HSI also mediated protection to a different IAV subtype in pigs (90, 174) and cotton rats (207), reducing viral titers and modulating pathology in infected tissues. Thus, HSI can contribute to decreased severity of a subsequent influenza infection by controlling viral titers and promoting viral clearance, diminished shedding and transmission.

ii. HSI in humans

HSI has not been directly tested or demonstrated in humans and proves to be controversial (63). Ideally, one would have to find an influenza sero-negative individual or know the history of influenza exposure to perform challenge experiments. However, this is really hard to come by in human studies. In theory, one could study HSI in pediatric cohorts, although there are confounding differences in the influenza susceptibility between adults and children (63). The majority of what is known about HSI in humans has been gleaned from epidemiological evidence, usually relating incidence rates of one IAV subtype to previous exposure to a different subtype, either through natural infection or vaccination. Interestingly, the occurrence of several pandemic events provided an avenue to study HSI in the context of two different IAV subtypes circulating in nature. During the onset of the 1957 pandemic, a cohort of factory workers in the former USSR were surveyed to determine if a previous infection

with the H1N1 subtype that circulated the previous year would affect their susceptibility to the emerging H2N2 subtype (200). They found that workers who were less likely to get sick with H2N2 had prior exposure to H1N1. A similar observation was seen in a retrospective analysis of the Cleveland Clinic Family Study in the 1950s. Adults who caught the flu in the prior year did not get sick or had relatively fewer symptoms when the H2N2 IAV emerged (61). In both these studies, there is an indirect indication that although there were no neutralizing antibodies to H2N2 present in these individuals, they were partially protected against H2N2 via HIS generated from previous encounters with a different IAV subtype (61). It is also thought that immunity to H2N2 may have provided protection during the onset of the 1968 pandemic, when H3N2 emerged, because these two subtypes share a common neuraminidase that may contain shared antigens that can contribute to cross-protection (187).

J. Thesis Objectives

The overall goal of this thesis is to characterize the breadth and depth of the influenza A specific $CD8^+$ and $CD4^+$ T cell responses in humans that are induced by natural infection and vaccination. **We hypothesize that a subset of the memory T cells generated from a previous encounter with influenza is cross-reactive and that these T cell responses may contribute to HSI to influenza with viruses of a different subtype.**

This thesis is presented in two parts:

CHAPTER III: Genome-wide screening of human T-cell epitopes to influenza A viruses

Question:

- What are the targets of the human T cell response to influenza?
- Are there cross-reactive T cell responses to specific viral components?

Approach:

- ELISPOT screening of human PBMC using peptides spanning all of the influenza viral proteins.
- Generating peptide-specific T lines to further characterize the functionality and phenotype of these T cells.

CHAPTER IV: A human $CD4^+$ T cell epitope in the influenza hemagglutinin is cross-reactive to influenza A subtypes and to influenza B virus

Question:

- Are there cross-reactive T cells specific to the influenza HA?
- Can we detect H2 HA-specific memory T cell responses in individuals who were previously exposed to H2N2?
- Are these H2 HA-specific responses cross-reactive to H5 HA?

Approach:

- ELISPOT screening using H2 HA peptides and comparing the profile of the IFN-γ responses in two groups: older donors who have previously encountered the H2N2 and younger donors who are naïve to H2N2.
- Characterization of HA specific responses by generating specific T cell lines.

CHAPTER II

MATERIALS AND METHODS

A. Influenza peptides and recombinant proteins

Peptides encompassing the entire sequence of all influenza viral proteins were obtained from the National Institutes of Health (NIH) Biodefense and Emerging Infections Research Resources Repository (BEI Resources; Table 2.1). They are 17 amino acids in length with overlapping 11 to 12 amino acids. The peptide length was a debated compromise in an effort to detect most $CD4^+$ and $CD8^+$ T-cell epitopes at a reasonable cost. The amino acid sequences of these peptides were based on vaccine strains of influenza A viruses (H1N1 and H3N2 or an antigenically indistinguishable strain from the vaccine strain when the amino acid sequences of the proteins were not available for the vaccine strain) and recent isolates of influenza virus A (H5N1), as indicated in Table 2.1. Because of the considerable difference between avian and human N1 NA amino acid sequences, peptide sets for both neuraminidases were synthesized. Polymerase B1-F2 peptides of both strains were also synthesized because of differences between the H1N1 and H3N2 strains. Peptides covering the pre-hemagglutinin protein (pre-HA) of influenza B/Nanchang/12/1998 were also synthesized. Control peptides for known major histocompatibility complex (MHC) class I and II epitopes of influenza A viruses were also provided by BEI Resources. Peptides spanning the HA of A/Japan/305/1957 (H2N2) were designed to correspond to BEI Resources' H5 HA

peptides and synthesized by AnaSpec, Inc. (San Jose, CA). A vaccinia virus B5R peptide ($B5R_{5-19}$) was used as a negative control and was also obtained from AnaSpec, Inc. All the peptides were reconstituted by dissolving in 100% dimethyl sulfoxide at a stock concentration of 10 mg/ml.

The CEF peptide pool was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, the National Institute of Allergy and Infectious Diseases (NIAID), NIH, and was used as a positive peptide pool control. This peptide pool contains MHC class I-restricted human T-cell epitope peptides of cytomegalovirus, Epstein–Barr virus, and influenza A virus (49).

Recombinant HA proteins of A/Singapore/1/1957 (H2N2) and A/Canada/RV444/04 (H7N3) were obtained from BEI Resources. Recombinant HA proteins from A/New Caledonia/20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2) and A/Vietnam 1203/2004 (H5N1) were obtained from Protein Biosciences (Meriden, CT; H1, H3 and H5). A recombinant vaccinia virus B5R protein was used as an irrelevant protein control and was provided by BEI Resources.

Table 2.1. Influenza peptides used in this study.

Influenza Protein	Abbreviation	Strain	No. of Peptides	BEI Catalog No.
Hemagglutinin	H1 HA	A/New Caledonia/20/1999 (H1N1)	94	NR-2602
Hemagglutinin	H3 HA	A/New York/384/2005 (H3N2)	94	NR-2603
Hemagglutinin	H5 HA	A/Thailand/4(SP-528)/2004 (H5N1)	94	NR-2604
Neuraminidase	aN1 NA	A/Thailand/4(SP-528)/2004 (H5N1)	74	NR-2607
Neuraminidase	hN1 NA	A/New Caledonia/20/1999 (H1N1)	78	NR-2606
Neuraminidase	N2 NA	A/New York/384/2005 (H3N2)	78	NR-2608
Matrix Protein 1	M1	A/New York/348/2003 (H1N1)	41	NR-2613
Matrix Protein 2	M2	A/New York/348/2003 (H1N1)	15	NR-2614
Nucleoprotein	NP	A/New York/348/2003 (H1N1)	82	NR-2611
Nonstructural Protein 1	NS1	A/New York/444/2001 (H1N1)	37	NR-2612
Nonstructural Protein 2	NS2	A/New York/348/2003 (H1N1)	19	NR-2615
Polymerase A	PA	A/New York/348/2003 (H1N1)	119	NR-2618
Polymerase B1	PB1	A/New York/348/2003 (H1N1)	126	NR-2617
Polymerase B1-F2	PB1-F2 (H1N1)	A/New York/348/2003 (H1N1)	8	NR-2685
Polymerase B1-F2	PB1-F2 (H3N2)	A/New York/504/1998 (H3N2)	16	NR-2685
Polymerase B2	PB2	A/New York/348/2003 (H1N1)	126	NR-2616
Synthesized by AnaSpec, Inc.				
Hemagglutinin	H2 HA	A/Japan/305/1957 (H2N2)	96	
Influenza B				
Pre-Hemagglutinin	B HA	B/Nanchang/12/1998	98	NR-2605

B. Influenza viruses and other viruses

For live virus infections, the following influenza virus strains were used: A/New Caledonia/20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), X-27 (reassortant of A/Rockefeller Institute/5/57 (HA) x A/NWS/34 (NA), subtype is H2N1), A/Hong Kong/483/1997 (H5N1), A/California /7/2009 NYMC (H1N1) and B/Malaysia/2506/2004. The H1N1 and H3N2 seasonal virus strains and the influenza B virus were a gift from Dr. Michel DeWilde and Dr. Robert Ryall of Sanofi Pasteur. The H5N1 strain was provided by Dr. Nancy Cox from the World Health Organization Influenza Reference Laboratory at the Centers for Disease Control and Prevention (CDC) and the reassortant H2N1 strain was obtained from BEI resources. The 2009 pandemic H1N1 strain was provided by Dr. Alexander Klimov of the CDC. The vaccinia virus WR strain was used as an unrelated virus control and was provided by Bob Marshall from the University of Massachusetts Medical School.

All experiments using the live H5N1 strain were performed by John Cruz in a biosafety level 3 laboratory of University of Massachusetts Medical School according to enhanced BSL3 guidelines (Federal Register / Vol. 74, No. 182 / Tuesday, September 22, 2009 / Notices).

C. Human PBMCs used in this study

Blood samples were obtained from healthy adult volunteers and their peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation using Ficoll–Hypaque (Sigma). DNA from either PBMC or autologous B lymphoblastoid cell lines (BLCLs) were extracted for HLA typing using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN). The HLA Class II typing for most of these donors was performed by either the HLA Typing Laboratory at the University of Massachusetts Medical Center or by the HLA Core Facility of the Center for Infectious Diseases and Vaccine Research (now part of the Division of Infectious Diseases and Immunology) at the University of Massachusetts Medical School.

The four donor PBMCs we used for our genome-wide screening of T cell epitopes in Chapter III were chosen based on either the availability of PBMCs for large-scale screening and/or their reactivity to the influenza peptides in the CEF peptide pool in enzyme linked immunosorbent spot (ELISPOT) assays that had been previously performed by Kim West in our laboratory. Donors 1, 3, and 4 received the influenza vaccine almost every year. Donor 2 never received any influenza vaccine. None of the four donors had a history of laboratory-confirmed influenza infection. Additional screening of the H1 and the H3 HA peptides was performed using PBMCs from 30 healthy hospital workers. These donors were recruited for a clinical study that was previously described elsewhere (40). The Day 0 bleed was used for the screening. For screening of B/HA peptides, additional donor PBMCs were collected from healthy volunteers through the weekly blood draw in our laboratory.

For the screening of H2 HA peptide pools described in Chapter IV, donors were classified into two age groups based on their birth year and relative to their exposure to the H2N2 subtype that circulated from 1957 to 1968. Older donor PBMCs (born on or before 1957) were originally obtained for a long-term vaccinia virus clinical study. These were comprised of hospital workers who did not report any febrile illness during blood draw. The younger donor PBMCs (born after 1968) were collected from healthy volunteers through the weekly blood draw in our laboratory. Additional donors were sought to further characterize a cross-reactive $CD4^+$ T cell peptide epitope that we identified in this study. We chose donors who had the HLA-DRB1*09 allele. PBMCs of these donors were previously collected for other studies in our laboratory and extra vials from these studies were used for the experiments described below.

D. IFN-γ ELISPOT assay

ELISPOT assays were performed as previously described (101). Briefly, cryopreserved PBMCs (2–2.5 x 10^6 cells per well) were seeded onto polyvinylidene difluoride membrane 96-well plates (Millipore, Bedford,MA) precoated with 5µg/ml anti-IFN-γ monoclonal antibody (clone D1K; Mabtech, Cincinnati, OH) in the presence or absence of peptide or peptide pools. Phytohemagglutinin (Sigma–Aldrich, St. Louis,MO;1:100),CEF peptide pool, and/or virus were used as positive controls. After 18–24 hours of incubation, cells were removed by washing with phosphate-buffered saline plus 0.05% Tween 20. Secondary biotinylated anti-IFN-γ monoclonal antibody

(clone 7-B6-1; Mabtech) was added at 2 μg/ml and the plates were incubated for two hours at room temperature. Plates were washed again and IFN-γ was detected with avidin–peroxidase (3420-2H, Mabtech) and substrate kit (NovaRed, Vector Laboratories, Burlingame, CA). The frequency of IFN-γ-producing cells was determined by using the ImmunoSpot S4 Pro Analyzer and the ImmunoSpot Academic V.4 Software (Cellular Technologies Ltd., Shaker Heights, OH). Experiments were usually performed in triplicate wells, with some repeat experiments done in duplicate wells.

E. Peptide pool design

For our genome-wide T cell epitope screening, we made peptide pools that contained 15 non-overlapping peptides and made two sets of peptide pools to facilitate the peptide screening. The first set of peptide pools (Table 2.2) contains all peptides of the surface glycoproteins of H1, H3, and H5 HA and avian N1 (aN1) and human N1 (hN1) and N2 NA. This set of peptide pools consists of 33 pools: pools 1–6 contain H1 HA peptides, pools 7–12 contain H3 HA peptides, pools 13–18 contain H5 HA peptides, pools 19–23 contain hN1NApeptides, pool 24–28 contains aN1NA peptides, and pool 29–33 contains N2 NA peptides. The second set of peptide pools (Table 2.3) consists of 38 pools and included all peptides of the internal viral proteins: NP and nonstructural protein 1 (NS1; pool 1–8), M1 and nonstructural protein 2 (NS2; pool 9–12), polymerase A (PA) and matrix protein 2 (M2; pool 13–21), and polymerase B1 (PB1) and polymerase B2 (PB2; pool 22–38). Three additional peptide pools were also made (Table 2.2 Pool 34-36).

These contained PB1-F2 peptides and H5 HA peptides representative of regions of amino acid sequence diversity among different strains of H5N1 viruses.

In later ELISPOT screening experiments using H2 and B HA peptides, we designed non-overlapping peptide pools containing 9 or 10 peptides per pool at a stock concentration of 1.1 or 1 mg/ml respectively (Table 2.4).

F. Depletion of $CD4^+$ or $CD8^+$ expressing cells from PBMCs

$CD4^+$ or $CD8^+$ expressing cell populations were depleted from PBMCs by negative selection using anti-CD4 or anti-CD8 antibody-coated magnetic beads from the MACS purification system (Miltenyi Biotec, Bergisch Gladbach, Germany) and processed according to the manufacturer's protocol. Depleted PBMCs were used in ELISPOT to determine the cell population producing IFN-γ.

Table 2.2. Peptide Pool Design: Set 1 – Hemagglutinins, Neuraminidases and PB1-F2

Pool #	Protein/BEI #	Peptide Number																Total
1	HA(1) NR2602	1	7	13	19	25	31	37	43	49	55	61	67	73	79	85	91	16
2		2	8	14	20	26	32	38	44	50	56	62	68	74	80	86	92	16
3		3	9	15	21	27	33	39	45	51	57	63	69	75	81	87	93	16
4		4	10	16	22	28	34	40	46	52	58	64	70	76	82	88	94	16
5		5	11	17	23	29	35	41	47	53	59	65	71	77	83	89		15
6		6	12	18	24	30	36	42	48	54	60	66	72	78	84	90		15
7	HA(3) NR2603	1	7	13	19	25	31	37	43	49	55	61	67	73	79	85	91	16
8		2	8	14	20	26	32	38	44	50	56	62	68	74	80	86	92	16
9		3	9	15	21	27	33	39	45	51	57	63	69	75	81	87	93	16
10		4	10	16	22	28	34	40	46	52	58	64	70	76	82	88	94	16
11		5	11	17	23	29	35	41	47	53	59	65	71	77	83	89		15
12		6	12	18	24	30	36	42	48	54	60	66	72	78	84	90		15
13	HA(5) NR2604	1	7	13	19	25	31	37	43	49	55	61	67	73	79	85	91	16
14		2	8	14	20	26	32	38	44	50	56	62	68	74	80	86	92	16
15		3	9	15	21	27	33	39	45	51	57	63	69	75	81	87	93	16
16		4	10	16	22	28	34	40	46	52	58	64	70	76	82	88	94	16
17		5	11	17	23	29	35	41	47	53	59	65	71	77	83	89		15
18		6	12	18	24	30	36	42	48	54	60	66	72	78	84	90		15
19	hNA(1) NR2606	1	6	11	16	21	26	31	36	41	46	51	56	61	66	71	76	16
20		2	7	12	17	22	27	32	37	42	47	52	57	62	67	72	77	16
21		3	8	13	18	23	28	33	38	43	48	53	58	63	68	73	78	16
22		4	9	14	19	24	29	34	39	44	49	54	59	64	69	74		15
23		5	10	15	20	25	30	35	40	45	50	55	60	65	70	75		15
24	aNA(1) NR2607	1	6	11	16	21	26	31	36	41	46	51	56	61	66	71		16
25		2	7	12	17	22	27	32	37	42	47	52	57	62	67	72		16
26		3	8	13	18	23	28	33	38	43	48	53	58	63	68	73		16
27		4	9	14	19	24	29	34	39	44	49	54	59	64	69	74		15
28		5	10	15	20	25	30	35	40	45	50	55	60	65	70	75		15
29	NA(2) NR2608	1	6	11	16	21	26	31	36	41	46	51	56	61	66	71	76	16
30		2	7	12	17	22	27	32	37	42	47	52	57	62	67	72	77	16
31		3	8	13	18	23	28	33	38	43	48	53	58	63	68	73	78	16
32		4	9	14	19	24	29	34	39	44	49	54	59	64	69	74		15
33		5	10	15	20	25	30	35	40	45	50	55	60	65	70	75		15
34	HA(5) NR2609	H1	H4	H7	H10	H13	H16	H19	P1	P4	P7	P10	P13	P16	P19	P22		15
35	and PB1-F2	H2	H5	H8	H11	H14	H17	H20	P2	P5	P8	P11	P14	P17	P20	P23		15
36	NR2865	H3	H6	H9	H12	H15	H18	H21	P3	P6	P9	P12	P15	P18	P21	P24		15

Table 2.3. Peptide Pool Design: Set 2 – Influenza Internal Proteins

Pool #	Protein/BEI #	Peptide Number															Total
1	NP NR2611	1	9	17	25	33	41	49	57	65	73	81	*7	*15	*23	*31	15
2	and NS1 NR2612	2	10	18	26	34	42	50	58	66	74	82	*8	*16	*24	*32	15
3	(with *)	3	11	19	27	35	43	51	59	67	75	*1	*9	*17	*25	*33	15
4		4	12	20	28	36	44	52	60	68	76	*2	*10	*18	*26	*34	15
5		5	13	21	29	37	45	53	61	69	77	*3	*11	*19	*27	*35	15
6		6	14	22	30	38	46	54	62	70	78	*4	*12	*20	*28	*36	15
7		7	15	23	31	39	47	55	63	71	79	*5	*13	*21	*29	*37	15
8		8	16	24	32	40	48	56	64	72	80	*6	*14	*22	*30		14
9	M1 NR2613 and	1	5	9	13	17	21	25	29	33	37	41	*4	*8	*12	*16	15
10	NS2 NR2615	2	6	10	14	18	22	26	30	34	38	*1	*5	*9	*13	*17	15
11	(with *)	3	7	11	15	19	23	27	31	35	39	*2	*6	*10	*14	*18	15
12		4	8	12	16	20	24	28	32	36	40	*3	*7	*11	*15	*19	15
13	M2 NR2614 (with *)	1*	10*	4	13	22	31	40	49	58	67	76	85	94	103	112	16
14	and PA NR2618	2*	11*	5	14	23	32	41	50	59	68	77	86	95	104	113	15
15		3*	12*	6	15	24	33	42	51	60	69	78	87	96	105	114	15
16		4*	13*	7	16	25	34	43	52	61	70	79	88	97	106	115	15
17		5*	14*	8	17	26	35	44	53	62	71	80	89	98	107	116	15
18		6*	15*	9	18	27	36	45	54	63	72	81	90	99	108	117	15
19		7*	1	10	19	28	37	46	55	64	73	82	91	100	109	118	15
20		8*	2	11	20	29	38	47	56	65	74	83	92	101	110	119	15
21		9*	3	12	21	30	39	48	57	66	75	84	93	102	111		14
22	PB1 NR2617 and	1	18	35	52	69	86	103	120	*11	*28	*45	*62	*79	*96	*113	15
23	PB2 NR2616	2	19	36	53	70	87	104	121	*12	*29	*46	*63	*80	*97	*114	15
24	(with *)	3	20	37	54	71	88	105	122	*13	*30	*47	*64	*81	*98	*115	15
25		4	21	38	55	72	89	106	123	*14	*31	*48	*65	*82	*99	*116	15
26		5	22	39	56	73	90	107	124	*15	*32	*49	*66	*83	*100	*117	15
27		6	23	40	57	74	91	108	125	*16	*33	*50	*67	*84	*101	*118	15
28		7	24	41	58	75	92	109	126	*17	*34	*51	*68	*85	*102	*119	15
29		8	25	42	59	76	93	110	*1	*18	*35	*52	*69	*86	*103	*120	15
30		9	26	43	60	77	94	111	*2	*19	*36	*53	*70	*87	*104	*121	15
31		10	27	44	61	78	95	112	*3	*20	*37	*54	*71	*88	*105	*122	15
32		11	28	45	62	79	96	113	*4	*21	*38	*55	*72	*89	*106	*123	15
33		12	29	46	63	80	97	114	*5	*22	*39	*56	*73	*90	*107	*124	15
34		13	30	47	64	81	98	115	*6	*23	*40	*57	*74	*91	*108	*125	15
35		14	31	48	65	82	99	116	*7	*24	*41	*58	*75	*92	*109	*126	15
36		15	32	49	66	83	100	117	*8	*25	*42	*59	*76	*93	*110		14
37		16	33	50	67	84	101	118	*9	*26	*43	*60	*77	*94	*111		14
38		17	34	51	68	85	102	119	*10	*27	*44	*61	*78	*95	*112		14

Table 2.4. Peptide pool design – H2 HAs and B HA

Pool #	Protein	Peptide Number										Total
H2-1	H2 HA (synthesized from Anaspec)	1	11	21	31	41	51	61	71	81	91	10
H2-2		2	12	22	32	42	52	62	72	82	92	10
H2-3		3	13	23	33	43	53	63	73	83	93	10
H2-4		4	14	24	34	44	54	64	74	84	94	10
H2-5		5	15	25	35	45	55	65	75	85		9
H2-6		6	16	26	36	46	56	66	76	86		9
H2-7		7	17	27	37	47	57	67	77	87		9
H2-8		8	18	28	38	48	58	68	78	88		9
H2-9		9	19	29	39	49	59	69	78	89		9
H2-10		10	20	30	40	50	60	70	80	90		9
BHA-1	B HA (BEI# NR2605)	1	11	21	31	41	51	61	71	81	91	10
BHA-2		2	12	22	32	42	52	62	72	82	92	10
BHA-3		3	13	23	33	43	53	63	73	83	93	10
BHA-4		4	14	24	34	44	54	64	74	84	94	10
BHA-5		5	15	25	35	45	55	65	75	85	95	10
BHA-6		6	16	26	36	46	56	66	76	86	96	10
BHA-7		7	17	27	37	47	57	67	77	87		9
BHA-8		8	18	28	38	48	58	68	78	88		9
BHA-9		9	19	29	39	49	59	69	78	89		9
BHA-10		10	20	30	40	50	60	70	80	90		9

G. Generation of bulk culture cell lines

To generate peptide-specific bulk culture lines, PBMCs (3 to 5 x 10^6 cells) were washed and resuspended in 2 ml of AIM/V-10% FBS supplemented with 1:100 sodium pyruvate (Gibco) and 1:1000 2-mercaptoethanol (Gibco). The corresponding influenza peptide was added at a final concentration of 10 µg/ml. Human recombinant interleukin (rhIL)-7 (Peprotech, Inc., Rockyhill, NJ) was also added to the culture (5 ng/ml) and incubated at 37 C. On day 3, human recombinant interleukin (rhIL)-2 (BD Discovery Labware, Bedford, MA; 25–50 U/ml) was applied, and the medium was replenished with AIM/V-10% FBS and rhIL-2 every 3 to 4 days. Bulk culture ^{51}Cr release assays were performed between days 10 and 13 of culture. The cultures were restimulated once with autologous PBMCs on day 14 to reduce nonspecific background lysis and to generate enough cells for the assays. To establish influenza A-specific T cell clones, a limiting dilution assay was performed as previously described (101). Briefly, PBMCs that had been stimulated in bulk culture for 14 days were plated at a concentration of 1, 3, 10, or 30 cells per well in 96-well round-bottom microtiter plates in 50 µl of AIM-V medium containing 10% FBS, 25 U IL-2, a 1:1,000 dilution of anti-CD3 monoclonal antibody 12F6 (gift from Dr. Johnson Wong), and 1 x 10^5 gamma-irradiated (3500 rads) allogeneic PBMCs/well. On day 7, 50 µl of fresh AIM-V medium with FBS and IL-2 was added, and on day 14, fresh medium with 1 x 10^5 gamma-irradiated allogeneic PBMCs/well and a 1:1,000 dilution of the anti-CD3 12F6 were added. The cells were assayed for cytolytic activity using ^{51}Cr release assays between days 21 and 28. Cells from wells with

influenza A peptide-specific cytolytic activity (specific killing of 15% and above at a peptide concentration of 10 µg/ml) were expanded to 48-well plates.

H. Preparation of antigen presenting cells (APCs) for ^{51}Cr release assays and intracellular cytokine staining (ICS)

For virus-infected targets, autologous B lymphoblastoid cell lines (BLCL) were established from donor PBMC by culture with Epstein–Barr virus in 24-well plates as previously described (82). BLCL target cells were infected with either of the following egg-adapted virus strains: A/New Caledonia/20/1999 IVR-66 or A/Wisconsin/67/2005X-161B. These virus strains were a gift from Dr. Michel DeWilde and Dr. Robert Ryall of Sanofi Pasteur. The optimal concentrations of the two strains were determined by John Cruz in preliminary experiments (unpublished results). For infections using recombinant vaccinia virus expressing influenza HA, BLCLs were infected at an MOI of 1. Infected cells were initially incubated for one hour in 300 ul PBS with 0.1% Bovine Serum Albumin (BSA) at 37°C. RPMI-10 was added to bring the volume up to 1mL and the cells were incubated for up to 18 hours at 37ºC. Virus-infected target cells were then radiolabeled for use in ^{51}Cr-release assays or used as APCs in ICS.

Peptide-pulsed targets were prepared using autologous BLCLs that were either radiolabeled for one hour in CTL assays or used directly in ICS assays. Peptide is added to the cells at a final concentration of 10 µg/ml per peptide, unless indicated otherwise.

I. Cytotoxic T cell assay by ^{51}Cr release

Cytotoxicity was measured as described previously (101). T-cell lines or bulk culture effector cells were added to $1.5x10^3$ ^{51}Cr-labeled target cells at various effector to target (E:T) ratios. Wells that had target BLCLs and media were used to determine the background (spontaneous) ^{51}Cr release, while wells that had target BLCLs and detergent (10% name of detergent) were used to determine the maximum ^{51}Cr release. After incubation for 4–6 hours at 37ºC, supernatants were harvested (Skatron Instruments, Sterling, VA), and % specific immune lysis was calculated as [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100. The % spontaneous lysis is given by the (spontaneous release/maximum release) x 100 and was <30% in all assays. All conditions were performed in triplicate wells. Unpulsed target cells were used as a negative control.

J. Surface and intracellular cytokine staining

Bulk culture lines or T cell lines derived from limiting dilution assays were used as effector cells in ICS. They were washed and resuspended at $5x10^5$ cells in RPMI 1640 medium supplemented with 10% FBS (RPMI 10). Autologous BLCLs were used as APCs at an E:T ratio of 10 and were added to the effector cells. These were incubated for 1 hour at 37 C in a 5% CO_2 incubator, followed by an additional five hours in the presence of Golgi plug (BD Biosciences, San Jose, CA). The cells were then washed with FACS buffer (2% FBS and 0.1% sodium azide in phosphate-buffered saline) and stained

using the Live/Dead aqua fixable dead cell stain kit (Invitrogen, Eugene, OR) to identify live and dead cells. Cells were then stained for surface markers such as CD3 (clone SK7, APC-Cy7; BD Biosciences, San Jose, CA) , CD8 (clone SK1, PerCP-Cy5.5 or clone RPA-T8, FITC; BD Biosciences, San Jose, CA), CD19 (clone SJ25C1, PE-Cy7; BD Biosciences, San Jose, CA) and/or CD56 (clone B159, PE-Cy7; BD Biosciences, San Jose, CA) and/or CD14 (clone M5E2, PE-Cy7; BD Biosciences, San Jose, CA), and CD4 (clone OKT4, Pacific Blue; eBiosciences, San Diego, CA or clone RPA-T4, APC; BD Biosciences, San Jose, CA) for 30 minutes at 4°C. After washing with FACS buffer, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) and stained for the intracellular cytokine IFN-γ (clone 4S.B3, PE or clone B27, Alexa Fluor 700; BD Biosciences, San Jose, CA) or tumor necrosis factor (TNF) -α (clone Mab11, APC; BD Biosciences, San Jose, CA) for 30 minutes at 4ºC. Cells were then washed with Permwash buffer (BD Biosciences, San Jose, CA) and resuspended in 1X BD Stabilizing Fixative (BD Biosciences, San Jose, CA) for flow cytometric analysis. Multiparameter flow cytometric analyses were performed using the BD FACSAria flow cytometer. The number of events collected per experiment varied from 150,000 to 300,000. List-mode data files were analyzed using FlowJo (Version 6.3 or 7.2, TreeStar 6, Inc., Ashland, CA).

K. Peptide binding assay

A fluorescence polarization assay was used to determine the binding affinities of HA peptides containing the fusion peptide sequence to the HLA-DR1 molecule. The reagents

used for the assay were provided by Dr. Lawrence Stern and Liusong Yin from the University of Massachusetts Medical School. The $HA_{306-318}$ peptide probe (Ac-PRFVKQNTLRLAT) was synthesized (21st Century Biochemicals, Marlboro, MA) and labeled with Alexa-488-tetrafluorophenyl ester (Invitrogen, Eugene, Oregon). Soluble recombinant HLA-DR1was prepared as previously described (68). Peptide-free HLA-DR1 (100 nM) was mixed together with the Alexa-488-HA peptide probe (25nM) and varying concentrations of unlabeled competitor peptide (from 0.08 to 20 nM). The mixtures were done in triplicate wells for each competitor peptide concentration in a 96-well format and incubated for three days at 37 C in binding buffer (pH 5.5) containing protease inhibitors and 0.5 mg/ml octylglucoside. Fluorescence was detected using the Alexa488-FP-DISS-one measurement protocol of the PerkinElmer 2030 Explorer multilabel plate reader. IC_{50} values were obtained by fitting a binding curve of the plots of percent dissociation of peptide probe versus the logarithmic value of the concentration of competitor peptide using GraphPad Prism Version 5.04.

L. Statistical analysis

Statistical analysis of the ELISPOT responses of PBMC to peptide pools was done using GraphPad Prism Version 5.04. One-way analysis of variance (one-way ANOVA) was performed to determine variations among the means of the different peptide pool responses. An unpaired student's t-test was performed comparing the mean of media only wells against a specific peptide pool response. P values of ≤ 0.01 were considered significant.

CHAPTER III

GENOME-WIDE SCREENING OF HUMAN T CELL EPITOPES TO INFLUENZA A VIRUSES

Characterization of epitope specific T cell responses is relevant in understanding the immune response to influenza infection and vaccination. Previous efforts to characterize the T cell response to influenza by our group (101) and others (76) were limited by focusing only on the conserved viral proteins or utilizing viral protein sequences derived from the A/PR/8 strain. Other groups also focus on a particular influenza viral protein such as HA or M1 (50, 243), or determine influenza-specific T cell epitopes restricted to a particular HLA allele (242, 244). Therefore, we sought to determine the baseline T cell memory responses to influenza by performing a genome-wide screening of T cell epitopes using synthetic peptides covering all viral proteins whose sequences are based on more recent circulating strains of influenza, including the avian H5N1. ELISPOT was used to quantitate the number of IFN-γ-producing cells in donor PBMCs that are specific for the influenza A viral proteins. This method of screening for T-cell epitopes directed to viral proteins had been used with much success (9), in particular with vaccinia virus (148).

A. Peptide Screening

We first sought to determine the optimal conditions in establishing a standard approach to screening IFN- γ responses using ELISPOT. We have several vials of

PBMC collected from Donor 1, thus preliminary experiments were performed using this donor's PBMC. Donor 1 is HLA-A*02:01 positive and was previously shown by Kim West in our laboratory to have T-cell responses specific to the influenza $CD8^+$ T cell epitope $M1_{58-66}$ (unpublished data), which is considered an immunodominant epitope (138). We initially determined the optimal peptide concentration to use in our ELISPOT screening to minimize false positives and negatives by using 17-mer peptides that contain known influenza T cell epitopes that Donor 1 is expected to respond to. We were able to detect IFN-γ responses to the M1 peptide containing the immunodominant $M1_{58-66}$ at all peptide concentrations but the spot forming cells/10^6 (SFC/10^6) were increased significantly when the peptide concentration was 2 μg/ml (Fig. 3.1). There is no apparent explanation as to why we see a significant difference in the SFC values for the M1 peptide epitope, since the DMSO used to reconstitute the peptides is essentially diluted (<0.1% of final volume in well). We also did not observe a peptide dose response for the M1 peptide epitope since there was no trend of decreasing SFC values as peptide concentration decreases. However, for the M1 peptide epitope, we consistently saw higher SFC values at 2 μg/ml compared to higher peptide concentrations (data not shown). IFN-γ responses were also optimal at a peptide concentration of 2 μg/ml for the other peptide epitopes tested, with higher concentrations of the peptide giving minimal SFC values (Fig. 3.1).

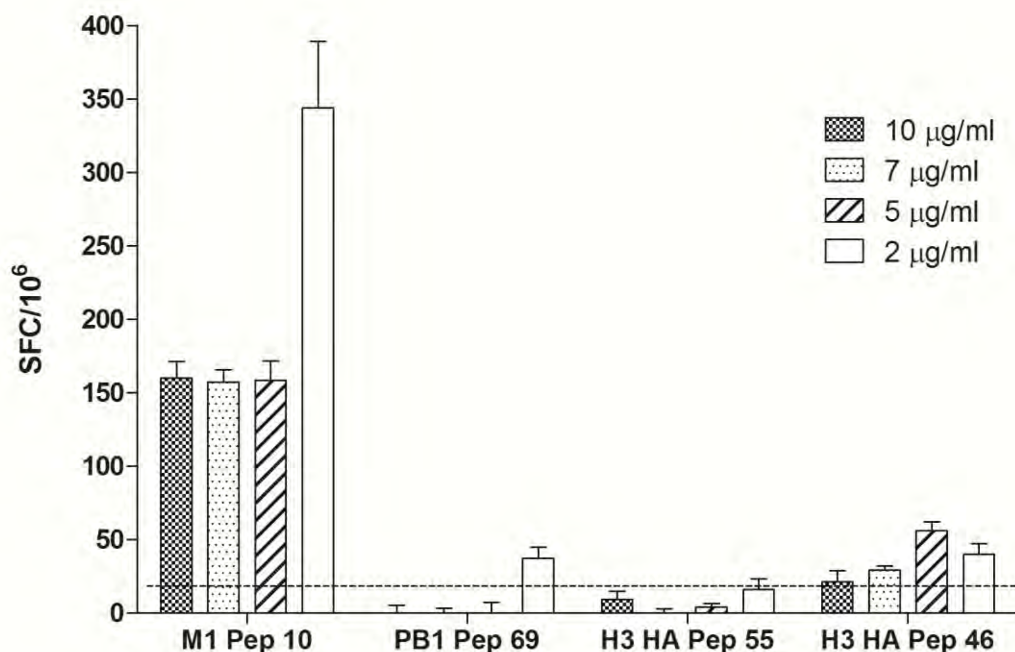


Figure 3.1. Determining the optimal peptide concentration for our ELISPOT screening. Donor 1 PBMCs were used to determine the minimal peptide concentration that would give positive IFN-γ responses in ELISPOT. 17-mer peptides containing known influenza epitopes were assayed at various concentrations. M1 Peptide 10 contains the HLA-A*0201 epitope $M1_{58-66}$, PB1 Peptide 69 contains the HLA-A*0201 epitope $PB1_{413-421}$, and H3 HA Peptide 55 contains the HLA-DR1 epitope H3 $HA_{322-333}$. H3 HA Peptide 46 is a candidate peptide that had IFN-γ responses in our initial experiment. SFC values are representative of one experiment and error bars are given by the standard deviation of the mean of triplicate wells. PHA stimulation was used as a positive control (mean = 2545 SFC/10^6).

We only had limited peptide amounts and donor PBMC to perform our screening, thus we decided not to use the matrix system of designing peptide pools. Instead, we explored the possibility of maximizing the number of peptides we can use in a pool and taking advantage of the overlapping nature of the synthetic peptides. The number of IFN-γ-producing cells did not differ significantly if we used five, 10, or 15 non-overlapping peptides in a pool (Table 3.1). The PBMC also responded to the CEF peptide pool, which contains HLA-A*02:01-restricted T cell epitopes to CMV, EBV and influenza (49), including the influenza epitope, $M1_{58-66}$. However, we saw a significant decrease in the SFC/10^6 values if we used a peptide pool that included overlapping peptides to $M1_{58-66}$ (Table 3.1). Based on our results, we established for our screening that 15 non-overlapping peptides in a pool at a concentration of 2 µg/ml per peptide would allow us to detect influenza-specific IFN-γ responses in ELISPOT using human PBMC.

We also wanted to determine the cut-off SFC for a positive response. There is no set standard to determine the cut-off number of spots in ELISPOT screening. In most cases, the number of spots in unstimulated or negative wells (background spots) is used to determine the cut-off. We arbitrarily determined the cut-off of a positive ELISPOT response at 20 SFC/10^6 based on the value given by the mean of the spot-forming cells (SFC) per 10^6 cells plus three standard deviations (SD) of the negative control wells in our donors (Table 3.2). For donors that had higher background spots, we adjusted the cutoff value for a positive response accordingly (see Donor 3, Table 3.2). In later analyses, peptide pools that had SFC values close to the arbitrary 20 SFC/10^6 cut-off

Table 3.1. ELISPOT results of optimization experiments for peptide screening.

Stimulation	[a]SFC/10^6	
	Experiment 1	**Experiment 2**
PHA	1810.7 ± 31.7	1741.3 ± 33.0
CEF peptide pool	230.7 ± 1.5	226.7 ± 5.5
Overlapping peptide pool	0.3 ± 0.6	0.3 ± 0.6
Non-overlapping peptide pool		
5 peptide per pool	37.3 ± 3.5	33.3 ± 4.9
10 peptides per pool	40.0 ± 6.1	30.7 ± 1.2
[b]15 peptides per pool-A	38.7 ± 2.1	34.7 ± 0.6
[c]15 peptides per pool-B	36.0 ± 1.0	32 ± 6.08

[a] SFC/10^6 values are given by the mean of three wells for each experiment and the standard error of the mean was calculated for each value.

[b] Pool includes M1 peptide 10 (containing $M1_{58-66}$) and peptides from H3 HA, NS1, NP, PB1 and M2.

[c] Pool includes M1 peptide 10 (containing $M1_{58-66}$) and non-overlapping M1 peptides.

Table 3.2. Background spots of negative control wells for Donors 1, 2, 3, and 4.

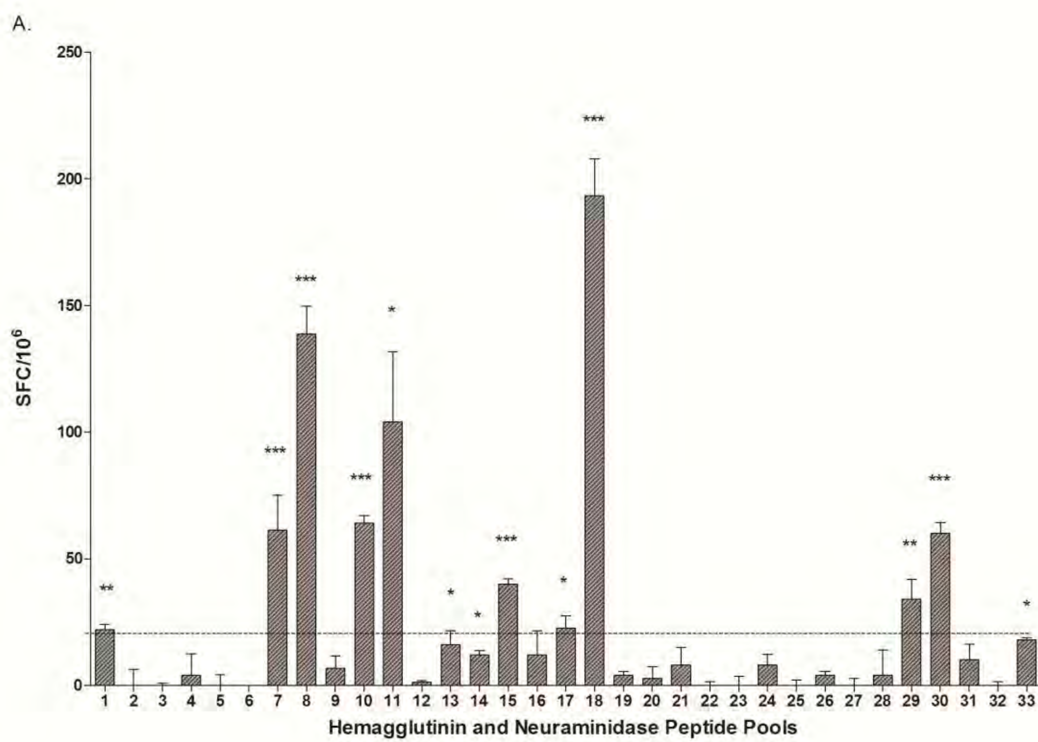
Experiment	SFC/10^6			
	Donor 1	**Donor 2**	**Donor 3**	**Donor 4**
1	3.5	12.5	17.5	2
2	3.5	5	16.5	1
3	2	5	18.5	2.5
4	2	3.5	32	1.5
5	1.5	3	21.5	4.5
6	2.5	4	24	13.5
7	9	6.5	17.5	
8	8	6	16.5	
9	4.5	0.5	26	
10	8		25.5	
Mean	4.5	5.1	21.6	4.2
S.D.	2.8	3.3	5.2	4.7
Cut-Off (Mean +3*S.D)	12.9	15	37.2	18.4

were considered positive if the p-value were determined by using an unpaired student's t-test was < 0.01.

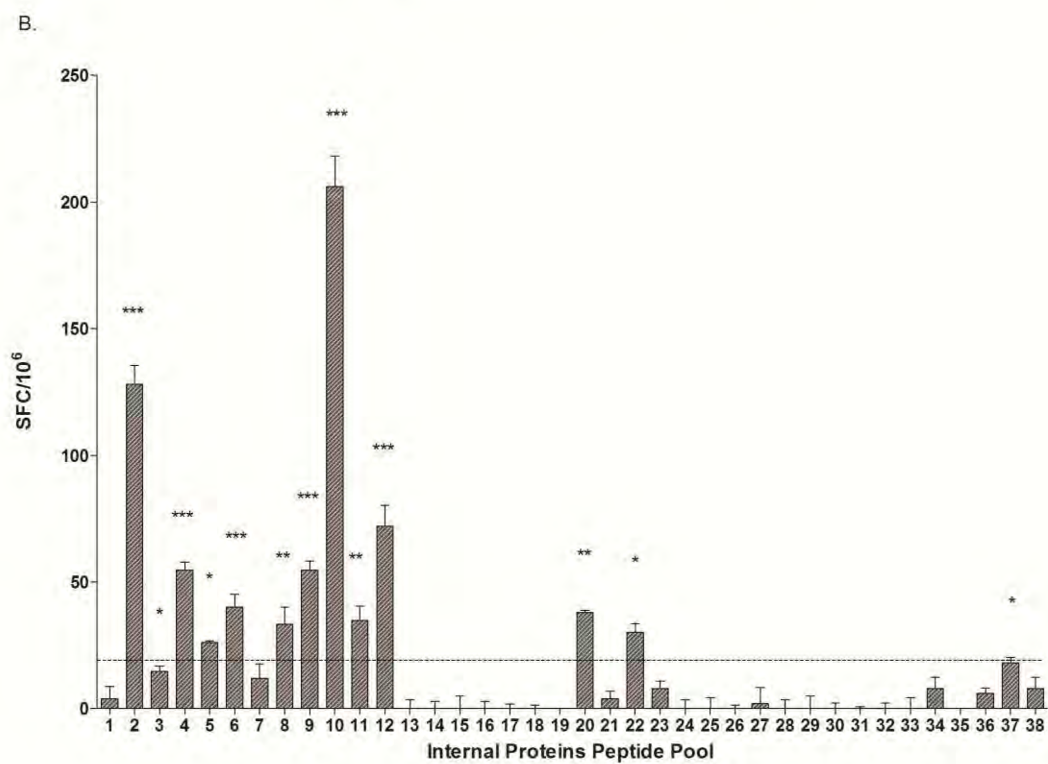
B. The PBMCs of Donor 1 have broad IFN-γ response to peptide epitopes on several influenza A proteins

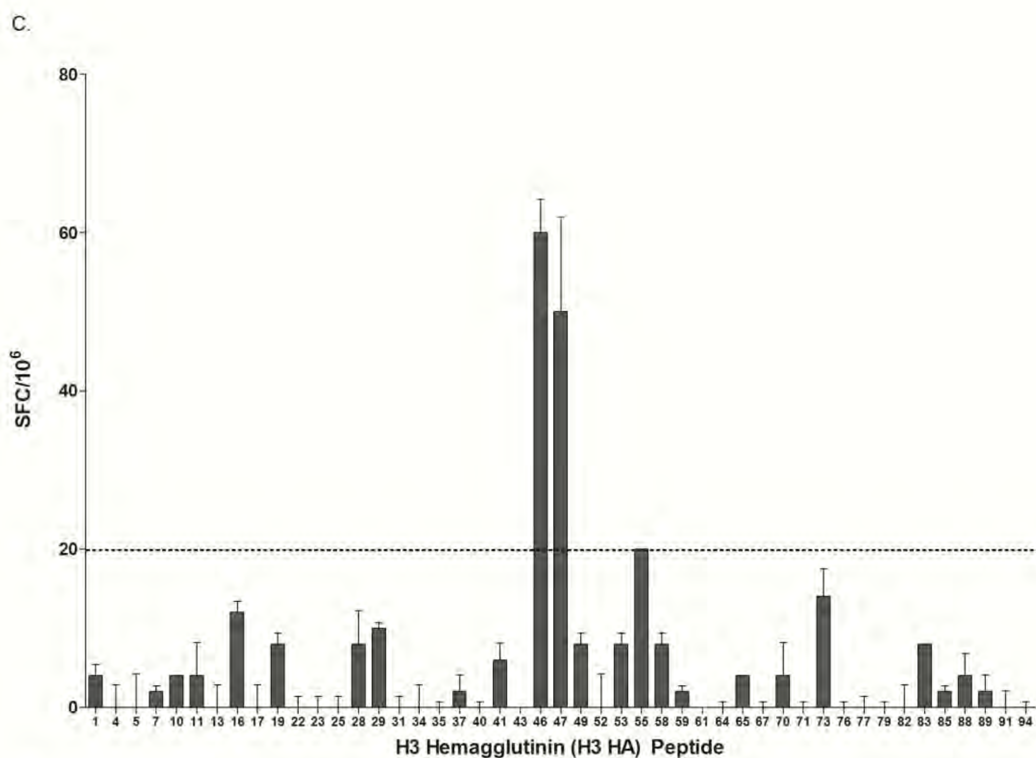
We performed a genome-wide screening of influenza T cell epitopes in Donor 1 using peptide pools that cover the entire influenza viral proteins. The PBMCs of donor 1 were broadly reactive to peptide pools from several influenza proteins, as indicated by the IFN-γ responses to peptide pools containing HA peptides (Fig. 3.2A; pools 1, 7, 8, 10, 11, 13, 14, 15, 17 and 18), NA peptides (Fig. 3.2A; pools 29, 30 and 33), NP peptides and NS1 peptides (Fig. 3.2B; pools 2, 3, 4, 5, 6, and 8), M1 and NS2 peptides (Fig. 3.2B; pools 9, 10, 11, and 12), PA peptides (Fig. 3.2B; pool 20), and PB1 and PB2 peptides (Fig. 3.2B; pools 22 and 37). IFN-γ responses to individual peptides from positive pools containing H3 HA and NS2 and M1 peptides are illustrated in Fig. 3.2C and D, respectively. In addition, donor 1 had positive responses to two pools containing PB1-F2 and H5 HA variant peptides (data not shown). We also detected several IFN-γ responses to H3 but only one pool tested positive for H1 HA. For the internal viral proteins, the majority of responses were seen in pools containing M1, NP, NS1, and NS2 peptides. The pool that contained the peptide with the HLA-A2-restricted M1 epitope M158-66 (Fig. 3.2B; pool 10) had the highest SFC value among all the peptide pools. The sum of the number of IFN-γ-producing cells responding to these positive peptides was 1108, which is 90.7% of

A.



B.




C.
80
60
40
20
0
SFC/10^6
1 4 5 7 10 11 13 16 17 19 22 23 25 28 29 31 34 35 37 40 41 43 46 47 49 52 53 55 58 59 61 64 65 67 70 71 73 76 77 79 82 83 85 88 89 91 94
H3 Hemagglutinin (H3 HA) Peptide

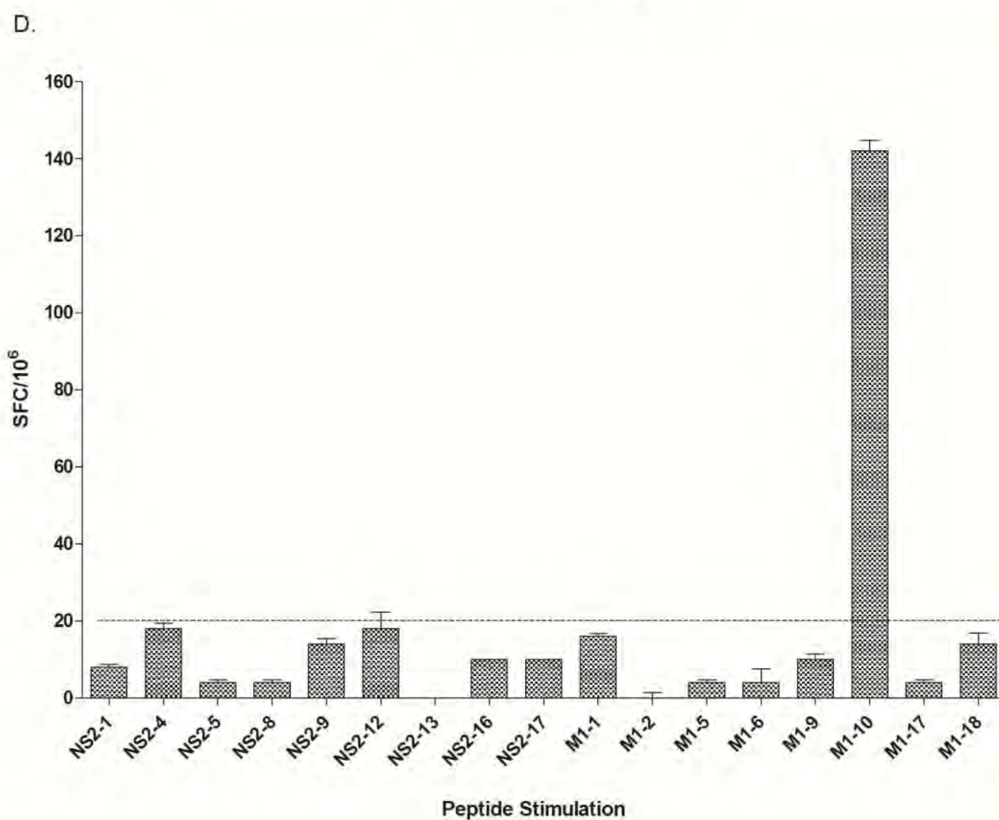

D.
160
140
120
100
80
60
40
20
0
SFC/10^6
NS2-1
NS2-4
NS2-5
NS2-8
NS2-9
NS2-12
NS2-13
NS2-16
NS2-17
M1-1
M1-2
M1-5
M1-6
M1-9
M1-10
M1-17
M1-18
Peptide Stimulation

Figure 3.2. IFN-γ response of Donor 1 PBMC to peptides from all influenza proteins. PBMC were first tested in ELISPOT against (A) Set 1 peptide pools (1–33), which include the hemagglutinin (H1 HA, H3 HA, and H5 HA) and neuraminidase (aN1 NA, hN1 NA, and N2 NA) peptides and (B) Set 2 peptide pools (1–38), which include peptides spanning all viral internal proteins. Positive pools were deconvoluted to test individual peptides. An example of such screening is illustrated in C and D. ELISPOT assays were performed using (C) individual peptides in Set 1 peptide pool 7, 10, and 11 consisting of H3 HA peptides and (D) NS2 and M1 peptides contained in Set 2 peptide pools 9 and 10. PHA stimulation was used as a positive control and was consistently above 2,000 SFC/10^6 in all experiments. Peptide pools and individual peptides were tested in triplicate wells with 200,000 to 250,000 cells per well. The final concentration of peptide used in all experiments was 2 µg/ml per peptide. The dotted line indicates the cutoff SFC value of positive responses for this donor (20 SFC/10^6) that determines which pools will be deconvoluted. Error bars indicate the standard deviation among triplicate wells and statistical significance was determined by using unpaired Student's *t* test (* = $p<0.01$, ** = $p<0.001$ and *** = $p<0.0001$).

the number of IFN-γ-producing cells responding to live influenza virus stimulation (1221 SFC/10^6 for H1N1 and 1219 SFC/10^6 for H3N2; Table 3.4).

C. IFN-γ responses to influenza A proteins in three other healthy adults

We selected three additional donors based on the availability of PBMCs for large-scale screening and their previous reactivity to the influenza peptides in the CEF peptide pool in preliminary ELISPOT assays. All three of these donors responded to peptides from several influenza A viral proteins as summarized in Figure 3.3. The PBMCs of Donor 2 exhibited less reactivity to influenza peptides, with responses to only four peptides (Fig. 3.3, white box). Positive peptide pools for each of the donors were deconvoluted to test the individual peptides and narrow down the peptide-specific response. Table 3.3 summarizes all the peptides that elicited IFN-γ responses from the four donor PBMCs. On average, the four donors' PBMCs responded to 21 different peptides from nine different viral proteins, including H3 and H5 HA. Responses to H3 and H5 HA were comparable, with SFC values ranging from 20 to 60 SFC per million cells. Most of these peptides do not contain a known epitope sequence based on our search using the Immune Epitope Database (IEDB; www.immuneepitope.org) (28, 155). We considered a peptide sequence to be a potential novel epitope if there was no record that the sequence was associated with positive T-cell data in the IEDB. We also considered both the amino acid sequence of the 17-mer peptide and the corresponding HLA allele of the donor's PBMCs that responded. Some of the peptides we identified have a record of some positive T-cell data in IEDB, but were not fully characterized with

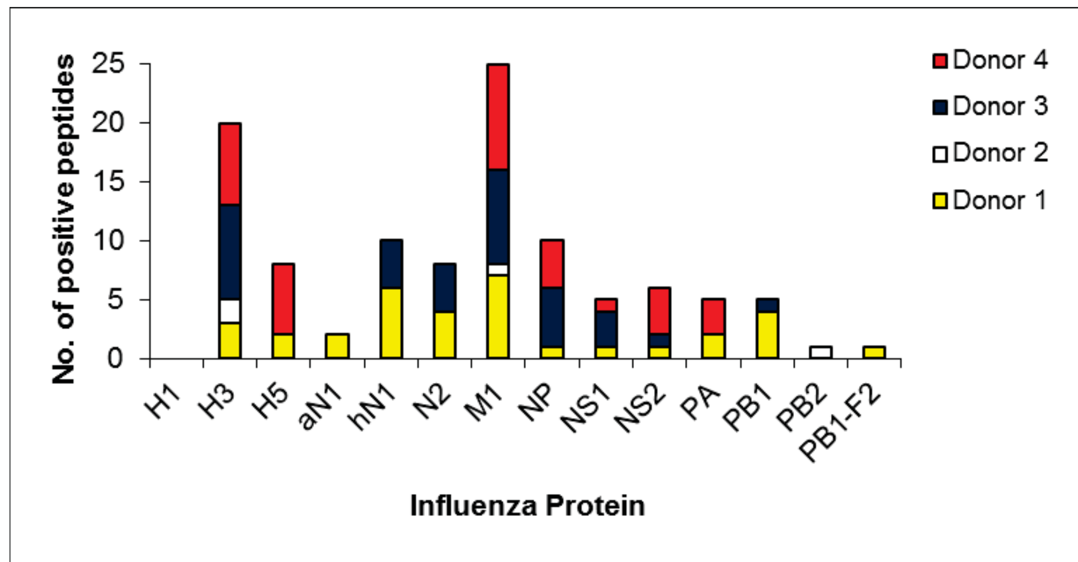


Figure 3.3. Broad T-cell responses to influenza viral proteins in PBMC from healthy adults. PBMCs from four healthy adults were screened for IFN-γ responses to all influenza proteins in ELISPOT assays. Each bar corresponds to the total number of peptides that elicited an IFN-γ response in all donor PBMC tested.

Table 3.3. Influenza peptides that gave a positive IFN-γ response in our ELISPOT screening using PBMC from Donors 1, 2, 3 and 4.

Protein	AA Position	Peptide Sequence[a]	Donor	SFC/10^{6}[b]	Comments[c]	CD4/CD8[d]
H3 HA	71-87	PHQILDGENCTLIDALL	3	37.5	Novel	ND
H3 HA	179-195	ALNVTMPNNEKFDKLYI	4	22.5	Novel	ND
H3 HA	209-225	SLYAQASGRITVSTKRS	4	42.5	Novel	ND
H3 HA	215-231	SGRITVSTKRSQQTVIP	3	195	Novel	CD4
H3 HA[e]	243-259	PSRISIYWTIVKPGDIL	2, 4	27.5, 80	Novel	CD4
H3 HA[e]	249-265	YWTIVKPGDILLINSTG	3, 4	127.5, 47.5	CD4 T cell epitope restriction not known	CD4
H3 HA	255-271	PGDILLINSTGNLIAPR	3	67.5	CD4 T cell epitope restriction not known	CD4
H3 HA	267-283	LIAPRGYFKIRSGKSSI	1	60	DRB1*1001; DQA1*0102; DQB1*0602	CD4
H3 HA	273-289	YFKIRSGKSSIMRSDAP	1	50	DRB1*1001; DQA1*0102; DQB1*0602	CD4
H3 HA	279-295	GKSSIMRSDAPIGKCNS	4	20	Novel	ND
H3 HA	285-301	RSDAPIGKCNSECITPN	3	75	Novel	ND
H3 HA	321-337	CPRYVKQNTLKLATGMR	1, 3	20, 52.5	DRB1*0101; DRB1*0401; DRB5	CD4

Protein	AA Position	Peptide Sequence[a]	Donor	SFC/10^6[b]	Comments[c]	CD4/CD8[d]
H3 HA	350-366	AIAGFIENGWEGMVDGW	2	25	Novel	CD4
H3 HA	391-407	NQINGKLNRLIGKTNEK	3, 4	75, 20	Novel	ND
H3 HA	505-521	DVYRDEALNNRFQIKGV	3	47.5	CD4 T cell epitope restriction not known	CD4
H3 HA	528-543	KDWILWISFAISCFLL	4	40	CD4 T cell epitope restriction not known	CD4
H5 HA	30-46	EQVDTIMEKNVTVTHAQ	4	35	Novel	ND
H5 HA	136-152	SSWSSHEASLGVSSACP	4	22.5	Novel	ND
H5 HA	151-167	SSACPYQRKSSFFRNVV	1	80	Novel	ND
H5 HA	207-223	YQNPTTYISVGTSTLNQ	4	27.5	Novel	ND
H5 HA	243-259	EFFWTILKPNDAINFES	4	55	Novel	CD4
H5 HA	351-367	AIAGFIEFFWQGMVDGW	4	20	Novel	ND
H5 HA	387-403	TQKAIDGVTNKVNSIID	4	32.5	Novel	ND
H5 HA	470-486	LQLKDNAKELGNGCFEF	1	34	Novel	ND
hN1 NA	13-29	ISIAIGIISLMLQIGNI	1	44	A*0201	CD8
hN1 NA	42-58	SQNHTGVCNQRIITYEN	1	38	Novel	ND
hN1 NA	309-325	NLDYQIGYICSGVFGDN	1	34	Novel	ND

Protein	AA Position	Peptide Sequence[a]	Donor	SFC/10[6b]	Comments[c]	CD4/CD8[d]
hN1 NA	434-450	NTTIWTSGSSISFCGVN	1	40	Novel	ND
hN1 NA[e]	452-468	DTANWSWPDGAELPFTI	1	32	Novel	ND
hN1 NA[e]	458-470	WPDGAELPFTIDK	1	28	Novel	ND
aN1 NA	1-17	MNPNKKIITIGSICMVT	1	38	Novel	ND
aN1 NA	24-40	LQIGNLISIWVSHSIHT	1	42	Novel	ND
N2 NA	13-29	VSLTISTICFFMQIAIL	1	44	Novel	ND
N2 NA[e]	176-192	IAWSSSSCHDGKAWLHV	1, 3	50, 120	Novel	CD4
N2 NA[e]	188-204	AWLHVCVTGDDKNATAS	1	22	Novel	ND
N2 NA	206-222	IYNGRLVDSIVSWSKEI	3	87.5	Novel	ND
N2 NA	236-252	TCTVVMTDGSASGKADT	3	47.5	Novel	ND
N2 NA	266-282	STLSGSAQHVEECSCYP	1, 3	24, 52.5	Novel	ND
M1[e]	1-17	MSLLTEVETYVLSIVPS	1, 4	34, 45	Novel	ND
M1[e]	7-23	VETYVLSIVPSGPLKAE	1	22	A*1101	CD8
M1[e]	13-29	SIVPSGPLKAEIAQRLE	1	26	DRB1*0101; DRB1*1501	CD4
M1	37-53	TDLEALMEWLKTRPILS	1	50	CD4 T cell epitope restriction not known	CD4

Protein	AA Position	Peptide Sequence[a]	Donor	SFC/10^{6}[b]	Comments[c]	CD4/CD8[d]
M1	43-59	MEWLKTRPILSPLTKGI	4	47.5	CD4 T cell epitope restriction not known; CW*0102	CD4
M1	55-71	LTKGILGFVFTLTVPSE	1	142	A*0201	CD8
M1[e]	91-107	NNMDRAVKLYRKLKREI	3, 4	157.5, 22.5	DRB1*0103	CD4
M1[e]	97-113	VKLYRKLKREITFHGAK	3	212.5	DRB1*0103	CD4
M1	121-137	AGALASCMGLIYNRMGA	3	42.5	B*3501	CD8
M1	139-155	TTESAFGLICATCEQIA	4	45	Novel	ND
M1	163-179	RQMVTTTNPLIRHENRM	4	20	DRB1*0103	CD4
M1	169-185	TNPLIRHENRMVLASTT	1	42	DRB1*0103; DRB1*1501; DRB5	CD4
M1[e]	187-203	KAMEQMAGSSEQAAEAM	3, 4	47.5, 37.5	DRB5	CD4
M1[e]	193-209	AGSSEQAAEAMEVASQA	4	37.5	Novel	ND
M1[e]	205-221	VASQARQMVQAMRAIGT	3	210	Novel	CD4
M1[e]	210-226	RQMVQAMRAIGTHPSSS	3, 4	142.5, 65	Novel	CD4
M1[e]	216-232	MRAIGTHPSSSTGLKND	3	115	Novel	ND
M1	228-244	GLKNDLLENLQAYAKRM	2	22.5	DRB5	CD4
M1	234-250	LENLQAYQKRMGVQMQR	1	40	DRB1*0103; DQw3, DQw1	CD4

Protein	AA Position	Peptide Sequence[a]	Donor	SFC/10^6[b]	Comments[c]	CD4/CD8[d]
M1	240-252	YQKRMGVQMQRFK	4	35	DQw1	CD4
NS1	13-29	CFLWHVRKQVADQDKGD	4	25	Novel	ND
NS1	120-136	DQAIMDKNIILKANFSV	1	24	A*0201	CD8
NS2[e]	13-29	LMRMSKMQLGSSSGDLN	4	35	Novel	ND
NS2[e]	19-35	MQLGSSSGDLNGMITQF	4	30	Novel	ND
NS2	37-53	SLKLYRDSLGEAVMRLG	1, 4	50, 35	Novel	ND
NS2	85-101	HKLKTTENSFEQITFMQ	4	37.5	Novel	ND
NP	55-71	RLIQNSLTIERMVLSAF	4	47.5	Novel	ND
NP[e]	109-125	VLYDKEEIRRIWRQANN	3	165	Novel	CD4
NP[e]	115-131	EIRRIWRQANNGDDATA	3	70	Novel	CD4
NP[e]	175-191	RSGAAGAAVKGVGTMVL	3	95	B*2705[f]	CD4
NP[e]	187-203	GTMVLELIRMIKRGIND	3	75	A*1101[f]	CD4
NP[e]	193-209	LIRMIKRGINDRNFWRG	3	102	Novel	CD4
NP	246-262	RNPGNAEIEDLTFLARS	4	22.5	Novel	ND
NP	258--274	FLARSALILRGSVAHKS	1	40	A3	CD8
NP	294-310	EGYSLVGVDPFKLLQTS	4	22	Novel	ND

Protein	AA Position	Peptide Sequence[a]	Donor	SFC/10^{6}[b]	Comments[c]	CD4/CD8[d]
PA	220-236	PPNFSCIENFRAYVDGF	1	22	A*0201	CD8
PA	310-326	CMRTFFGWKEPTVVKPH	4	32	Novel	ND
PA	516-532	DVVNFVSMEFSLTDPRL	1	20	Novel	ND
PA	576-592	KWGMEMRRCLLQSLQQI	4	36	Novel	ND
PB1	406-422	GMMMGMFNMLSTVLGVS	1	26	A*0201	CD8
PB1	447-463	FALIVNAPNYAGIQAGV	1	44	Novel	ND
PB1[e]	537-553	NDLGPATAQMALQLFIK	1	32	*B7*	CD8
PB1[e]	548-564	LQLFIKDYRYTYRCHRG	1	36	Novel	ND
PB1-F2 (H3N2)	65-81	KNPTQGSLRTHALKQWK	1	48	Novel	ND
PB2	203-219	VAYMLERELVRKTRFLP	2	30	Novel	ND

[a] Underlined amino acid sequences are previously published T cell epitopes (also see comments). Amino acid/s highlighted in yellow reflects an amino acid change in our peptide sequence compared to the previously published epitope.

[b] SFC values are representative of one ELISPOT experiment with three replicate wells per donor.

[c] The HLA class I and class II alleles described in the comments are the restricting MHC allele of the underlined amino acid sequence.

[d] T-cell phenotype was determined by either IFN-γ ELISPOT using CD4 or CD8 depleted donor PBMC or IFN-γ ICS using peptide-stimulated bulk culture cells as effectors.

[e] This peptide contains amino acid residues that overlaps with another peptide and is identified by the same donor.

[f] This peptide contains a known CD8 T-cell epitope, but the peptide-stimulated bulk culture we generated has a CD4 T-cell phenotype.

ND (not determined).

regard to their HLA restriction or minimal epitopes (indicated in Table 3.3). About 60% of these peptides gave "moderate" IFN-γ responses (SFC between 31 to 99 SFC/10^6) compared with the number of IFN-γ-producing cells responding to the "immunodominant" HLA-A2-restricted $M1_{58-66}$ epitope (Fig. 3.4).

To determine if the IFN-γ responses we saw to the peptides were representative of the IFN-γ responses elicited during a viral infection, we took the sum of the SFC values to the peptides for each donor and compared it the SFC values during live virus stimulation. Table 3.4 lists the sum of the number of IFN-γ-producing cells responding to these positive peptides for donors 2, 3, and 4, as well as the SFC values for live virus stimulation of these donors' PBMCs. By taking the sum of the responses after stimulation with the complete set of the peptides spanning all viral proteins, we find that our total SFC values for all the viral proteins are comparable with that of live virus stimulation in ELISPOT.

D. The phenotype of IFN-γ producing cells

To determine the T-cell population that produced IFN-γ after peptide stimulation, we depleted $CD4^+$ or $CD8^+$ cells from the donors' PBMCs. We then performed ELISPOT using the peptide of interest to stimulate the $CD4^+$- or $CD8^+$-depleted cells. Peptides that had SFC values greater than 60 SFC/10^6 in Table 3.3 were first analyzed to ensure that there are enough peptide-specific precursor cells. Except for the $M1_{58-66}$ epitope, all of the IFN-γ-producing cells that responded to the peptides in ELISPOT were $CD4^+$ cells, as

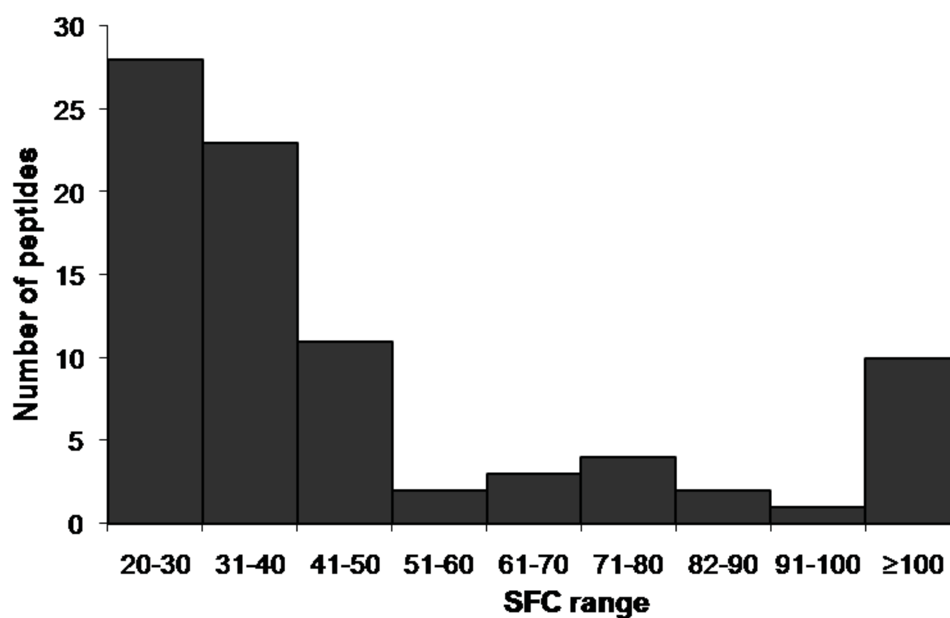


Figure 3.4. Distribution of SFC values of positive peptides in ELISPOT assays. The SFC values for each peptide that gave a positive IFN-γ response were plotted in a histogram chart to determine the frequency of IFN-γ-producing cells among four donor PBMCs.

Table 3.4. Total SFC values corresponding to IFN-γ responses in ELISPOT to influenza peptide stimulation or live virus infection.

	Total SFC values[a]					
	H1 and hN1 peptides	**H3 and N2 peptides**	**H5 and aN1 peptides**	**Internal protein peptides**	**H1N1 virus infection**	**H3N2 virus infection**
Donor 1	202	264	180	620	1221	1219
Donor 2	0	52.5	0	52.5	ND[b]	420
Donor 3	0	985	0	1825	1182	1478
Donor 4	0	273	193	714	1033	1135

[a]Total SFC values are representative of one ELISPOT experiment, with triplicate wells per peptide or virus stimulation.

[b]ND, not done.

indicated by a drastic decrease in SFC values when $CD4^+$cells are depleted from whole PBMCs (Table 3.5).

For the other candidate peptide epitopes, short term bulk culture cell lines using donor PBMCs were set up and ICS was performed on days 12–14 to determine the phenotype of the IFN-γ-producing cells. Fig. 3.4 illustrates representative plots indicating the phenotype of IFN-γ-producing cells from peptide-stimulated bulk culture using donor 3 PBMCs. More than 87% of IFN-γ-producing T cells expressed the CD4 surface marker upon cognate peptide stimulation in ICS (Fig. 3.4).

E. Characterization of the influenza-specific T cell lines generated from limiting dilution assay

We generated bulk culture lines by stimulating donor PBMCs with peptides of interest to further characterize the peptide-specific responses we identified in our ELISPOT screening. We selected peptides that potentially contain novel epitopes by searching for MHC class I and class II binding motifs within the peptides using two prediction algorithms, HLA Peptide Binding Predictions (http://www-bimas.cit.nih.gov/molbio/hla_bind/) (153) and SYFPEITHI (http://www.syfpeithi.de) (169). We used these peptides to stimulate donor PBMC and generate short-term bulk culture cell lines. After two weeks in culture, the peptide specificity of these cell lines was determined by using a standard ^{51}Cr release assay by looking at their ability to kill autologous BLCL targets pulsed with the cognate peptide. Although cytotoxicity may not be a major function of virus-specific $CD4^+$ T cells, our group had previously

Table 3.5. Phenotype of IFN-γ-producing cells after $CD4^+$ or $CD8^+$ cell depletion.

Peptide	SFC/10^6		
	Whole PBMCs	CD4-depleted PBMCs	CD8-depleted PBMCs
DONOR 1			
[a]$M1_{58-66}$	401.7±32.3	281.7±4	0±2.5
[b]H3 $HA_{322-334}$	8.3±0	6.7±2.1	25±5.5
H3 $HA_{273-289}$	46.7±1.2	8.3±2.1	65±1.2
$M1_{234-250}$	53.3±5.9	1.3±1.5	57.3±6.6
$N2_{176-192}$	34.7±3.2	0±0.6	68±4.7
DONOR 3			
$M1_{91-107}$	90.7±2.1	0±0.6	164.2±2.1
$M1_{97-113}$	124±10.4	27.5±4	152.5±18.3
$M1_{205-221}$	68±8.2	7.5±1	174.2±4
$M1_{210-226}$	97.3±9.3	7.5±2	235±5
$M1_{216-232}$	22.7±0.6	0.8±0.6	50±5
$N2_{176-192}$	44±2	0.8±1.2	135±5
$NP_{109-125}$	74.7±1.5	7.5±1	95±5
$NP_{115-131}$	30±3.5	0.8±1.2	45±9.2
$NP_{175-191}$	22±3.5	0.8±1.2	20±12
$NP_{187-203}$	37.3±2.9	2.5±1.7	42.5±5.7
$NP_{193-209}$	52±4	4.2±1.5	102.5±8.5
H3 $HA_{249-265}$	25±8.5	2.5±1.4	25±0

[a]$M1_{58-66}$ is an HLA-A2-restricted immunodominant epitope. It is used here as a control peptide for positive CD8 T-cell responses in HLA-A2 donors.

[b]H3 $HA_{322-334}$ is an HLA-DR1-restricted epitope. It is used here as a control peptide for positive CD4 T-cell responses in HLA-DR1 donors.

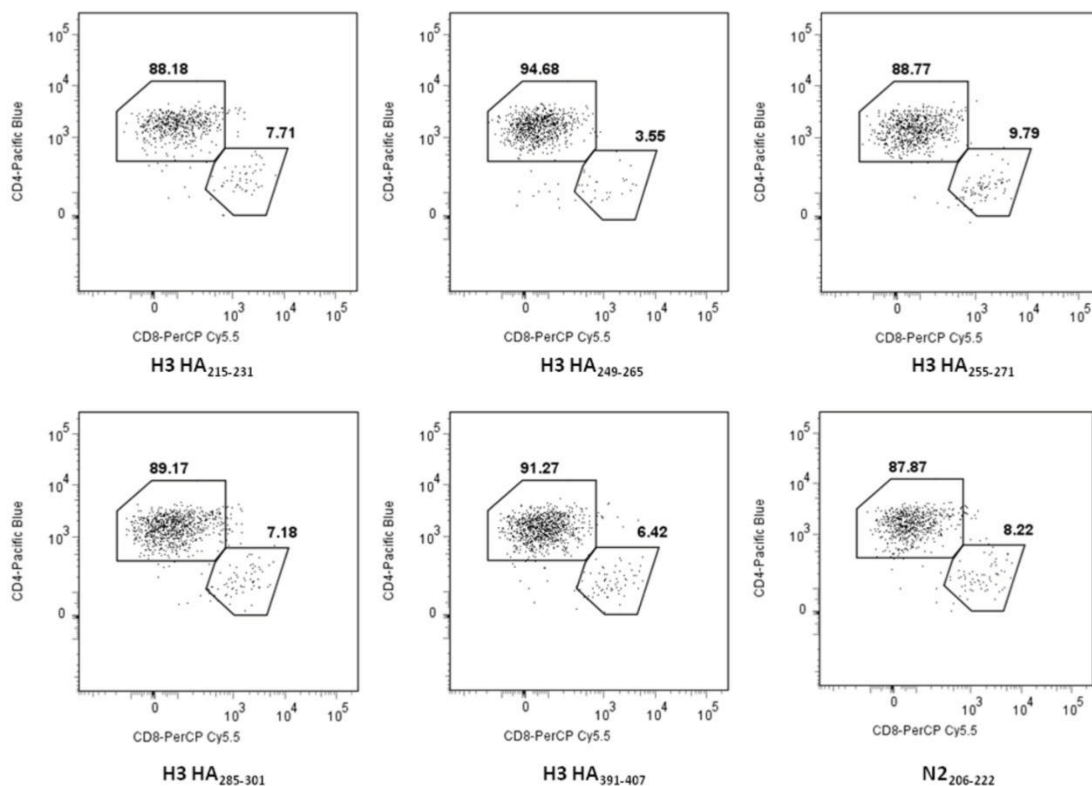


Figure 3.5 Flow cytometry to determine the phenotypes of IFN-γ producing cells in Donor 3 PBMCs. Peptide-stimulated short term bulk culture cells using donor 3 PBMC were used as effectors in an ICS. The peptides illustrated here were used to initially stimulate whole PBMC and establish the bulk culture. The final peptide concentration was 10 µg/ml in all experiments.

established both $CD8^+$ and $CD4^+$ T cell lines using a standard ^{51}Cr release assay (58, 71, 83, 101, 102, 141). Peptide-stimulated bulk culture lines that had a specific immune lysis of ≥15% in ^{51}Cr-release assays (E:T ratios of 10, 30, and 90 were tested) were used in a limiting dilution assay to generate peptide-specific T-cell lines. We were able to generate T-cell lines that are specific to peptides H3 $HA_{267-283}$, H3 $HA_{350-366}$, $M1_{205-221}$, and $M_{191-107}$, as well as to known T-cell epitopes contained in peptides H3 $HA_{321-337}$ (PRYVKQNTLKLAT, $HA_{322-334}$) restricted by HLA-DR1 and $M1_{55-71}$ (GILGFVFTL, $M1_{58-66}$) restricted by HLA-A2. A ^{51}Cr-release assay was performed to determine the ability of the T-cell lines to kill targets pulsed with decreasing doses of cognate peptide. T-cell lines specific to H3 $HA_{267-283}$, H3 $HA_{350-366}$ and $M1_{205-221}$ were able to kill peptide-pulsed targets at a peptide concentration of 10 μg/ml (≥15% specific lysis, Table 3.6).

We also determined the surface expression of CD4 or CD8 of these T-cell lines by flow cytometry. The T-cell lines that were specific to H3 $HA_{267-283}$ (1-3E2), H3 $HA_{350-366}$ (2-10D8), and $M1_{205-221}$ (3-1C9) were $CD4^+$ (Table 3.6), whereas the M1-$M1_{55-71}$-specific T-cell line was $CD8^+$, as expected (data not shown). We also performed IFN-γ ICS by pulsing autologous BLCLs with the cognate peptide and adding the appropriate T-cell line prior to Golgi plug application (Fig. 3.6). T-cell lines were able to produce IFN-γ upon cognate peptide stimulation of autologous APCs, comparable with the virus-infected control, although only a low percentage of the 2-10D8 T-cell line produced IFN-γ after stimulation with either live influenza A virus or the peptide.

Table 3.6. Recognition of influenza virus peptides by CTL lines derived from limiting dilution assay using peptide-stimulated PBMCs.

Cell Line[b]	Protein	Epitope (AA position)	CD4/CD8[c]	% CD4 T-cells producing IFN-γ[d]	HLA restriction[e]	% Specific Immune Lysis[a]			
						10μg/ml peptide	1 μg/ml peptide	0.1 μg/ml peptide	0.001 μg/ml peptide
1-3E2	H3 HA	267-283	CD4	84.5	DRB1*01	59.2	38.0	31.7	31.5
2-10D8	H3 HA	350-366	CD4	0.73[f]	N.D.	30.2	8.5	12.9	8.4
3-1C9	M1	205-221	CD4	27.6	N.D.	55.7	35.5	37.9	7.4

[a] E:T ratio was 10:1; values are representative of one experiment.
[b] Cell lines were tested in CTL assay at least twice in triplicate wells.
[c] T cell phenotype was determined by flow cytometry shown in Figure 3.6. Purity of the T cell population is >98%.
[d] IFN-γ producing cells were identified by ICS. Cell lines were added to either virus or peptide-stimulated autologous BLCLs. PMA/ionomycin was used as a positive control.
[e] HLA restriction was determined by CTL assay against various BLCLs expressing different combinations of MHC class II molecules and by pre-treating target cells with anti-HLA-DR, -DQ, and –DP antibodies.
[f] For this T-cell line, 62.4% of CD4 T-cells produced IFN-γ upon PMA/ionomycin stimulation, but only 0.22% of CD4 T-cells produced IFN-γ upon H3N2 virus infection.

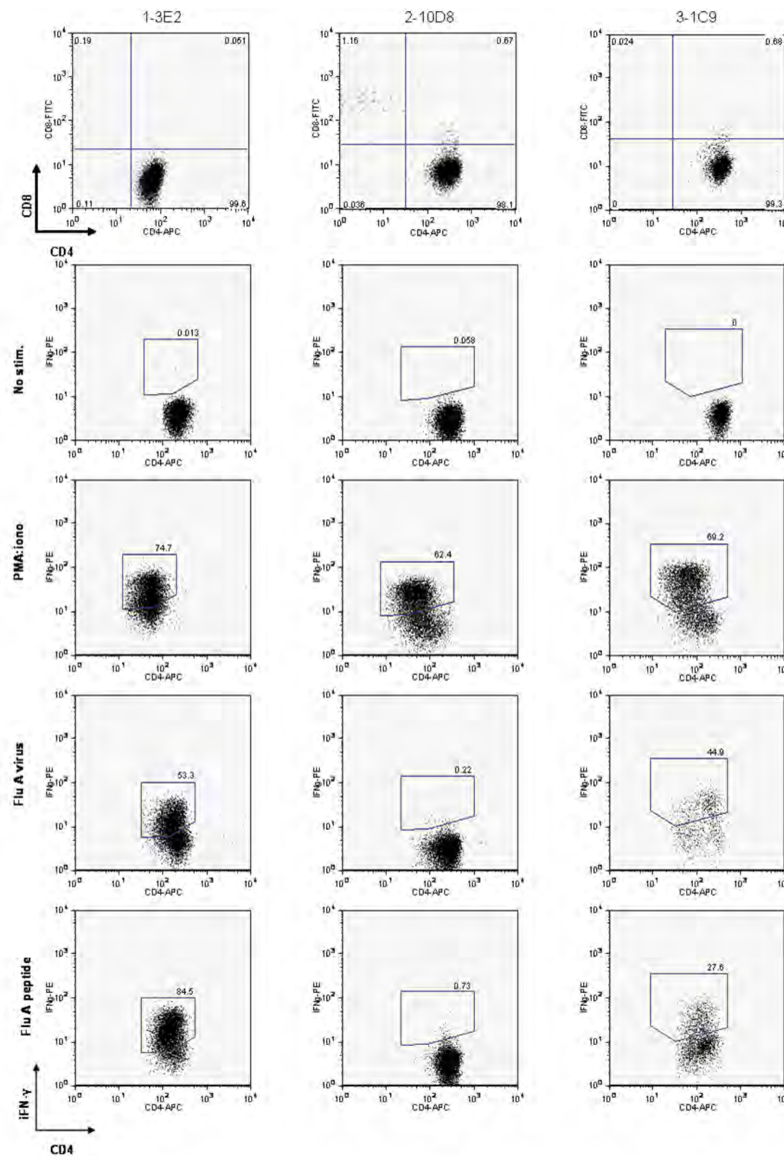


Figure 3.6. Influenza specific T cell lines generated from limiting dilution assay are CD4$^+$ and secrete IFN-γ. T cell lines were stimulated by culturing them with either virus-infected or peptide-pulsed autologous BLCLs as APC for six hours after Golgiplug application. (A) The CD3$^+$ LDA^{low} CD14$^-$CD19$^-$ gated population were analyzed for their CD4 and CD8 surface expression. (B) The CD4$^+$ T cells were analyzed for their IFN- γ expression after peptide or virus stimulation. PMA:ionomycin was used as a positive control. Virus-infected APCs used for the 1-3E2 and 2-10D8 T cell lines were infected with A/Wisconsin/67/2005X-161B (H3N2) virus, while APCs used for the 3-1C9 T cell line were infected with A/New Caledonia/20/1999 IVR-66 (H1N1) virus.

F. H1 HA and H3 HA T cell responses in 30 additional donors

The human T-cell responses to influenza hemagglutinin are not well-characterized. Since we detected several HA-specific T-cell responses in the first four donors, we decided to analyze HA-specific T-cell responses using the PBMCs of 30 hospital workers. These donor PBMCs were collected as part of a clinical study performed by our group (40). Using the same ELISPOT strategy we previously employed, Laura Orphin in our laboratory screened pre-vaccination PBMCs from these donors to determine their baseline HA-specific T cell responses. We did not include the H5 HA peptides from BEI because of the limited number of PBMCs. Fifteen out of the 30 donors have memory responses to at least one HA peptide. All fifteen responders had memory responses specific to the H3 HA, while five of them responded to H1 HA. These results are consistent with our initial screening using four donor PBMCs where majority of the HA responses that we detected are to the H3 HA.

Table 3.7. Summary of H1 and H3 HA peptides that gave a positive IFN-γ response in our ELISPOT screening using PBMC from additional 30 donors.

Protein	AA Position	Sequence[a]	No. of Positive Donors	SFC/10[6b]
H3 HA	13-29	LVFAQKLPGNDNSTATL	1	62.5
H3 HA	37-53	PNGTIVKTITNDGIEVT	2	50, 107.5
H3 HA	43-59	KTITNDQIEVTNATELV	2	30, 37.5
H3 HA	59-75	VQSSSTGGICDSPHQIL	1	57.5
H3 HA	83-99	IDALLGDPQCDGFQNKK	1	52.5
H3 HA	107-123	SKAYSNCYPYDVPDYAS	2	40, 50
H3 HA	119-135	PDYASLRSLVASSGTLE	1	22.5
H3 HA	131-147	SGTLEFNNESFNWTGVT	1	37.5
H3 HA	155-171	CKRRSNNSFFSRLNWLT	1	20
H3 HA[c]	209-225	SLYAQASGRITVSTKRS	4	20, 25, 330, 167.5
H3 HA[c]	215-231	SGRITVSTKRSQQTVIP	2	37.5
H3 HA[c]	243-259	PSRISIYWTIVKPGDIL	5	33.3, 37.5, 40, 42.5, 75
H3 HA[c]	249-265	YWTIVKPGDILLINSTG	4	27.5, 27.5, 32.5, 32.5
H3 HA[c]	255-271	PGDILLINSTGNLIAPR	4	30, 22.5, 35, 50
H3 HA	267-283	LIAPRGYFKIRSGKSSI	1	20
H3 HA	291-307	GKCNSECITPNGSIPND	1	22.5
H3 HA	321-337	CPRYVKQNTLKLATGMR	3	50, 35, 67.5
H3 HA	344-360	TRGIFGAIAGFIENGWE	1	50
H3 HA	368-384	GFRHQNSEGIGQAADLK	1	27.5
H3 HA	386-402	TQAAINQINGKLNRLIG	2	27.5, 32.5
H3 HA	397-413	LNRLIGKTNEKFHQIEK	1	22.5
H3 HA	403-419	KTNEKFHQIEKEFSEVE	1	57.5
H3 HA	409-425	HQIEKEFSEVEGRIQDL	1	57.5
H3 HA	421-437	RIQDLEKYVEDTKIDLW	1	90

Protein	AA Position	Sequence[a]	No. of Positive Donors	SFC/10^{6b}
H3 HA	433-449	KIDLWSYNAELLVALEN	3	35, 27.5, 57.5
H3 HA	439-455	YNAELLVALENQHTIDL	2	22.5, 35
H3 HA	457-473	DSEMNKLFERTKKQLRE	1	55
H3 HA	463-479	LFERTKKQLRENAEDMG	1	20
H3 HA	481-497	GCFKIYHKCDNACIGSI	3	62.5, 22.5, 80
H3 HA	505-521	DVYRDEALNNRFQIKGV	1	85
H3 HA	528-543	KDWILWISFAISCFLL	1	50
H3 HA	550-566	FIMWACQKGNIRCNICI	1	47.5
H1 HA	37-53	LEKNVTVTHSVNLLEDS	1	27.5
H1 HA	156-172	GKSSFYRNLLWLTGKNG	1	35
H1 HA	262-278	GNLIAPWYAFALSRGFG	1	60
H1 HA	416-432	LERRMENLNKKVDDGFL	1	40
H1 HA	434-450	IWTYNAELLVLLENERT	1	25
H1 HA	458-474	VKNLYEKVKSQLKNNAK	1	37.5
H1 HA	464-479	KVKSQLKNNAKEIGNG	2	65, 75
H1 HA	480-496	CFEFYHKCNNECMESVK	1	45
H1 HA	510-526	KLNREKIDGVKLESMGV	1	35
H1 HA	527-543	YQILAIYSTVASSLVLL	1	20
H1 HA	545-560	SLGAISFWMCSNGSLQ	1	42.5
H1 HA	550-565	SFWMCSNGSLQCRICI	1	20

[a] Underlined sequences are known influenza HA epitopes. Amino acids highlighted in yellow reflect an amino acid change in our peptide sequence compared to the previously published epitope.

[b] SFC values are representative of one ELISPOT experiment with three replicate wells.

[c] This peptide contains amino acid residues that overlaps with another peptide and tested positive for IFN-γ response using PBMC from the same donor.

G. Influenza B HA screening

The IBV is a component of the trivalent vaccine given annually, and IBV co-circulates with IAV during most influenza seasons. The T cell responses to IBV are also not well-characterized. We thought of extending our ELISPOT screening using B HA peptides because we were able to detect several responses to the IAV HA proteins than previously reported. Therefore, we performed a similar screening using peptides comprising the IBV pre-HA protein. PBMCs from six additional healthy donors with no prior respiratory or influenza-like illness at the time of blood drawing were tested against pools containing 9 or 10 B/HA peptides at a final peptide concentration of 2 μg/ml. All donors had detectable IFN-γ responses to at least one IBV HA pool (Fig. 3.8). Our results indicate that there is a robust memory T cell population specific to the IBV HA protein that is generated from exposure to IBV through either natural infection or vaccination.

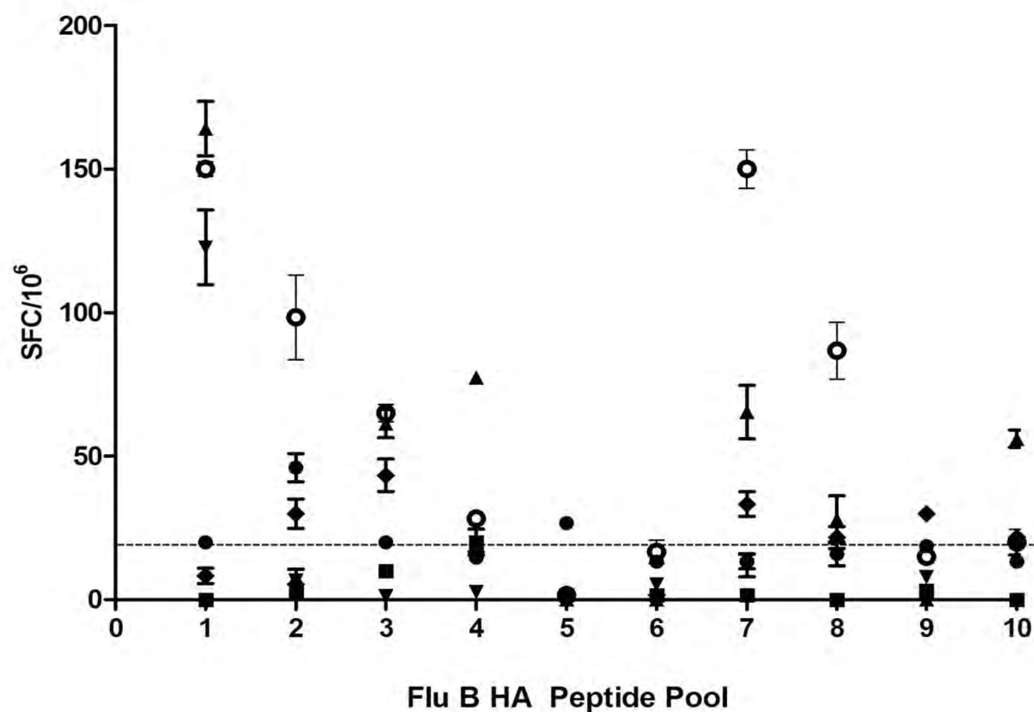


Figure 3.7. T cell responses to the influenza B HA peptide pools. Donor PBMCs were screened against peptide pools spanning the entire IBV B/HA. Each legend represents an individual donor. PHA stimulation is used as a positive control and is ≥ 2000 SFC/10^6 for all donor PBMC. Error bars represent the standard deviation in triplicate wells. (n = 6 donors).

H. Chapter Summary

We performed a comprehensive screening of peptides covering all influenza A virus proteins to determine the breadth and depth of the T cell response to influenza at baseline levels. Our results confirm previous reports that the T cell response to influenza is broadly directed to several viral proteins. We found that the surface glycoproteins, HA and NA, which are major components of inactivated vaccines, had many T cell epitopes. Majority of the responses that we detected were $CD4^+$ T cell responses, probably due to the bias given by the length of the peptides that we used. In addition, we found several $CD4^+$ T cell epitopes in the HA protein and confirmed the abundance of this HA-specific T cell response by screening an additional 30 donors. Healthy adults also have a robust memory T cell response to the IBV HA. Genome-wide screening using overlapping peptides covering all viral proteins is useful in identifying T cell epitopes and is complementary to the approach based on predicted binding peptides to well-studied HLA alleles.

CHAPTER IV

CHARACTERIZATION OF A CROSS-REACTIVE CD4 T CELL RESPONSE TO INFLUENZA A AND B HEMAGGLUTININS

We hypothesized that prior exposure to an IAV subtype generates memory T cells that can potentially contribute to HSI to a subsequent IAV of a different subtype. Our group (102) and others (50, 180) previously identified H5 HA T cell responses in healthy individuals. These individuals were not exposed to avian influenza, thus this T cell response to H5 HA must be cross-reactive. Among the different HA subtypes, H2 and H5 HA are the closest based on their phylogeny (67). Because H2N2 only circulated from 1957-1968, this provided an opportunity to address the hypothesis that prior immunity to H2 HA may contribute to this cross-reactive T cell response.

A. Screening of H2 HA and comparisons between older and younger donors

Individuals who were previously exposed to the H2N2 virus would have a memory pool of H2 HA specific T cell responses. Therefore, we sought to see if there are differences in the H2 HA responses in individuals born before the 1957 pandemic and those who were born after 1968, when H2N2 was last detected in the human population. IFN-γ ELISPOT assays were done to quantify the H2 HA memory T cells using H2 HA peptide pools to be able to compare the responses between older (born before 1957) and younger donors (born after 1968). We expected to detect responses to H2 HA in the PBMC of the older group but not in the younger donors. Indeed, seven of the 11 older

individuals' T cells had IFN-γ responses to at least one peptide pool (Fig. 4.1A), although for most donors, the SFC values are modestly above our cut-off for a positive IFN-γ response (20 SFC/10^6) as described in Chapter III. In our screening of T cell responses to the H2 HA peptides, we did not expect younger donors to have IFN-γ responses to the H2 HA peptides. However, four out of seven younger donors responded to a particular H2 HA peptide pool. Donors YD01 and YD04 had responses to peptides in the H2 HA peptide pool 7, while donors YD02 and YD06, had responses to peptides in the H2 HA peptide pool 9 (Figure 4.1B). These younger donors were born well after 1968, when H2N2 was last detected in the human population. When we tested the individual peptides in these pools, the PBMC of donors YD02 and YD06 both had IFN-γ responses to H2 $HA_{339-355}$ (Fig. 4.2A), while YD04 had responses to H2 $HA_{387-403}$ (Fig. 4.2B). Based on these results, we hypothesize that the IFN-γ responses we saw in the PBMC of these donors may be due to cross-reactive memory T cells generated by exposure to more recently circulating seasonal influenza strains of the H1N1 and H3N2 subtypes.

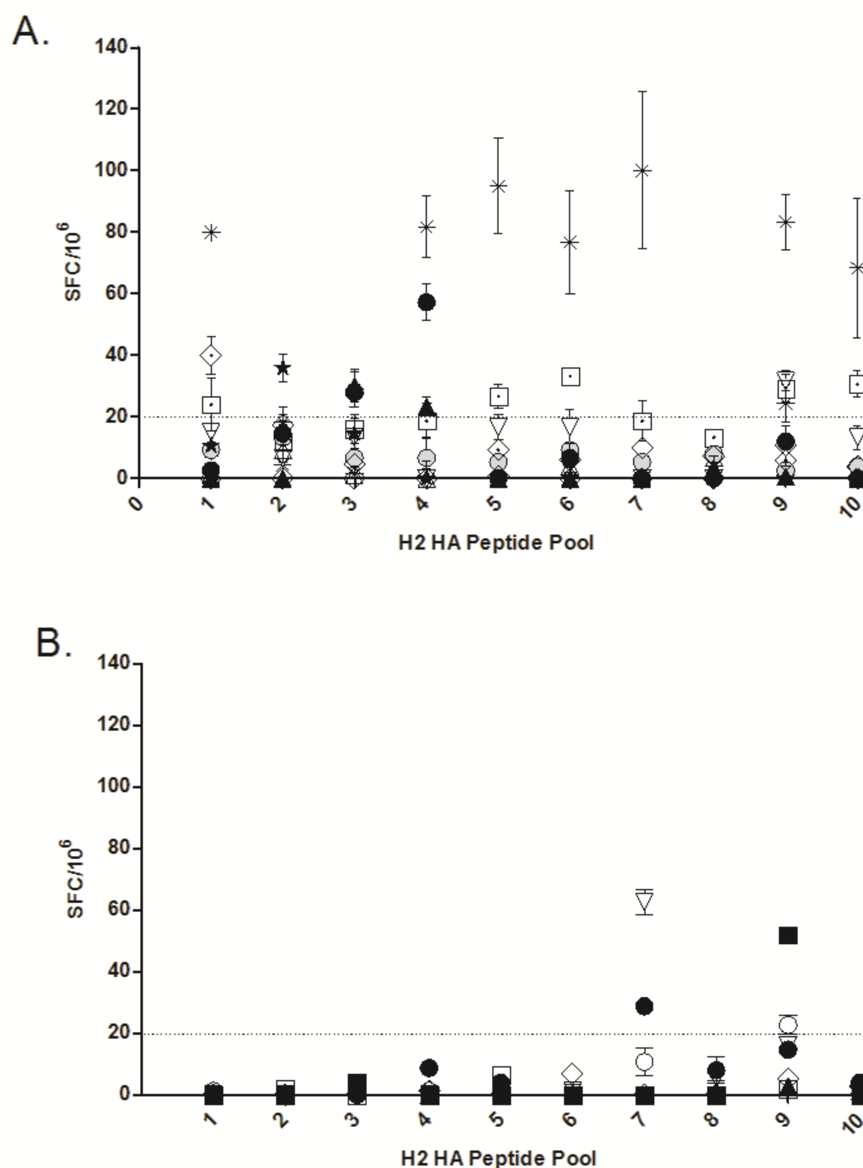


Figure 4.1. IFN-γ responses of various donor PBMCs to H2 HA peptide pools. (A) Older (n=11) and (B) younger donor PBMCs (n=7) were tested in ELISPOT against H2 HA peptide pools to quantify and compare the IFN-γ responses to H2 HA. Each peptide pool contains 9-10 non-overlapping peptides spanning the H2 HA and each point represents an individual donor. Due to limited PBMCs collected from each donor, the ELISPOT peptide pool screening was only done once. Error bars represent the standard deviation in triplicate wells. SFC values for the positive control, PHA, were between 1245 -3664 SFC/10^6.

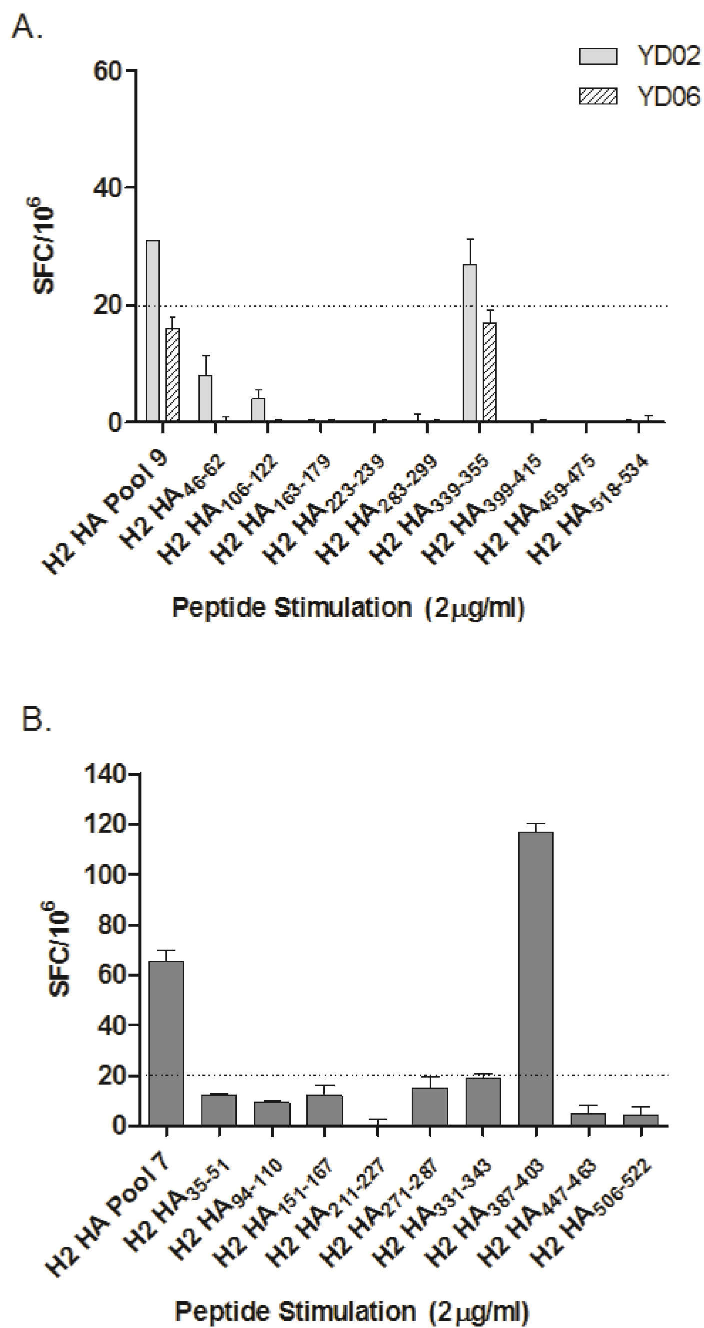


Figure 4.2. Deconvolution of positive H2 HA peptide pools. (A) YD02 and YD06 PBMC were screened against the individual peptides in H2 HA Pool 9, while (B) YD04 PBMC was screened against the individual peptides in H2 HA Pool 7. Error bars represent the standard error of the mean of triplicate wells.

B. The IFN-γ response to the H2 $HA_{339-355}$ peptide is well-conserved in other HA subtypes and is mediated by $CD4^+$ cells

To determine if the H2 HA response we saw in YD02 and YD 06 was due to a cross-reactive memory T cell response to H1 and/or H3 HA, we tested the corresponding peptides in the H1 and H3 HAs. We also included the corresponding peptides in H5 HA to see if this T cell response is also cross-reactive to H5. Our ELISPOT screenings show that the donors' T cells responded to these peptides (Fig 4.3A), especially to the peptides that contained the RGLFGAIAGF amino acid sequence (see Table 4.1 for sequence of HA peptides). This sequence maps to the N-terminus of the HA2 subunit, which spans the fusion peptide of the influenza HA (48).

The $CD4^+$ or $CD8^+$ expressing cells from YD02 PBMC were depleted by using MACS to identify the cell population that is responding to the peptides. We were only able to perform the experiment with donor YD02 PBMC because we had limited PBMC from YD06. IFN-γ was produced in the presence of $CD4^+$ cells in the PBMC, but not with $CD8^+$ cells (Figure 4.3B). The SFC values are also relatively increased when $CD8^+$ cells are depleted due to enrichment of $CD4^+$ cells, indicating that the peptides are presented by the MHC Class II molecule.

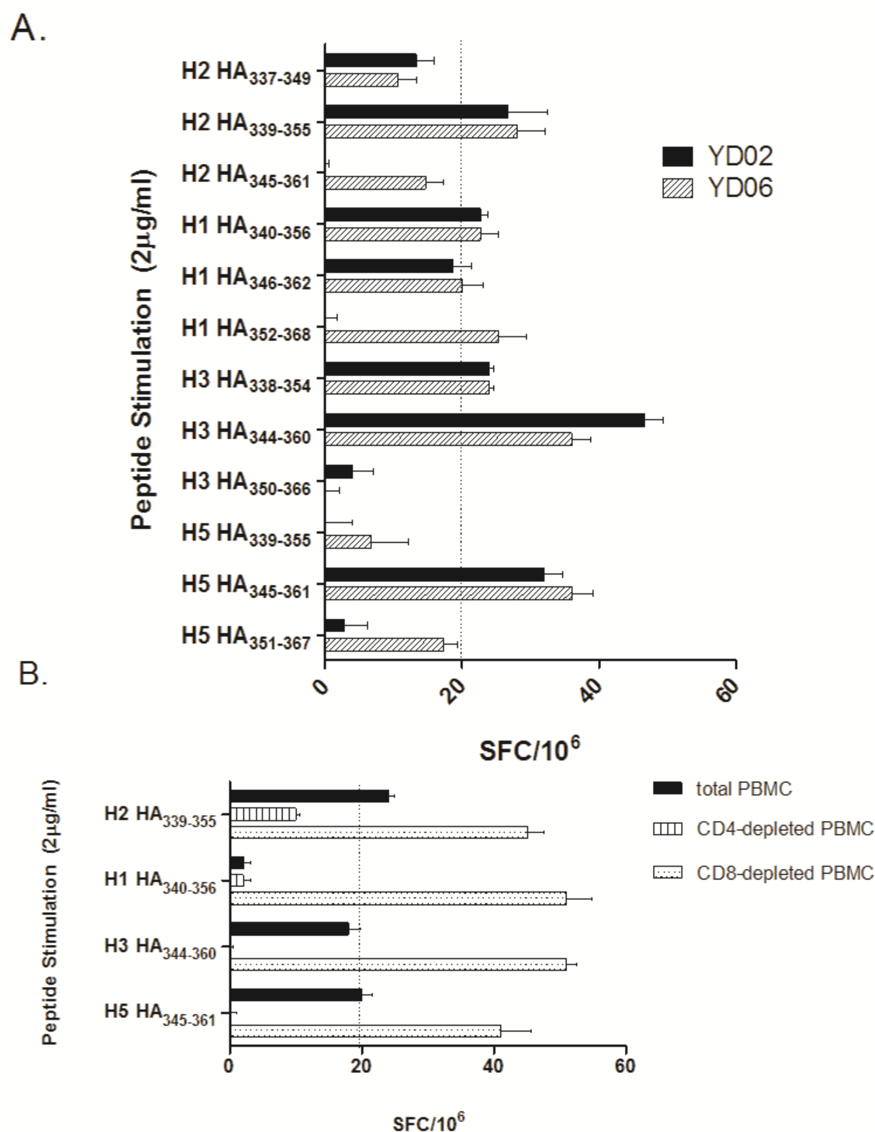


Figure 4.3. IFN-γ responses to corresponding peptide sequences in other A/HAs. (A) YD02 and YD06 PBMCs were screened against the corresponding 17-mer peptides in H1, H3 and H5 HAs that include a part or the entire RGLFGAIAGF amino acid sequence in ELISPOT. Data is representative of two independent ELISPOT experiments. (B) YD02 PBMCs were depleted with either CD4 or CD8 expressing cells by negative selection using magnetic beads and were used in ELISPOT to determine the phenotype of IFN-γ responding cells. SFC values shown are adjusted for background spots given by the negative control SFC (2-3 SFC/10^6). SFC values for the positive control, PHA, were greater than 2,000 SFC/10^6 in all experiments.

C. Characterization of *in vitro* generated cell line specific to H2 $HA_{339\text{-}355}$

To further characterize the T cell response to H2 $HA_{339\text{-}355}$, we generated bulk culture lines by stimulating PBMC from YD02 with the peptide. A limiting dilution assay at one, three or 10 cells per well in a 96-well plate was then set up to isolate T cell line(s) specific to H2 $HA_{339\text{-}355}$. We used a standard ^{51}Cr release assay to select for peptide specific T cell lines from the limiting dilution plates as described in Chapter III. Selected T-cell lines that showed peptide specificity in our initial experiments were propagated and restimulated. We found one T cell line that was able to kill autologous target BLCLs pulsed with H2 $HA_{339\text{-}355}$ in a dose-dependent fashion (Figure 4.4A). When we performed surface staining, greater than 97% of the live, $CD3^+$cells were $CD4^+$ (Figure 4.5). A bulk culture line was also set-up by using YD04 PBMC and the H2-$HA_{387\text{-}403}$ peptide for stimulation, but we were not able to generate a specific T cell line after limiting dilution assay.

The $CD4^+$ T cell line was tested against target cells pulsed with corresponding peptides in H1, H3 and H5 HAs (Figure 4.4B). As expected, this H2 $HA_{339\text{-}355}$-specific T cell line was able to lyse those peptide-pulsed target cells. The pattern of lysis is also consistent with the IFN-γ responses to the peptides in ELISPOT shown in Figure 4.3A (summarized in Table 4.1). We also tested the H2 $HA_{339\text{-}355}$ T cell line against target cells pulsed with various recombinant HA proteins in a ^{51}Cr release assay (Fig. 4.6). The T cell line recognized both recombinant HA from human influenza strains (H1, H2 and H3) and avian influenza HAs (H5 and H7).

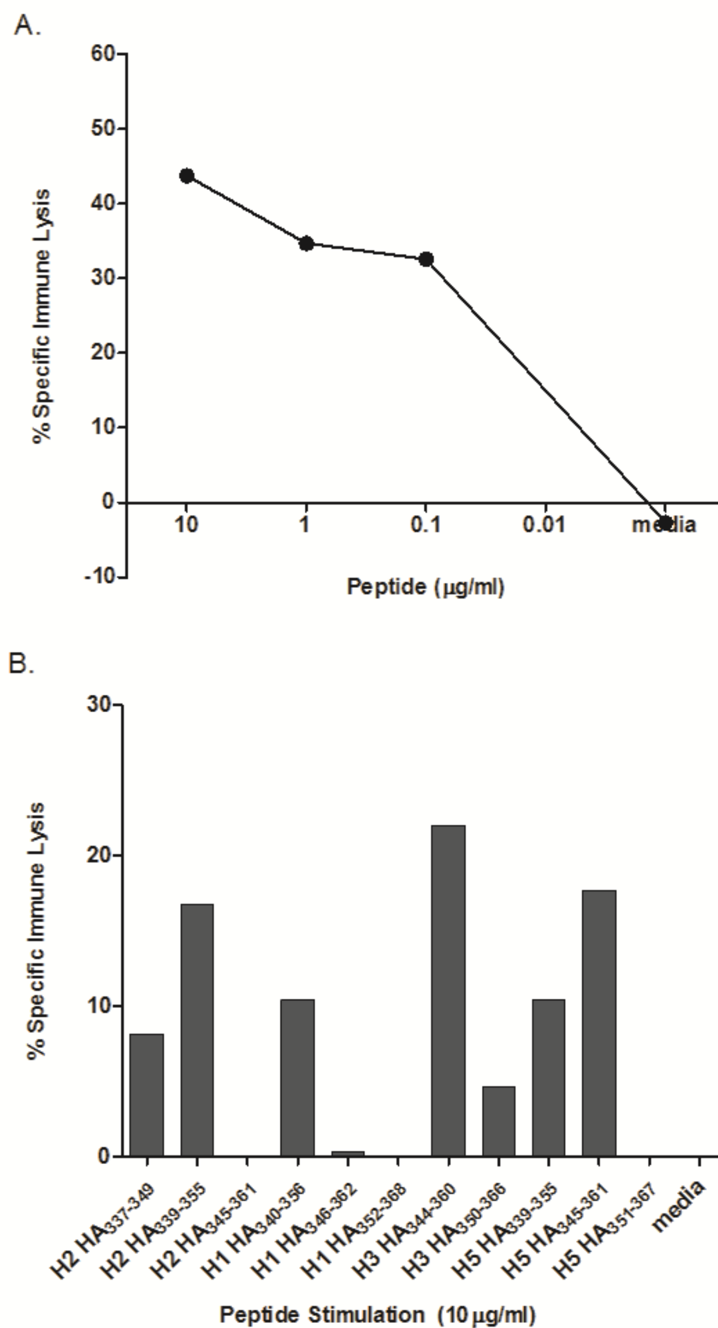


Figure 4.4. Characterization of the H2 $HA_{339\text{-}355}$-specific T cell line using a standard ^{51}Cr release assay. (A) A dose response of the T cell line to H2 $HA_{339\text{-}355}$ peptide was done using a standard 51Cr release assay. (B) The T cell line was also used as effector cells against autologous BLCLs pulsed with the corresponding peptides in other A/HAs.

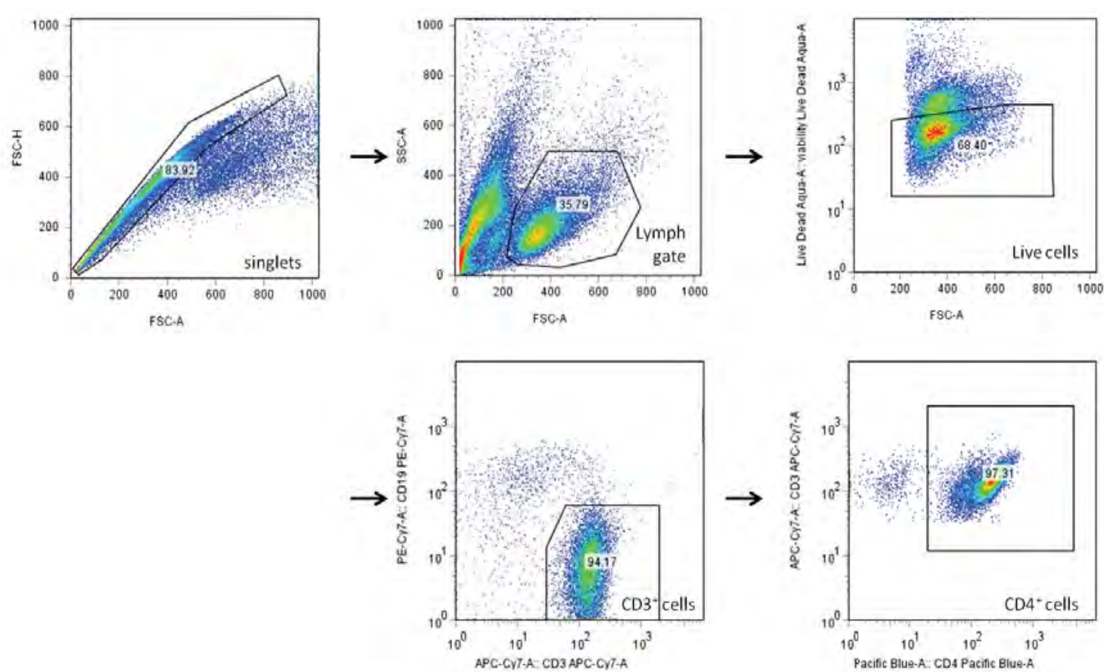


Figure 4.5. The H2 $HA_{339\text{-}355}$ T cell line is a $CD4^{+}$ T cell line. In this surface staining, cells were first gated for singlets (FSC-H vs. FSC-A) and lymphocytes (SSC-A vs. FSC-A). The lymphocyte gate is further analyzed for their uptake of the Live/Dead Aqua stain to determine live versus dead cells and their expression of CD3 and CD19, taking only the live, healthy T cell population ($LDA^{-/low}$, $CD3^{+}$, $CD19^{-}$). CD4 surface expression is then determined from this gated population.

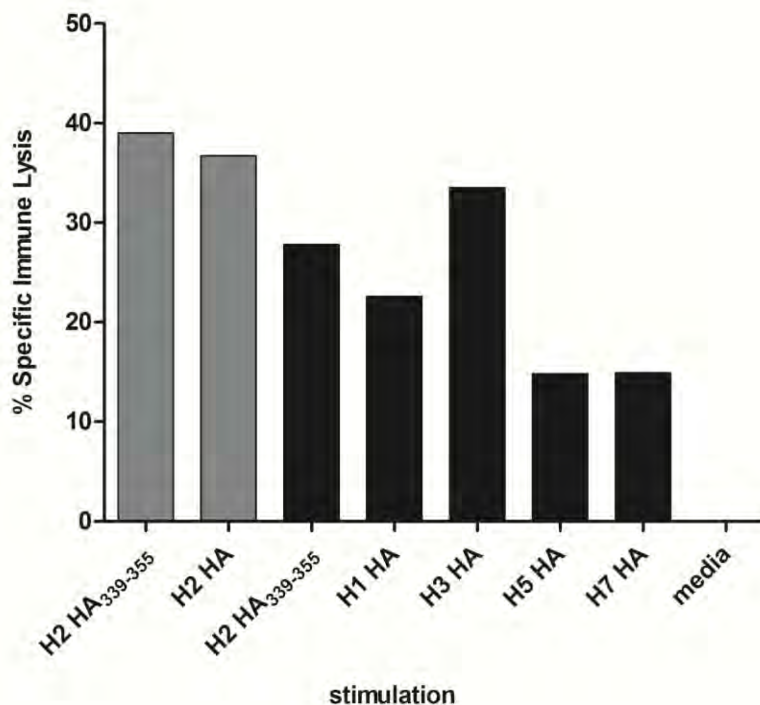


Figure 4.6. Ability of the H2 $HA_{339-355}$-specific T cell line to recognize recombinant HA protein. Autologous BLCLs were treated with recombinant HA protein at a final concentration of 10μg/ml and used as targets in a standard ^{51}Cr release assay using the H2 $HA_{339-355}$-specific T cell line as effector cells. The recombinant H2 HA protein was tested in a separate experiment (light gray bars). The H2 $HA_{339-355}$ was used as a positive control.

Table 4.1. Summary of peptides in several of influenza A/HA subtypes and B/HA containing the conserved RGLFGAIAGF sequence of the fusion peptide and the corresponding responses of donor YD02 T cells in ELISPOT and ^{51}Cr release assays.

Flu A Virus Strain	Peptide Name	Peptide Sequence	YD02 PBMC in ELISPOT[a]	T cell line from YD02 in ^{51}Cr release[b]
A/Japan/305/1957 (H2N2)	H2 $HA_{337-349}$	IESRGLFGAIAGF	-	-
	H2 $HA_{339-355}$	SRGLFGAIAGFIEGGWQ	+	+
	H2 $HA_{345-361}$	AIAGFIEGGWQGMVDGW	-	-
A/NewCaledonia/20/1999 (H1N1)	H1 $HA_{340-356}$	IQSRGLFGAIAGFIEGG	+	-
	H1 $HA_{346-362}$	FGAIAGFIEGGWTGMVD	-	-
	$H1HA_{352-368}$	FIEGGWTGMVDGWYGYH	-	-
A/New York/384/2005 (H3N2)	H3 $HA_{338-354}$	NVPEKQTRGLFGAIAGF	+	N.D.
	H3 $HA_{344-360}$	TRGLFGAIAGFIEGGWE	+	+
	H3 $HA_{350-366}$	AIAGFIENGWEGMVDGW	-	-
A/Thailand/4(SP-528)/2004 (H5N1)	H5 $HA_{339-355}$	RERRRKKRGLFGAIAGF	-	-
	H5 $HA_{345-361}$	KRGLFGAIAGFIEGGWQ	+	+
	H5 $HA_{351-367}$	AIAGFIEGGWQGMVDGW	-	-
B/Nanchang/12/1998 (Influenza B)	$B/HA_{354-370}$	PAKLLKERGLFGAIAGF	-	-
	$B/HA_{360-376}$	ERGLFGAIAGFIEGGWQ	+	+

[a] Summary of ELISPOT results from Figure 2A. A plus sign (+) is defined as ELISPOT responses greater than or equal to 20 SFC/10^6.

[b] Summary of CTL results from Figure 3B. A plus sign (+) is defined as greater than or equal to 15% specific lysis in ^{51}Cr release assays.

N.D. Not determined.

D. The H2 $HA_{339\text{-}355}$ T cell line recognize autologous target cells infected with various influenza A viruses

To determine if the T cell line can kill virus-infected targets, we infected autologous BLCLs with seasonal H1N1 and H3N2 strains, including a 2009 pandemic H1N1 strain. Target cells were also infected with a reassortant H2N1 strain. All of the virus-infected target cells were lysed specifically by the H2 $HA_{339\text{-}355}$ T cell line (Fig. 4.7A). In a separate experiment, the avian H5N1 infected target cells were also lysed by the H2 $HA_{339\text{-}355}$ T cell line (Fig. 4.7 A, gray bars). This indicates that this peptide epitope was processed and presented by APCs during infection *in vitro*.

E. The H2 $HA_{339\text{-}355}$ T cell line can also recognize target cells infected with influenza B virus

The fusion peptide sequence is well-conserved in the influenza HA, including the B/HA (48). Therefore, we asked if this T cell line is able to recognize this sequence in the influenza B/HA as well. Indeed, the T cell line killed both peptide-pulsed or B virus infected targets (Fig. 4.7B), indicating that this $CD4^+$ T cell epitope is also presented in the context of an influenza B virus infection.

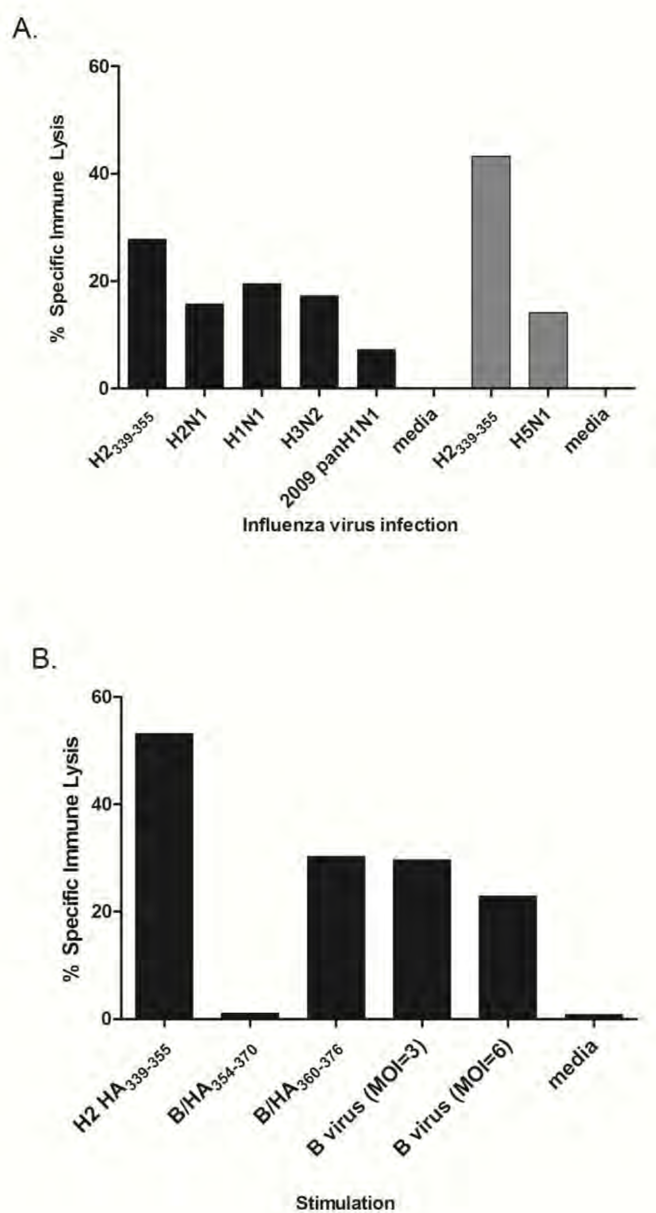


Figure 4.7. Recognition of influenza virus infected target cells by the H2 HA$_{339-355}$ T cell line. (A) Autologous BLCLs were infected with several strains of IAV representing relevant IAV subtypes and were used as target cells in a ^{51}Cr release assay. (B) Responses to the corresponding B/HA peptides and B virus infection were also determined in a ^{51}Cr release assay. H2 HA$_{339-355}$-pusled target cells were used as a positive control.

F. The H2 $HA_{339\text{-}355}$ T cell line produces IFN-γ and TNF-α

Influenza-specific $CD4^+$ T cells have been shown to produce a variety of cytokines, including IFN-γ and TNF-α (136). We performed an intracellular cytokine staining assay to identify the cytokines produced by the H2 $HA_{339\text{-}355}$ T cell line upon stimulation with either peptide-pulsed or virus-infected target cells. The live $CD3^+CD4^+$ T cells (>95%) produced both IFN-γ and TNF-α when they are stimulated with autologous BLCLs that are pulsed with the fusion peptide epitope, but not with the negative control HLA-A2 restricted $M1_{58\text{-}66}$ epitope peptide (Fig. 4.8A). We also infected autologous BLCLs with seasonal and pandemic H1N1 and H3N2 strains, a reassortant H2N1, and influenza B strain and used them to stimulate the H2 $HA_{339\text{-}355}$ T cell line. As with peptide stimulation, the $CD4^+$ T cells were double-positive for IFN-γ and TNF-α with more than 50% of the cells producing both cytokines in response to viral infection (Figure 4.8B). This response is specific only to influenza, since the T cell line did not respond to peptide stimulation using a vaccinia B5R epitope or to vaccinia virus (MOI of 1) infected APCs in ICS (Fig. 4.8C).

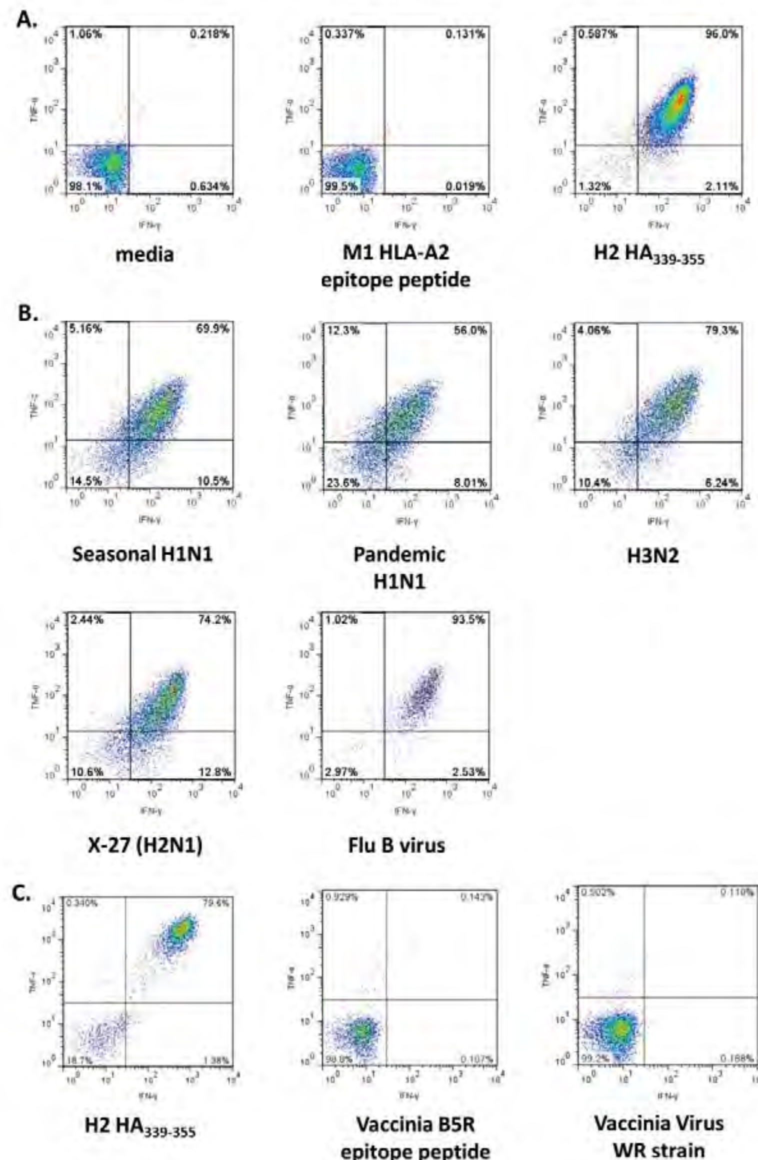


Figure 4.8. Cytokine profile of the H2 $HA_{339\text{-}355}$-specific T cell line upon stimulation with peptide-pulsed or virus-infected autologous BLCLs. The H2 $HA_{339\text{-}355}$-specific T cell line was incubated with either (A) peptide-pulsed (10µg/ml) (B) influenza virus-infected or (C) vaccinia B5R epitope-pulsed or vaccinia virus-infected BLCLs for 5-6 hours in the presence of Golgi plug. The T cells were then stained with surface marker and intracellular cytokine fluorophore-conjugated antibodies to determine the cytokine profile after stimulation. These plots were gated for live, $CD3^+$, $CD19^-$, $CD4^+$ cells. The cytokine response to peptide shown in (A) is representative of one out of three experiments. Responses to viral infected targets, including vaccinia virus infection, were determined in a single experiment.

G. Determining the HLA restriction of the H2 $HA_{339\text{-}355}$ –specific T cell epitope

To determine the HLA-restriction of the T cell line, we first tested it against mismatched BLCLs, initially focusing on HLA-DR alleles. The two donors who responded in our ELISPOT screening shared the HLA-DRB1*09 allele. We performed a ^{51}Cr release assay using peptide-pulsed BLCLs expressing a variety of HLA class II alleles as target cells (Fig. 4.9A). As expected, the T cell line was able to lyse the autologous targets cells expressing HLA-DRB1*03 and HLA-DRB1*09:01 even at a peptide concentration of 0.1μg/ml (Fig. 4.9A). It also lysed target BLCLs that expressed the HLA-DRB1*09:01, although to a lesser extent compared to the autologous targets, but not BLCLs that have mismatched HLA-DR alleles. Our results suggest that this epitope is restricted by HLA-DRB1*09. We did not see comparable % specific immune lysis in both BLCLs that expressed the HLA-DRB1*09:01. Aside from HLA-DR, there are two other MHC Class II molecules that are expressed and can present antigens in humans. Therefore, to eliminate the possibility that this epitope is restricted by a different HLA Class II molecule, we pre-treated target autologous BLCLs with various concentrations of blocking antibodies to HLA-DR, HLA-DP or HLA-DQ and used them in a ^{51}Cr release assay to further investigate the restriction of this T cell epitope. The % specific immune lysis was reduced by more than 70% (from 37.82% to 9.54%) when target cells were treated with blocking antibodies to HLA-DR at a concentration of 5 μg/ml, but not with anti-HLA-DQ or anti-HLA-DP (Fig. 4.9B), although the % reduction of specific immune lysis by the anti-HLA-DQ at a concentration of 0.625 μg/ml is close

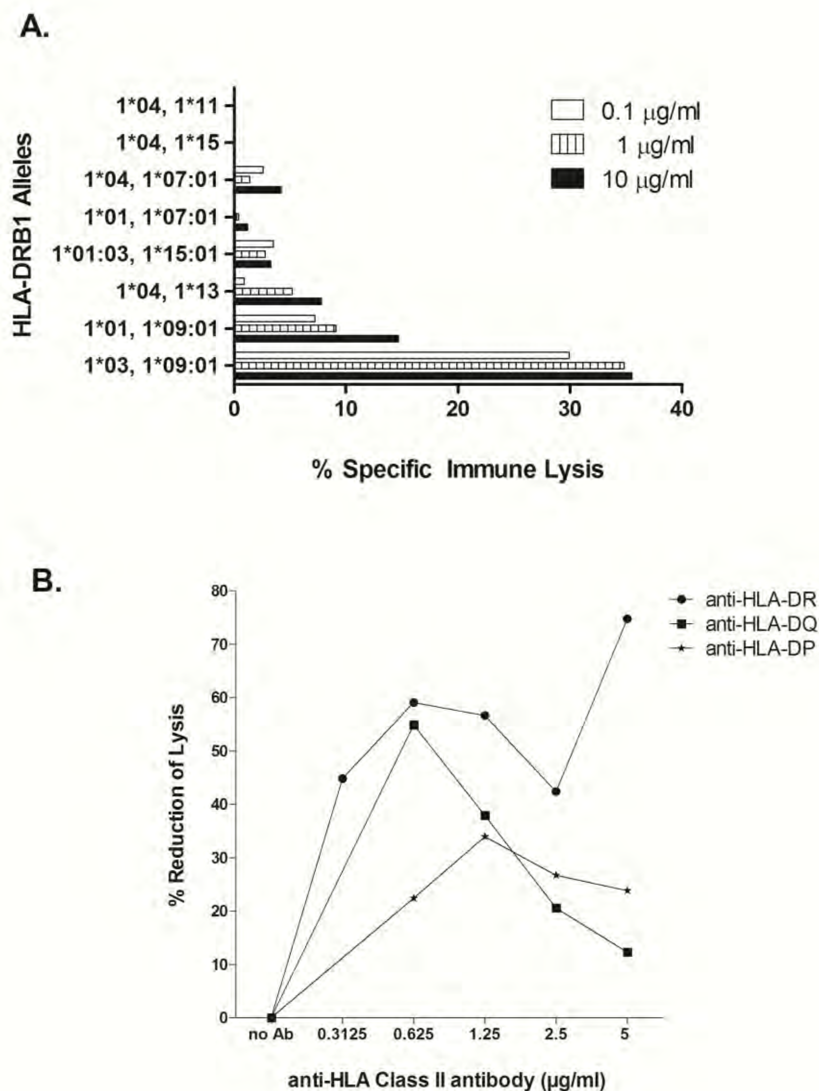


Figure 4.9. Determining the HLA restriction of the H2 $HA_{339-355}$ T cell epitope. (A) The H2 $HA_{339-355}$ T cell line was used effector cells against various mismatched BLCL targets in a ^{51}Cr release assay. A dose response to the H2 $HA_{339-355}$ peptide was also determined. The HLA-DRB1 alleles expressed by each BLCL are listed on the y-axis. (B) Target BLCLs were incubated with anti-HLA-DR, -DQ or -DP antibodies prior to the addition of peptide and effector cells in a ^{51}Cr release assay. The % reduction of lysis was determined by ($\%SIL_{no\ Ab}$ -$\%SIL_{anti\text{-}HLA\ Ab}$)/($\%SIL_{no\ Ab}$) *100, where % SIL is the specific immune lysis value for that particular antibody treatment. Data is representative of at least three separate experiments for each blocking antibody treatment.

to 60%. This indicates that the restriction of this peptide epitope is not solely restricted by HLA-DR and that the donor's particular HLA haplotype may contribute to promiscuity of this epitope.

H. *Ex vivo* responses to the H2 $HA_{339-355}$ epitope in donor PBMCs with the HLA-DRB1*09 allele

The two donors, YD02 and YD06, that responded to the H2 $HA_{339-355}$ peptide in our initial ELISPOT experiments both express HLA-DRB1*09 allele. We therefore tested additional donor PBMCs that have the HLA-DRB1*09 allele. All of the five additional HLA-DRB1*09 expressing donors' cells had *ex vivo* IFN-γ responses to the HA peptides containing the RGLFGAIAGF sequence of the fusion peptide (Table 4.3).

An MHC Class II binding motif prediction algorithm (http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html) from the IEDB (155) identified "IESRGLFGAIAGFIE" as a top 4.52% binder to the HLA-DRB1*09:01 molecule as well as "ESRGLFGAIAGFIEG" (top 4.98%) and "SRGLFGAIAGFIEGG" (top 5.32%) among peptides in the HA protein of the A/Japan/305/1957 (H2N2) strain. We also ran a similar prediction query using the NetMHCIIpan-2.1 server (http://www.cbs.dtu.dk/services/NetMHCIIpan/), which ranks the predicted binding motifs against a set of 200,000 random natural peptides (145). "IESRGLFGAIAGFIE",

Table 4.2. Healthy adult donors have ex-vivo IFN-γ responses to the fusion peptide epitope.

Donor	HLA Class II typing	[a]SFC/10^6				
		H1	H2	H3	H5	B/HA
YD02	DRB1*03, **DRB1*09:01:02**	22.7±1.73	26.7±2.65	46.7±2.65	32±5.86	14±1.53[b]
YD06	DRB1*01, **DRB1*09:01:02**	22.7±4.04	28±3.21	36±2.89	36±4.16	N.D.
YD08	DRB1*04, DRB1*13	22.2±2.65	60±4.04[b]	66.7±2	22.2±3	7.4±2.31
YD09	**DRB1*09**, DRB1*12	33.3±4	46.7±5.51	41.7±3.06	31.7±3.06	48.3±6.56
YD10	**DRB1*09**, DRB1*14	101.7±4.24	96±10.50	84.7±11.79	71.2±7	81.4±9.17
YD11	DRB1*01, **DRB1*09**	68±7.55	40±2.65	72±7	68±3.60	34.7±3.51
YD12	DRB1*04:06, **DRB1*09:01**	103.3±20.43	56.7±2.08	53.3±6.43	40±7	48.3±2.89
YD13	DRB1*04, **DRB1*09**	N.D.	66.7±6.11	N.D.	30±3.46	N.D.

[a] The SFC/10^6 values indicate the IFN-γ response of that donor to the HA peptide containing the conserved RGLFGAIAGF sequence. The cut-off for a positive response is 20 SFC/10^6.

[b] Performed in a separate experiment.

N.D. Not done.

"ESRGLFGAIAGFIEG", and "SRGLFGAIAGFIEGG" are all in the top 16% of predicted binders to HLA-DRB1*09:01 (IC_{50} between 231-238 nM). Donor YD08 also had *ex vivo* responses to the H2 $HA_{339-355}$ epitope, but did not have the HLA-DRB1*09 allele (Table 4.3), suggesting the promiscuity of this epitope.

I. Binding of the $CD4^+$ T cell epitope containing the conserved fusion peptide sequence to HLA-DR1

We also performed a fluorescence polarization assay to determine the binding affinity of the H2 $HA_{339-355}$ peptide to the HLA-DR molecule. Since the HLA-DRB1*09 molecule was not available for the assay we used HLA-DRB1*01:01 (HLA-DR1) molecule, which has a similar peptide binding motif in position 1 (55, 202) . We used overlapping H5 HA peptides containing parts of the fusion peptide sequence to determine the optimal 17-mer that can bind to HLA-DR1 (Fig. 4.10). The H5 $HA_{345-361}$ (KRGLFGAIAGFIEGGWQ) had an IC_{50} of ~ 893 µM, which indicates that this 17-mer sequence can bind, although modestly compared to $HA_{306-318}$, to HLA-DR1 (Table 4.3). This peptide sequence was also predicted to be a strong binder HLA-DRB1*01:01 (top 16%; IC_{50} = 28.1 nM) using the NetMHCIIpan-2.1 server (http://www.cbs.dtu.dk/services/NetMHCIIpan/) (145) and belongs to the top 9.36% binders to HLA-DRB1*01:01 using the IEDB MHC Class II binding motif prediction algorithm (http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html) (155).

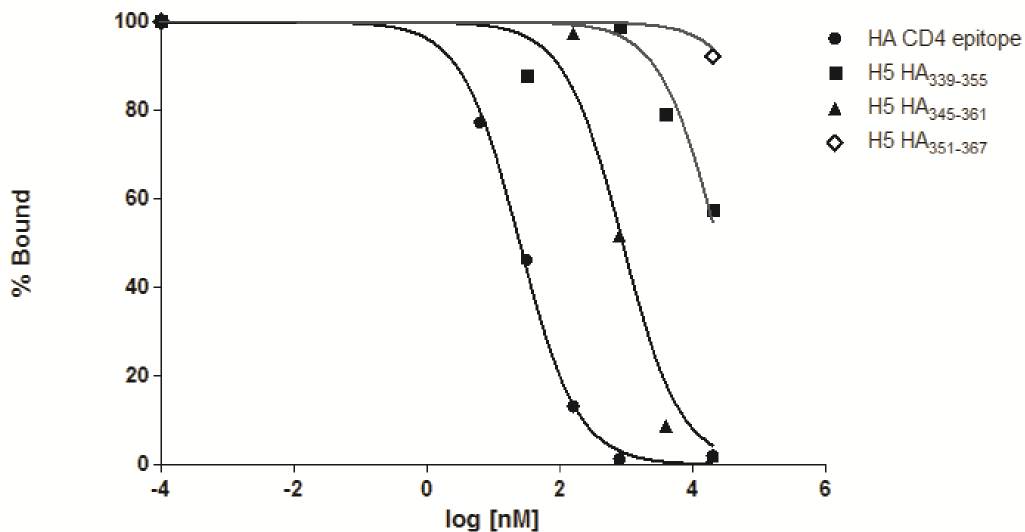


Figure 4.10. Binding of HA peptides to the HLA-DR1 molecule. Various concentrations of unlabeled HA peptides containing the fusion peptide sequence were mixed with Alexa-488-conjugated HA CD4 T cell epitope ($HA_{306-318}$) to determine their binding affinity to recombinant soluble HLA-DR1. Unlabeled HA CD4 T cell epitope peptide was used as a positive control for binding to HLA-DR1. Values shown represent the mean of triplicate wells for a single experiment.

Table 4.3. IC_{50} values determined from Figure 4.10.

Peptide	[a]IC_{50} (nM)
HA CD4 epitope (H3 $HA_{306-318}$)	25.22±0.03
$H5_{339-355}$	24139±0.44
$H5_{345-361}$	893±0.09
$H5_{351-367}$	314640±0.39

[a]IC50 values were determined by fitting to a competition binding equation using GraphPad Prism v5.04.

J. Chapter Summary

We established a human $CD4^+$ T cell line recognizing a cross-reactive epitope that is conserved among the different HA types of influenza A, and also the HA of influenza B. The epitope is located in the fusion peptide sequence of the influenza HA and contains the conserved RGLFGAIAGF sequence. Our *in vitro* experiments show that the $CD4^+$ T cell response to this epitope is characterized by the production of IFN-γ and TNF-α. This epitope is recognized by individuals who have the HLA-DRB1*09 allele. This epitope may also be promiscuous, as it was recognized by donor PBMC that did not have the HLA-DRB1*09 allele. In addition, this epitope is able to bind the HLA-DR1 molecule in a biochemical assay. To our knowledge, this is the first influenza $CD4^+$ T-cell epitope in the HA protein that is cross-reactive to influenza A and B viruses. The implications of a cross-reactive T cell response to HA are discussed in Chapter V.

CHAPTER V

DISCUSSION

A. The T cell response to influenza is broad and directed to several influenza proteins

We performed a comprehensive screening of peptides covering all influenza A virus proteins. We confirmed previous reports by us (101) and others (10, 23, 76, 229) that the T-cell responses to influenza are broadly directed to multiple epitopes on several viral proteins. We found that surface glycoproteins, HA and NA, which are major components of inactivated vaccines, also had several T-cell epitopes. Overall, HA and M1 had more T-cell epitopes than other viral proteins, and those that we have done depletion experiments or by surface staining of peptide-stimulated bulk cultures with were of the $CD4^+$ phenotype. Recently, we (39) and others (89) reported that trivalent inactivated influenza vaccine can induce T-cell responses and that these T-cell responses may be targeting epitopes on HA, NA, and M1. Therefore, healthy adults have a broad and diverse memory T cell pool to influenza. This is in agreement with the diversity of the T cell responses observed in human subjects to HIV(http://www.lanl.gov), CMV(213) and poxviruses (105, 148). This memory T cell pool is generated by prior exposure to influenza by either natural infection and/or vaccination.

There have been previous studies that looked at T-cell responses to all of the influenza proteins in humans, both by our group and others (76, 101), but they were limited by either focusing on well-conserved peptide sequences or by using the sequences

of the A/PR8, which was isolated more than 70 years ago and is not a circulating strain. Other groups that identify T cell responses to influenza focus on a particular influenza viral protein such as HA or M1 (50, 180, 243, 244), or determine influenza-specific T cell epitopes restricted to a particular HLA allele (242, 244). The majority of the human T cell epitopes previously identified were to the internal proteins of influenza A. This is most likely due to the conservancy of the amino acid sequence of these viral proteins among the different strains. Thus, previous efforts may have underestimated the breadth and depth of the T cell response to influenza. In this thesis, we have employed a broader approach to genome-wide screening of T cell epitopes to influenza in humans (134) and this has been done by other groups as well (10).

Although we have identified T cell responses to influenza that are directed to almost all of the viral components, there were limitations to the approach we used for epitope screening. For several of the peptides that we identified, we were unable to determine whether T cells responding to a given peptide were $CD4^+$ or $CD8^+$ T cells. Depletion of PBMCs with either CD4 or CD8 expressing cells prior to ELISPOT is a useful method to determine the T cell population responding to a particular peptide and we were able to do so for PBMC from Donor 1 and Donor 3. However, this was limited by the amount of PBMCs we have collected from our donors. In hindsight, depletion of PBMCs prior to screening of individual peptides from positive peptide pools determined by the initial ELISPOT experiments may have provided us more information as to the phenotype of the responding T cells. We also did not use *in vitro* amplification of influenza A-specific T cells to avoid skewing the relative frequency of the T cells specific to each epitope. As

a result, the frequency of most peptide-specific T cells in PBMCs was not high enough to determine the phenotype by performing ICS experiments. For the PBMC of Donor 3, we were able to determine the phenotype of responding T cells by stimulating the PBMC with peptide and maintaining short term bulk cultures that we used in ICS. Again, we were limited by the PBMC that we have, since generating bulk cultures for each particular peptide would require a significant number of cells.

Based on the ICS of Donor 1 PBMCs stimulated with live influenza A viruses (A/New Caledonia/20/1999 (H1N1) and A/Wisconsin/67/2005 (H3N2)), it is estimated that approximately 80% of T cells responding to a live virus infection are $CD8^+$ T cells (data not shown). This would indicate that majority of the T cell specificities present in response to a live virus infection are of the $CD8^+$ phenotype. However, all peptides for which we were able to perform further experiments with were recognized by $CD4^+$ T cells, with the exception of the $CD8^+$ T-cell epitope, $M1_{58\text{-}66}$. This could be explained by the length of peptides we used for the screening. 17-mer peptides may have stimulated $CD4^+$ T cells better than $CD8^+$ T cells, thus the sensitivity of detecting $CD4^+$ T cells has been relatively higher. Ideally, we should have included influenza-naive donors as a negative control to demonstrate that T cells responding to influenza peptides have been generated against influenza virus exposure from either natural infection or vaccination. However, it is practically impossible to find influenza-naive adults (we cannot obtain large volumes of blood for genome-wide screening experiments from very young influenza-naive children).

Assarsson *et al*. recently reported that PB1 was the major target for both $CD4^+$ and $CD8^+$ T-cell responses (10). In our screening, we found that the HA (especially H3) and M1 were the major targets of T-cell responses. Our peptides were 17-mer overlapping by 11 amino acids covering all influenza A viral proteins. In their study, they used synthetic peptides based on the minimal epitopes predicted to have high-affinity binding to HLA class I or class II molecules and to be highly conserved based on prediction algorithms (10). The use of minimal epitope peptides in the assays is likely to increase the sensitivity of detecting responding T cells, especially in the case of $CD8^+$ T cells. This is true in their study, where they were able to detect more $CD8^+$ T cell epitopes compared to our results. The use of longer peptides covering all viral proteins is probably less sensitive in detecting specific $CD8^+$ T cells, but it may detect T cells recognizing atypical T-cell epitopes or epitopes restricted by MHC class I or class II molecules whose binding motif predictions are not available (for example, HLA-DP and DQ alleles and rare HLA-A, -B, and -C alleles). These differences in the peptide sets may explain why screenings by Assarsson *et al*. (10) and our study presented here produced different results.

The HLA restriction of the epitope candidate peptides identified in this screening has not been experimentally determined, thus we were unable to test whether these epitopes could be identified by prediction algorithms. However, in deciding which peptides to use in establishing peptide-specific T cell lines, we used prediction algorithms to determine binding motifs using the HLA alleles expressed by the donor PBMC that responded to a particular peptide as a determinant of MHC restriction. We think that our

results are complementary to those of Assarsson *et al*. (10) and that both approaches may be used, if possible, to identify T-cell epitopes on a virus.

IFN-γ ELISPOT has been used to determine T cell responses to influenza and other viral pathogens including vaccinia virus (148), HIV (208) and herpes simplex virus (HSV)-2 (162). Although IFN-γ production represents majority of the T cell response to a particular pathogen, other cytokines may also characterize the T cell response. One of the $CD4^+$ T cell lines that we generated from a limiting dilution assay using cytotoxicity as a readout, 2-10D8, produced very minimal IFN-γ after stimulation with autologous BLCL pulsed with the cognate peptide or infected with influenza A virus (Chapter III, Fig. 3.6 B). This cytotoxic $CD4^+$ T cell line recognized a peptide epitope in HA that was initially identified in IFN-γ ELISPOT assays (Chapter III, Table 3.3; 25SFC/10^6). We have not evaluated this T cell line for its ability to produce other cytokines. This suggests that measurement of IFN-γ production alone may also underestimate the T cell response to influenza.

Other cytokines can be used to evaluate the T cell responses to influenza in ELISPOT. IL-2 is an important cytokine required for T cell proliferation and is produced by activated T cells. In a screening of CD4 T cell epitopes to influenza using HLA-DR transgenic mice, IL-2 ELISPOT was used to determine the *ex vivo* T cell responses after intranasal influenza infection (172). Another relevant cytokine to look at is IL-10, which has anti-inflammatory properties that may be able to mediate tissue damage and pathology in the course of influenza infection. Both $CD8^+$ and $CD4^+$ effector T cells have been shown to simultaneously produce large amounts of IL-10 and IFN-γ during

acute influenza infection in mice (211). In addition, virus-specific IL-10$^+$ CD4$^+$ memory T cells were readily detected 26 days post infection and were still detectable at day 9 (35). IL-10 producing human CD4$^+$ T cells that are specific to the M1 protein and have T-regulatory characteristics have also been described recently (156). Thus, a more thorough identification and characterization of the T cell response to influenza will have to assess the production of other cytokines in addition to IFN-γ, such as IL-2 and IL-10.

B. The T cell responses to influenza A hemagglutinins

In our genome-wide screening, HA and M1 proteins were the major targets of the T cell response to influenza. We expected to see that majority of the T cell responses would be towards the internal proteins of influenza because they are highly conserved. It was interesting to see that despite the propensity of the HA gene to mutate and thus, potentially generate escape mutations in T cell epitope sequences, a significant portion of the memory T cell responses were still directed to epitopes in the HA protein. Because we only screened four individuals, we determined if we would see a similar robust HA response by screening more donors' PBMC. The abundance of T-cell responses against HA was confirmed by analyzing the PBMCs of 30 additional healthy donors by screening with HA peptides covering H1 and H3 HA in IFN-γ ELISPOT. More responses to H3 HA were seen than to H1 HA (53.3% of the donors responded to H3 and 16.7% to H1); thus, it is probably not surprising that we did not detect H1 HA-specific responses among the four healthy donors we previously screened. These may reflect (1) the epidemiology of currently circulating influenza A strains during the time when PBMC samples were

collected, (2) the history of prior infections with H3N2 versus H1N1 viruses in these individuals, or (3) higher virulence of H3N2 strain than H1N1 strain, all of which are factors that are difficult to ascertain. A surveillance of influenza and pneumonia-related cases in the United States spanning fifteen years reveal that the H3N2 subtype has a higher severity index, causing more illness and increased mortality compared to the co-circulating H1N1 or IBV (197), which is consistent with the third factor.

The amino acid sequence identity between the HA and NA of A/New Caledonia/20/99 (H1N1) and A/Wisconsin/67/2005 (H3N2) is 40% for the HA (AAP34324 and ABW80978) and 42% for the NA (CAD57252 and ABP52004), respectively. This suggests a low probability of identifying subtype cross-reactive T-cell epitopes in these proteins. However, in two of the four healthy adult donors whose PBMCs were screened we detected T cells responding to the peptides encoded by the H5 HA gene in IFN-γ ELISPOT assays. Although the frequencies of these T cells were not high, they were comparable with those responding to the peptides encoded by the H3 HA gene. Other groups have also identified cross-reactive memory T-cell responses to avian H5N1 proteins in healthy individuals who were previously infected or exposed to seasonal influenza, as well as in individuals who received seasonal influenza vaccination (77, 79, 115, 128, 180), including our group (102). The donors included in these studies were unlikely to have been exposed to H5N1. Most of the cross-reactive responses they identified were toward the internal proteins M1 and NP (115, 128), which is expected because the internal proteins are highly conserved, even among the different subtypes.

They were also able to identify cross-reactive responses to the HA (128, 180) and NA (77) proteins.

Roti and colleagues (180) reported the presence of $CD4^+$ T cells recognizing epitopes encoded by H5 HA gene in healthy individuals, who were unlikely to have been exposed to the H5N1 virus, although *in vitro* amplification of specific T cells was needed to detect them, suggesting a low frequency of these H5 HA cross-reactive T cells. These donors who had H5 HA-specific responses were old enough to have been exposed to H2N2 and H2 and H5 HA are the most related HA. However, they reported that none of the H5 HA epitopes identified was uniquely cross-reactive to H2 HA. Their findings suggest that exposure to H2N2 viruses is not essential for cross-reactivity to H5 HA. Except for two peptides ($H5_{151-167}$ and $H5_{243-259}$ in Table 3.3) that have a four- to eight-amino-acid overlap with the H5 HA epitopes identified by them, the H5 HA peptides that our donors' PBMCs responded to are different. We performed a multiple alignment between the H5 HA peptide we identified and the corresponding sequence in H1, H2 and H3 HAs (Fig. 5.1). As expected, H2 and H5 HAs have more sequence similarity, and there was some level of homology with the H1 HA, but not with H3 HA in peptides that span the HA2 subunit of H5 HA ($HA_{347-568}$, underlined in Fig. 5.1). We also did not observe responses to the H1 and H3 HA peptides corresponding to the eight H5 HA peptides in these donors. Therefore, we sought to detect H2 HA responses in donors who have been exposed to H2N2 and determine if these are cross-reactive to H5 HA. We detected H2 HA-specific T cell responses in both older and younger donors, suggesting

H5 30-46	EQVDTIMEKNVTVTHAQ	H5 207-223	YQNPTTYISVGTSTLNQ	H5 387-403	TQKAIDGVTNKVNSIID
H2	*M****L*R*******K	H2	***VG**V********K	H2	**N*FN*I******V*E
H1	DT***VL********SV	H1	*HTENA*V**VS*HYSR	H1	**N**N*I******V*E
H3	TI*K**TNDQIE**N*T	H3	*AQASGR*TVS*KRSQ*	H3	**A**NQING*L*RL*G
H5 136-152	SSWSSHEASLGVSSACP	H5 243-259	EFFWTILKPNDAINFES	H5 470-486	LQLKDNAKELGNGCFEF
H2	DR*TQ*TTT-*G*R**A	H2	**S**L*DMW*T*****	H2	M*****V**********
H1	***PN*TVT-***AS*S	H1	NYY**L*E*G*T*I**A	H1	S**KN****I*******
H3	FN*TGVTQN-*T****K	H3	SIY***V**G*ILLIN*	H3	K***E**EDM*****KI
H5 151-167	SSACPYQRKSSFFRNVV	H5 351-367	AIAGFIEGGWQGMVDGW		
H2	*R**AVS*NP*****M*	H2	**********T******		
H1	*AS*SHN*****Y**LL	H1	**********T******		
H3	****KRRSNN***SRLN	H3	*******N**E******		

Figure 5.1. Multiple alignment of cross-reactive H5 HA peptide epitopes and their corresponding sequence in H1, H2 and H3 HA. Alignment was determined using the align sequences analysis tool of the Influenza Research Database (http://www.fludb.org/brc/analysis_landing.do?decorator=influenza).

that H2N2 exposure is not essential for H5 HA cross-reactivity, as previously proposed by Roti and colleagues (180).

Many of the novel HA T cell epitopes we detected and those previously defined are located on conserved segments of the HA protein sequence, with the majority of them clustering at the C terminus. A previous study correlated the H3 three-dimensional structure and the epitopes that had been identified in mice and humans and found that the dominant epitopes to HA are primarily located in conformationally stable segments of the C-terminal region (126). In addition, a recent study using HLA-DR1 transgenic mice identified a diverse HA-specific, HLA-DR1- restricted $CD4^+$ T cell response, with the majority of epitopes located in conserved HA regions (172). Although we were not able to determine the MHC restriction of the HA-specific T cell responses we identified in ELISPOT (except for those that responded to Donor 1 and 3, Chapter III), we suspect that they are of the $CD4^+$ phenotype. As mentioned above, our ELISPOT screening strategy lends an unintentional bias towards detecting $CD4^+$ T cell responses due to the length of the peptides we used for screening.

The $CD4^+$ T cell epitope PKYVKQNTLKLAT, was one of the first human $CD4^+$ T cell epitopes identified (122), and initially was thought to be immunodominant (123, 249). It has been used in several studies to characterize and describe the $CD4^+$ T cell responses to influenza (30, 52, 74, 129, 164, 189). Subsequent studies have shown that a number of HA-derived $CD4^+$ T cell epitopes can be recognized in infected individuals (75) and healthy adults (12, 180). Several HA-specific $CD4^+$ T cell epitopes have also been identified by using transgenic mice expressing HLA-DR1 (172) and HLA-DR4

(242). A study that looked at the CD4$^+$ T cell memory phase in influenza infection using HLA-DRI transgenic mice found that although the overall memory response to influenza remains diverse and directed to several influenza proteins, they saw a modest but reproducible shift towards HA-derived epitopes (173). Our data and these studies suggest that a significant portion of the CD4$^+$ T-cell responses to influenza are directed to the HA, and most of them are in regions that are structurally and functionally conserved. A possible explanation could be that repeated exposure to different virus strains through infection or immunization may selectively stimulate T cells specific to the epitopes located in conserved regions of HA.

C. Cross-reactive CD4$^+$ T cell epitope to Influenza A and B HA

We established a human CD4$^+$ T cell line recognizing a cross-reactive epitope that is conserved among the different HA types of influenza A, and also the HA of influenza B. The epitope is located in the fusion peptide sequence of the influenza HA. We also found adult donors' T cells had *ex vivo* IFN-γ responses to the peptides that contain the RGLFGAIAGF sequence (H2 $HA_{340-349}$) of the fusion peptide by ELISPOT. There were, however, notable differences in the T cell responses to the RGLFGAIAGF sequence-containing peptides form different HAs. It would seem that the flanking residue in either the N-terminus or C-terminus of the conserved RGLFGAIAGF sequence may influence how these peptides are recognized (Chapter IV, Table 4.1). These differences can be attributed to the effect of flanking residues on the affinity of the peptide-MHC to the T cell receptor (42, 145). The differences in the T cell responses to the HA peptides can

also be due to the peptide registry that is bound to the MHC pocket. A detailed study binding motif of HLA-DRB1*0901 indicate that the binding of antigenic peptides depends on the interactions between pocket 6 and 9 (100). We speculate that the different overlapping peptides containing the conserved sequence may have different peptide registries, allowing for one peptide to bind more efficiently than the other.

Analysis of evolutionarily conserved sequences in the different influenza A viral components reveal that the FGAIAGFIE sequence of the fusion peptide is the only region in the HA protein that is 98-100% conserved in influenza viral strains of the different human and avian influenza subtypes that circulated between 1997 to 2006 (91). There is also significant conservation between the fusion peptide sequence of the HAs of IAV and IBV (118). The fusion peptide plays a critical role in triggering fusion and destabilizing target membranes during the fusion process (48), thus there is strong functional restraint against mutation in this portion of the HA sequence. A previous study using a mouse model showed that the stability of the fusion peptide sequence enhanced the immunogenicity of CD4 T cell epitopes adjacent to the fusion domain (168). Our findings show that the fusion peptide itself contains a $CD4^+$ T cell epitope as well.

The $CD4^+$ T cell epitope that we describe here is likely to be restricted by HLA-DRB1*09, although it could possibly bind to other HLA-DR molecules as well, as previously shown for the $HA_{306\text{-}320}$ $CD4^+$ T cell epitope (249). In fact we have shown that this $CD4^+$ T cell epitope peptide can bind to the HLA-DR1 molecule in a biochemical assay (Fig. 4.10). Moreover, all seven of the HLA-DRB1*09 donors' PBMC have *ex vivo* IFN-γ responses to the fusion peptide epitope in ELISPOT. The

ethnic origin of the HLA-DRB1*09 allele is Caucasoid and Oriental (www.ebi.ac.uk/cgi-bin/imgt/hla/get_allele.cgi?DRB1*09:01:02) and recent studies show that this allele is frequently present in East and Southeast Asian populations (152, 165, 178, 250), in particular the Han Chinese, which comprise more than 90% of the population of mainland China. In a high resolution genotyping of the HLA-DRB1 locus of individuals from Jiangsu Province, China, HLA-DRB1*09:01 is the most frequent allele (15.26%) (165). It would be interesting to determine the contribution of this fusion peptide-specific $CD4^+$ T cell response to influenza immunity in this population. We also detected *ex vivo* T cell responses to the fusion peptide in one donor who is not HLA-DRB1*09. In addition, our experiments using blocking antibodies to HLA-DQ (at 0.625 µg/ml) showed a reduction of specific lysis to some extent, thus we cannot rule out completely that the epitope is restricted by HLA-DP or –DQ. The HLA Class II typing for donor YD02, from which the T cell line was derived from, is HLA-DRB1*0901, HLA-DRB1*03, HLA-DQB1*02, HLA-DPB1*05:01 and HLA-DPB1*28:01. Using the T cell epitope prediction tool of IEDB, the 15-mer "RGLFGAIAGFIEGGW" is among the 11.21% top binders to the HLA-DQA1*05:01/DQB1*02:01 allele and is among the 18.17% top binders to the HLA-DRB1*03:01 allele. This suggests that this peptide epitope could also be presented by HLA Class II molecules encoded by HLA-DP or DQ alleles. We were not able to obtain the HLA-DQ and HLA-DP typing of the donor PBMC that we used to detect *ex vivo* responses to the fusion peptide. It would be interesting to determine the contribution of the genotypic combination of HLA class II haplotypes to the fusion peptide-specific T cell response.

This $CD4^+$ T cell epitope is also conserved in the HA of influenza B viruses. Influenza A and B viruses are almost the same in structure by electron microscopy, with both having the same number of gene segments that encode for the viral proteins (181). At the amino acid level, however, sequence similarities are only 12 to 37% for all proteins except for basic polymerase 1, which is 61% similar (Table 1.1; (98)). Influenza B has a larger genome, and its membrane channel protein is quite different from influenza A (181). Although a number of $CD4^+$ and $CD8^+$ T cell epitopes in influenza A viruses have been identified using different strategies (IEDB, www.immuneepitope.org), only a few T cell epitopes have been identified in influenza B viruses. An HLA-A*0201-restricted $CD8^+$ T cell epitope located in the NP (NP_{85-94}) has been studied most extensively (175, 176, 195). There are also two HLA-B8-restricted $CD8^+$ T cell epitopes also located in the NP (NP_{30-38} and $NP_{262-271}$) that have been identified (177) and one HLA-DRB1*0101-restricted $CD4^+$ T cell epitope that is located in the HA ($HA_{308-320}$) (177). These epitopes were identified by generating peptides from these two viral proteins based on prediction algorithms and using them to stimulate CTL responses in PBMC from a limited number of donors. Robust *ex vivo* responses to the IBV HA pools were detected in ELISPOT (Fig. 3.7) suggesting that B/HA-specific T cell responses are elicited through natural infection or vaccination. Further studies to determine the role of these memory T cell responses should be done since IBV contributes to the burden of influenza disease (133, 150) and is a major component of the influenza vaccination that is currently being administered. The identification of a cross-reactive T cell epitope that is

shared by both IAV and IBV provides an interesting avenue to explore how such a cross-reactive T cell response may contribute protection to both influenza virus types.

D. The relevance of cross-reactive $CD4^+$ T cell responses in influenza infection

A memory T cell pool is generated after initial encounter with influenza. The presence of cross reactive T cells in the memory pool that can recognize a subsequent, but different, influenza subtype might be of an advantage. They can proliferate more rapidly and are present at higher frequencies than antigen-specific naïve T cells allowing them to dominate the response to secondary (or tertiary) infection (234). Our data suggest that a significant portion of the memory T cell response to influenza is comprised of $CD4^+$ T cells, most of which are subtype cross-reactive. The potential role of these cross-reactive T cells in HSI is summarized in Figure 5.2.

$CD4^+$ T cells may play an important role in HSI than previously appreciated. Immunization with either A/H1 N1 or A/H3N2 of β_2-microglobulin -/- mice that lack MHC Class I expression and do not have $CD8^+$ T cells, are protected against a heterosubtypic challenge as indicated by the viral titers in the lung and survival after challenge, but depletion of $CD4^+$ T cells in these knockout mice partially abrogated this protection implying the contribution of $CD4^+$ T cells to HSI (62). Cross-reactive $CD4^+$ T cells also play important roles in generating robust antibody responses to influenza (182) and adoptive transfer of $CD4^+$ T cell clones specific to HA, NA, M1 or NP of A/PR8 in athymic mice one day after infection enhanced the anti-HA antibody response (183).

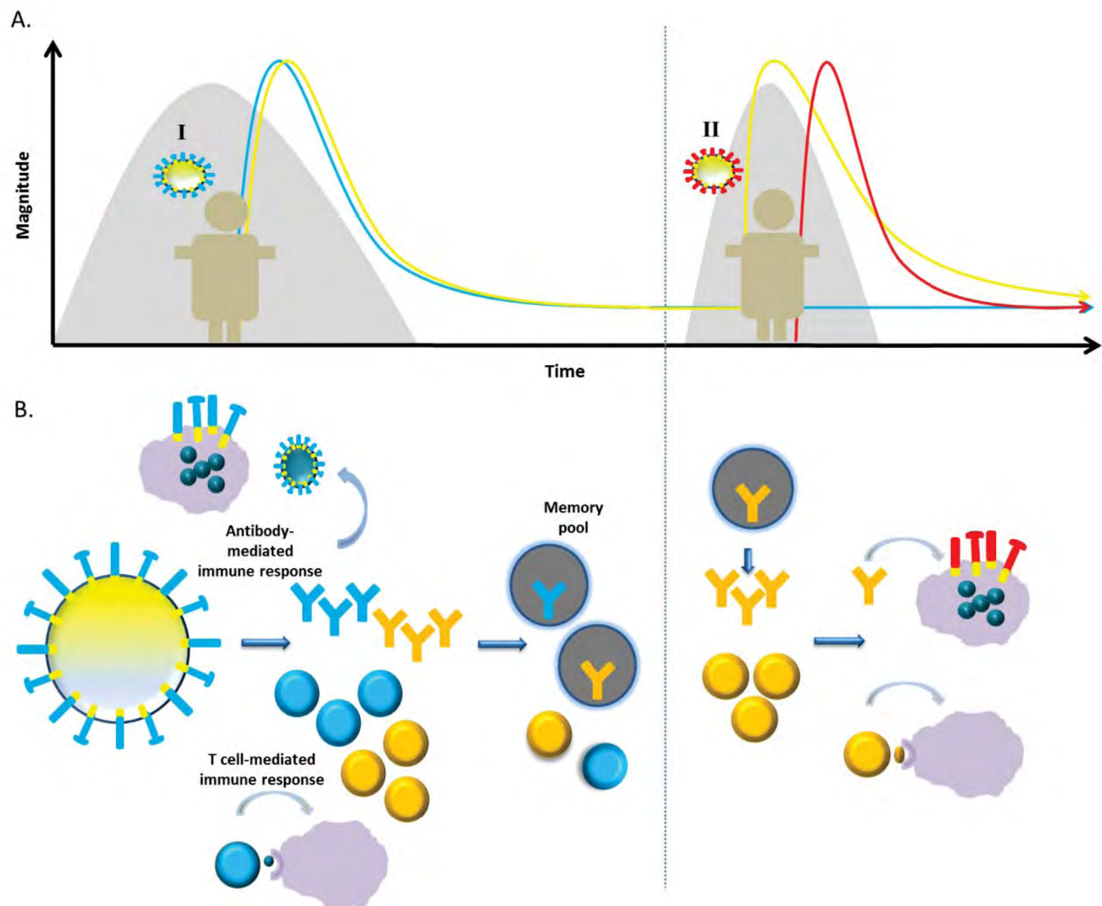


Figure 5.2. A simplified model of the role of cross-reactive CD4$^+$ T cells in heterosubtypic immunity to influenza. (A) After initial encounter of an IAV of a given subtype (I), effector CD4$^+$ T cells that are both subtype specific (blue) and cross-reactive (yellow) are generated. They act in concert with components of the innate and humoral arms of the immune response (not shown) to resolve the infection (gray background). During a second encounter with an IAV of a different subtype (II), subtype specific CD4$^+$ T cells are generated at later stages of the infection (red), but memory CD4$^+$ T cells that are subtype cross-reactive (yellow) can be readily expanded at earlier stages of infection, potentially contributing to partial protection to subtype II resulting to a less severe disease (gray background). The immune mechanisms by which partial protection can occur are illustrated in B. (B). Cross-reactive T cells generated from a previous encounter with subtype I can provide T cell help to antibodies and exhibit intrinsic effector functions upon encounter with subtype II. Vaccination strategies that can stimulate these subtype cross-reactive T cells should be considered.

Thus, cross-reactive $CD4^+$ T cells can contribute to protection by promoting the production of anti-HA antibodies.

The question remains: if cross-reactive $CD4^+$ T cells are restimulated every time we get exposed to influenza antigens, why are they not preferentially expanded and therefore dominate the response during a subsequent infection or repeated vaccination? This is not an easy question to address, since the immune response to influenza is characterized by both humoral and cell-mediated responses, not to mention a potent innate immune response that can affect the outcome of the adaptive immune response. A possible hypothesis is that the immunodominant responses directed to the conserved internal proteins and to $CD8^+$ T cell epitopes may overwhelm the cross-reactive T responses directed to these $CD4^+$ T cell targets, including the conserved HA epitope we describe here. Because $CD8^+$ T cells target internal proteins which are highly conserved, the presence of these conserved antigens during a subsequent infection may preferentially expand these cross-reactive $CD8^+$ T cells. The cross-reactive $CD8^+$ T cell memory population could also be present at higher frequencies. Both scenarios may allow certain immunodominant responses to dominate the memory response to influenza. It is worth determining how we can boost these cross-reactive $CD4^+$ T cell responses to these conserved regions in the HA and to study the mechanism by which they contribute to protection against influenza.

E. Implications to vaccine design

The gold standard of influenza vaccination has always been the generation of neutralizing antibodies targeting HA. However, with recent developments in the field, including our results, it seems that the most effective way to approach the design of a universal influenza vaccine is to be able to stimulate both humoral and cell-mediated arms of the immune response. Several studies in other viral models of infection have shown that a broad-based T cell response that is directed against several epitopes seems to be appealing (24, 139). With a broad-based cell-mediated immune response, the virus is less likely to accumulate mutations that may lead to immune evasion. This is applicable in the case of influenza virus, since it accumulates several mutations through antigenic shift and drift. It may also be relevant to consider vaccination strategies that will enhance cross-reactive $CD4^+$ T cell responses since they have to potential be key effectors themselves while providing help to antibody producing B cells and $CD8^+$ T cells. The challenge remains to be the polymorphism of the HLA expression in humans, which causes the differential ability of various MHC molecules to present viral epitopes. One way to approach this is to take advantage of the presence of MHC supertypes. MHC class I or II molecules belonging to the same supertype can bind the same epitope peptide expanding the population coverage by the single epitope (55, 192, 194). Given the different studies on human T cell epitopes to influenza compiled and curated by the IEDB, we can begin to look at the distribution of T cell epitopes restricted by a particular MHC supertype and determine ways in which we can stimulate these T cells in individuals with the MHC supertype.

The fusion peptide epitope that we have characterized here is cross-reactive to both IAV and IBV. Although natural infection or standard vaccination may not induce strong T and B cell responses to this highly conserved epitope (21, 114), it is worth determining how we may be able to induce these cross-reactive $CD4^+$ T cells by vaccination. As with any vaccination strategy, careful evaluation of the role of these fusion peptide-specific, cross-reactive T cells *in vivo* is required. The fusion peptide sequence is highly conserved in IAV and IBV but not influenza C and is also quite different from class I glycoproteins expressed by other viruses such as HIV and parainfluenza virus type 1 (48). However, a BLAST search of the first 12 amino acids (GLFGAIAGFIEG) of the influenza fusion peptide against known human protein sequences deposited in the NCBI database reveal that a few host proteins, mostly transport proteins (e.g. tricarobxylate transport protein, citrate transporter protein and solute carrier family 25), contain a similar sequence, "G-FGAIAG." This has important implications in inducing T cell responses to this peptide epitope as a vaccination strategy, since we do not want to induce a population of auto-reactive T cells. There is evidence of viral epitopes that are cross-reactive to self-antigens, such as a human CMV $CD4^+$ T cell epitope that is recognized by auto-reactive glutamic acid decarboxylase (GAD65)-specific T cells (94). To our knowledge, there is no known auto-reactive epitope containing the fusion peptide sequence. Further studies are needed to determine if the fusion peptide $CD4^+$ T cell response that we describe here are potentially auto-reactive.

We described a cross-reactive $CD4^+$ T cell response specific to the fusion peptide of HA that is characterized by the production of pro-inflammatory cytokines IFN-γ and

TNF-α *in vitro*. The *ex vivo* responses of healthy donors to the fusion peptide were also characterized by IFN-γ production. There is also the possibility that instead of lending protective immunity, this cross-reactive T cell response may actually promote immunopathology instead, as seen in some models of heterologous immunity, wherein a T cell memory response to a particular virus is able to mediate the immune response to a subsequent infection by an unrelated virus (reviewed in (190, 234). How this cross-reactive response to the fusion peptide mediates protection remains to be determined, and could be challenging to ascertain in humans.

Recent identification and characterization of several human cross-reactive monoclonal antibodies to epitopes located in the more conserved HA2 domain or the stalk region of HA have been reported (45, 57, 210, 238). This has stimulated a novel approach of generating influenza virus vaccines based on the stalk region (including the fusion peptide) where a "headless" HA lacking the HA1 subunit is used as an immunogen that elicits immune sera with broader reactivity (205). The presence of the cross-reactive $CD4^+$ T cell epitope in the fusion peptide may be helpful to induce higher levels of cross-reactive antibody responses, at least for individuals with the HLA-DRB1*09 allele (and other alleles with a similar peptide binding motif). Several groups have also shown that these cross-reactive antibodies can be detected in human sera (44, 108, 221). Specific activation of these helper T cells should be considered in designing or improving vaccination strategies to influenza. $CD4^+$ T cell epitopes also tend to be more promiscuous than $CD8^+$ T cell epitopes due to the nature of MHC class II molecules (84).

This may be advantageous when considering epitopes to be included in an improved vaccine for influenza since more than one HLA molecule can present the peptide.

A peptide-conjugate vaccine based on the fusion peptide of the precursor HA of the B virus (using the outer membrane protein complex (OMPC) of *Neisseria meningitides* as conjugate) was shown to elicit protection in mice against lethal challenge of various strains of influenza B and can potentially be extended to influenza A strains (21). However, they also show naïve wild type mice that survived an IBV challenge do not have significant antibody titers to the fusion peptide suggesting that immunity to the cleavage site is not usually elicited during natural influenza virus infection (21). A similar study using the fusion peptide conjugated to keyhole limpet hemocyanin (KLH) yielded similar results (203). Both studies did not evaluate the T cell response to the peptide-conjugate vaccine. A more recent study examined the presence of HA2-specific antibodies in acute and convalescent sera from adults with confirmed H3N2 infection (204). They found that a third (15/45) of the subjects had antibodies specific to the N-terminal residues 1-38 of HA2, which includes the fusion peptide sequence. They also confirm the previous report that the first few residues of HA2 are weak natural immunogens. Nonetheless, it is interesting to note that a B cell epitope (11) and an HLA-A2 restricted $CD8^+$ T cell epitope (76) have been previously described. More recently, $CD8^+$ T cells specific to the fusion peptide (H1 $HA_{344-353}$) were detected at modest frequencies in vaccinated human subjects one year after vaccination (114). These data indicate that the fusion peptide of the influenza HA is a relevant target of the immune response to influenza, which can be exploited to contribute to protective immunity.

F. Overall summary and conclusions

In this thesis, we have shown that the human T cell immune response to influenza is broad and directed to several viral proteins, mostly to the HA and M1. Although there were subtype specific T cell responses to the HA and NA, we identified several cross-creative T cell epitopes not only to the more conserved internal proteins, but also to the more divergent HA protein. We also saw T cell responses to the H5 HA in individuals who have not been previously exposed to H5N1 viruses. These subtype-cross-reactive H5 HA responses were generated by prior exposure to seasonal influenza subtypes either by natural infection and/or vaccination and were not particularly attributed to T cell memory from H2 HA, contrary to our initial thought. In addition, the subtype-cross-reactive T cell responses to HA that we saw were directed to highly conserved regions. Among them, we identified and characterized an HA $CD4^+$ T cell epitope that is highly conserved in both IAV and IBV HA. This T cell epitope is probably restricted by the HLA-DRB1*09:01 allele, although it also can bind to HLA-DRB1*01:01 in a biochemical assay.

Although we did not perform animal experiment to test whether the cross-reactive T cell responses that we identified here are able to contribute to HSI to influenza of a different subtype, existing literature on cross-reactive T cell responses to influenza as discussed above suggests that cross-reactive T cells can provide partial protection to a subsequent influenza infection. Further characterization of these cross-reactive T cells in

the context of human influenza infection and vaccination and in consideration of the epidemiology of the virus is quite challenging, but is important in our understanding of the adaptive immune response to influenza. Our knowledge of the T cell response to influenza, including the results we have described in this thesis, has implications for the improvement of current vaccination strategies and in the design of a universal influenza vaccine.

CHAPTER VI

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