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Nelson B Moseley II Date

Characterization of Human T Cell Responses to 2010-2011 Influenza Vaccines

By
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Doctor of Philosophy

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Characterization of Human T Cell Responses to 2010-2011 Seasonal Influenza Vaccines

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B.S, Auburn University, 1999

Advisor: John D. Altman, Ph.D.

An abstract of a dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy Program in Immunology and Molecular Pathogenesis Graduate Division of Biological and Biomedical Sciences.

Date: October 27, 2011

ABSTRACT

Characterization of Human T Cell Responses to 2010-2011 Seasonal Influenza Vaccines

By Nelson B. Moseley II

The ultimate goal of influenza-specific T cell research is to develop effective methodologies for exploiting T cell immunity in influenza vaccination. Theoretically, this may be achieved by boosting CD4 T cell populations that provide help for influenza-specific CD8 T cells and B cells or by directly inducing the expansion of CD8 T cells specific for highly conserved influenza epitopes. Interest in this area has been fueled by numerous studies that demonstrate pre-existing T cell immunity against both seasonal and antigenically variant influenza strains in the general population, raising the question of whether it is possible to enhance influenza vaccine efficacy by targeting these populations. The suitability of this approach is difficult to assess, because human T cell responses to influenza vaccination are not well understood. To address this issue, we performed an investigation to characterize human T cell responses to 2010-2011 seasonal influenza vaccines. To accomplish our objective, we first developed a recombinant vesicular stomatitis virus (rVSV)-based quantitative T cell assay that was demonstrated to be an effective and practical alternative to conventional T cell assay methodologies, and we optimized this system for use in human influenza-specific T cell studies. Using the rVSV system, we measured vaccine-induced CD4 and CD8 T cell responses in adult donors following the administration of trivalent inactivated influenza vaccine (TIV) or live, attenuated influenza vaccine (LAIV). We investigated responses against various external and internal proteins across multiple influenza A strains. Pre-existing T cell populations specific for external and internal influenza proteins were observed in most donors. Although we noted modest vaccine-associated CD4 T cell responses specific for external proteins in specific donors, trial members as a group did not demonstrate significant boosting of baseline influenza external protein-specific CD4 T cell responses. LAIV was more effective than TIV in generating CD4 T cell responses specific for internal proteins while both vaccines were ineffective in boosting external or internal protein-specific CD8 T cell responses. Overall, we conclude that current seasonal influenza vaccine formulations are poor inducers of T cell responses and propose that new technologies specifically engineered to target T cells will likely be warranted in order to establish effective and long-lived influenza-specific T cell immunity.

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Chapter 3

Chapter 1: Introduction

1.1 Background and classification

Influenza is a virus that causes acute, febrile disease that is annually responsible for approximately 500,000 deaths worldwide (1). Many viral illnesses that are problematic in developing countries, such as measles, dengue, and yellow fever, do not represent major health burdens in the United States. This is not the case with influenza. Pneumonia and other respiratory and circulatory complications associated with seasonal influenza account for nearly 300,000 hospitalizations in the U.S. each year with an estimated annual medical cost in excess of $10 billion (2). In addition to the challenges involved in controlling seasonal influenza, there remains a constant threat that reassortment events among circulating influenza strains could result in pandemic influenza. This fear was realized as recently as two years ago when the emergence of the 2009 swine-origin H1N1 virus resulted in the World Health Organization (WHO) declaring the first influenza pandemic of the 21st century. 2009 H1N1 is estimated to have caused approximately 12,500 deaths in the U.S. alone (3).

Influenza viruses belong to the *Orthomyxoviridae* family, which consists of five genera: Influenza A, B, and C, Isavirus, the causative agent of infectious salmon anemia (4), and Thogotovirus, a tick-borne virus that is known to infect and cause mild disease in humans (5). All Influenza genera are known to infect humans; in fact, for influenza B viruses, humans appear to be the only natural

host (6). Influenza A viruses infect a range of mammals, including humans, dogs, cats, horses, swine, and a variety of avian species, such as ducks, quail, chickens, and turkeys (6-9). It is generally accepted that human influenza viruses are derived from mutated and reassorted variants that originated in avian species, probably water fowl (10,11). Influenza A viruses are further classified into subtypes based on the sequences of hemagglutinin (HA) and neuraminidase (NA) glycoproteins that extend from the surface of the virion. Currently, 16 HA (H1-H16) and 9 NA (N1-N9) molecules have been classified, and although only H1-3 and N1-2 viruses historically cause widespread infection of humans, avian influenza HA subtypes, such as H5, 7, and 9, cause sporadic, yet often severe, human infection (12,13). Influenza C viruses have traditionally been considered human pathogens, but there have been reports of Influenza C isolation from swine (14).

1.2 Transmission and pathology of influenza viruses

It is generally accepted that transmission of influenza occurs primarily through an aerosol route. Infected individuals exhale droplets of virulent respiratory secretions that settle in the airways of the new host and initiate infection. This mode of transmission typically occurs over distances of 1m or less (15,16). Transmission through direct contact is also possible. There have been occasional concerns regarding the ability of influenza to be transmitted over distances greater than 1m via an airborne route, but there has been no conclusive supporting evidence (17). The period of time between exposure to influenza and onset of illness is highly variable, from 1-7 days, and depends on

various factors, such as the age of the host, immune status, infectious dose, etc. (18). A typical influenza infection in an adult will induce a fever between 100-105°F about 24 hrs. following onset. Additional symptoms commonly include a dry, unproductive cough, chills, malaise, sore throat, and headache (19,20). These symptoms usually subside by one week after onset, although general malaise, cough, and weakness may persist for an additional few weeks (21-23). Young children and the elderly experience higher morbidity and mortality than adult populations who have robust, fully developed immune systems. Although the course of disease and clinical manifestations are similar for all groups, susceptible populations often experience more severe symptoms, increased opportunistic infections, and gastrointestinal complications (24-26).

1.3 Influenza Pathogenesis

In humans, the lower airways of the lungs endure most of the pathological changes associated with influenza infection, although the effects of inflammation can be traced throughout the entire respiratory tract. Ciliated columnar epithelial cells are believed to be the major centers of viral replication. Analysis of these populations reveals that they become vacuolated and swollen in the 24 hrs. following infection. Eventually, infected cells lose their cilia, become necrotic, and desquamate (27). Immunopathology, mediated by the infiltration of monocytes and neutrophils into inflamed tissues, has also been shown to occur (28). Regeneration of damaged epithelial layers typically begins 5-7 days after onset, a process often requiring four weeks to complete. However, in cases of severe

infection, the repair process may require more than six weeks to restore normal respiratory function (29,30).

1.4 Influenza virus morphology and genome structure

Influenza viruses typically have a spherical morphology but also commonly exist in a long, filamentous form. The virion is surrounded by a lipid envelope, which is lined by a network of matrix proteins (M1). The M1 scaffolding is essential for viral assembly as well as budding from host membranes (31). M1 interacts with the ribonucleoprotein (RNP) at the core of the viral particle. The RNP is the transcriptional unit of the virus, and is composed of an RNA genome in a complex with four viral proteins, polymerase subunit A (PA), polymerase subunit B1 (PB1), polymerase unit B2 (PB2), and a nucleoprotein (NP). An additional viral protein, NS_2, is known to associate with the M1 scaffolding, but the biological function of this protein and this interaction has yet to be clearly defined (32,33). Protruding from the viral envelope are two types of external influenza proteins, trimers of hemagglutinin (HA) and tetrameric complexes of neuraminidase (NA; 34) that coat the surface of the virion at an approximate ratio of 5:1. Among influenza viruses, C viruses do not express NA. Influenza C viruses encode only one external protein, HEF. This protein was designated HEF as it is capable of hemagglutination, esterase, and fusion activity (35), essentially making it capable of performing the functions of both HA and NA in influenza A and B viruses. M2 is a transmembrane protein on the surface of the virion that serves as an ion channel for influenza A and C viruses. B viruses differ from A and C viruses in that they express two transmembrane ion channel proteins,

BM2 and NB. Both function in a manner analogous to M1 from influenza A and C viruses, and although it is not yet understood why B viruses express two ion channel proteins, one of the proteins (BM2) seems to be required for viral replication (36-37). The precise role of NB has yet to be determined.

Influenza A and B genomes consist of eight linear, non-segmented RNA gene segments that are listed in order based on descending nucleotide lengths: Segment 1 codes for PB2, 2) PB1, 3) PA, 4) HA, 5) NP, 6) NA (and NB for B viruses), 7) M_1/M_2 (BM2 for B viruses) and 8) NS_1/NS_2. Influenza C viruses have only seven gene segments, lacking segment 6 (NA). Approximately half of the influenza genome is comprised of the three polymerase genes (PA, PB1, and PB2). Segments 1-6 each code for a single protein in A and C viruses with segment 6 coding for both NA and NB proteins in influenza B viruses. For A and C viruses, segment 7 encodes two proteins, M_1 and M_2, which are generated as linear and spliced mRNA transcripts, respectively. Segment 7 of B viruses encodes two overlapping ORFs, M_1 and BM2, both of which are produced as linear, unspliced mRNAs. For all influenza genera, segment 8 encodes two proteins, NS_1 and NS_2. NS_1 is transcribed as a linear mRNA, and NS_2 is spliced. NS_1 is a nonstructural protein that does not appear in the viral particle, but it is readily detected in infected cells and has been demonstrated to perform critical functions in the viral life cycle (38,39).

1.5 Reassortment of influenza viruses

The life cycle of influenza begins with the binding of HA molecules to sialic acid (SA) receptors on the surface of an infectable cell. Numerous cell surface glycoproteins have terminal SA receptors that are joined by glycosidic linkage to galactose (Gal) residues, and the nature of this linkage is used to identify SA receptors. The most common influenza HA receptors are SAα2,3Gal and SAα2,6Gal. Avian influenza viruses prefer α2,3 linkages. This preference has evolved due to selective pressure, as avian species primarily express α2,3 receptors on airway epithelial cells and in the lung (40). In contrast to avian influenza, SA receptor expression in the upper airways of humans is dominated by α2,6 linkages, and there is a corresponding preference for α2,6 linkages in human influenza viruses.

Avian influenza viruses are introduced into human populations following genetic reassortment between avian and human influenza viruses. It is currently thought that swine serve as intermediate hosts for this reassortment, in large part because swine harbor both avian and human viruses (10,41,42). In support of this, epithelial cells in the swine trachea express an abundance of both α2,3 and α2,6 linkages (43), and swine are commonly referred to as “mixing vessels” for the generation of humanized viruses from avian influenza. Some avian species, such as quail and chicken have also been shown to express α2,3 and α2,6 linkages in the trachea (44), and these animals may also serve as reservoirs for influenza virus reassortment.

1.6 Antigenic variation and pandemic influenza

The scientific community has enjoyed successful vaccination campaigns for a number of viral pathogens, such as smallpox, measles, and poliovirus, primarily because these viruses do not routinely experience significant antigenic change. Accordingly, a single vaccination for these viruses will typically yield decades of protection (45). In contrast, influenza viruses undergo constant antigenic variation and effective control requires annual vaccine campaigns aimed at immunizing against circulating seasonal strains. The high level of antigenic variation observed in influenza viruses is driven by two distinct mechanisms, antigenic drift and antigenic shift.

Antigenic drift in influenza is the accumulation of single amino acid substitutions in HA and NA surface glycoproteins. Although all influenza genera are subject to antigenic drift, influenza A viruses have been demonstrated to have the highest mutation rates (46). Over time, error-prone viral RNA polymerases introduce mutations that alter protein structure and render host anti-HA/NA antibodies incapable of neutralizing drifted strains, an obvious problem given that antibodies against these surface glycoproteins are generally considered to be indispensable in achieving protection from influenza infection (47-50). Antigenic drift leads to the emergence of variant influenza strains via positive selection by host antibodies that neutralize non-drifted strains. This process eventually results in the generation of variants that are particularly successful in evading host responses and thus potentially capable of initiating

epidemics (51,52). In addition to the obvious dangers posed by antigenically drifted strains, this phenomenon is capable of adversely affecting annual vaccine campaigns. An example of this occurred during the 2003-04 seasonal influenza campaign. WHO officials recommended A/Panama/2007/99 as the H3N2 component of the seasonal vaccine. Antigenic drift in HA of circulating influenza strains generated an A/Fujian/411/02-like virus that was a poor antigenic match for the A/Panama/2007/99 vaccine strain. As a result, vaccinated individuals elicited H3N2 HA-specific antibody responses that offered only minimal protection upon exposure to A/Fujian-like viruses, which were the dominant H3N2 in circulation that year (53).

Antigenic shift occurs when genomic reassortment events swap entire gene segments between two separate viruses. This manner of antigenic variation introduces new HA and NA subtypes into the human population, and although this variation mechanism is far less frequent than antigenic drift, the consequences of a novel, humanized reassortant strain are potentially devastating. Antigenic shift generates pandemic influenza strains that vary considerably in amino acid sequence compared to previously circulating viruses, substantially increasing the susceptibility of human populations possessing antibodies that are incapable of neutralizing shifted strains.

Four major antigenic shift events occurred in the 20th century. The emergence of the 1918 H1N1 Spanish influenza virus (1918 H1N1) marked the

beginning of the most lethal influenza pandemic on record. The exact mortality of 1918 H1N1 is unknown, but has recently been reported to be on the order of 50 million worldwide and up to 1 million in the U.S. alone (54,55). Two additional major antigenic shifts occurred in 1957 (Asian influenza; H2N2) and 1968 (Hong Kong influenza; H3N2) and were followed by global pandemics. These strains were much less virulent than 1918 H1N1 and caused far fewer casualties (approximately 1.5-2.5 million total; 56,57). H1N1 reemerged as the dominant circulating strain in 1977 and caused local epidemics in the Soviet Union (hence the name, “Russian influenza”) and China. This pandemic was primarily confined to individuals younger than 20 years old, because older populations exposed to or vaccinated against H1N1 before the appearance of H2N2 in 1957 were protected by pre-existing levels of cross-reactive serum antibodies. As a result, 1977 H1N1 was a relatively mild pandemic (60,61). Multiple sources have suggested that 1977 H1N1 was introduced into the population as the result of a laboratory accident (58,59). The 2009 swine-origin H1N1 pandemic strain (2009 H1N1) was not the product of a antigenic shift to a new subtype, as H1N1 has been a common human subtype for nearly a century, but it was resultant from reassortment events that introduced a strain of novel composition with a highly divergent HA (62).

1.7 History of influenza vaccines

At the time of the 1918 Spanish influenza pandemic, it had not yet been established that a virus was the causative agent of influenza, although there was

speculation by numerous experts that viral infection was the most likely explanation (63-65). The extraordinary severity of the pandemic dramatically hastened influenza research, which led to the eventual isolation of virus from influenza-infected swine and humans in the early 1930s (66). Within a few years, egg-adapted strains were isolated, allowing researchers to easily propagate influenza virus in embryonated chicken eggs, and these strains became the foundation for seasonal influenza vaccines. The first human influenza vaccine trial was conducted in 1937, it was observed that vaccine recipients generated high levels of serum antibodies (67). Large human trials conducted by the military in the 1940s led to FDA approval of the first influenza vaccines. Original vaccine preparations were inactivated by a variety of chemical means, such as formaldehyde and β-propriolactone to render the virus incapable of establishing infection yet antigenically intact (68,69). While these whole-virus preparations were determined to be immunogenic, they led to a high number of adverse reactions, particularly among young children (70). To address this issue, vaccine manufacturers began to treat inactivated vaccines with detergents or solvents, which disrupt viral particles without altering the antigenic properties of the virus. This treatment resulted in what are termed split-virion vaccines, which are enriched for HA and NA proteins (71). For reasons probably related to additional purification steps during manufacturing, split-virion vaccines have been shown to result in fewer adverse reactions than their whole-virus counterparts, and in the U.S., inactivated influenza virus vaccines have been exclusively administered in this form since 2003 (72,73).

Cold-adapted influenza viruses (CAIV) were first characterized in 1967 and gained immediate interest as potential vaccine vectors (74,75). The first CAIV developed was produced via serial passage of A/Ann/Arbor/06/60 (A/AA/06/60; H2N2) in embryonated chicken eggs at progressively lower temperatures. This led to the recovery of viruses that grew well at both 25°C and 33°C. Additional passaging in eggs at 25°C produced A/AA/06/60 strains that grew well at 25°C but poorly at 33°C, resulting in an A/AA/06/60 variant that was both cold-adapted (*ca*) and temperature-sensitive (*ts*; 76,77). Similar techniques were subsequently used to generate a *ca*, *ts* influenza B variant from B/Ann Arbor 01/66 (78,79). These strains were tested for use as human influenza vaccine candidates and were determined to be safe and efficacious (80,81). Both the *ca* and *ts* attenuations are present in FluMist, a live, attenuated influenza vaccine that was approved by the FDA in 2003 (82).

1.8 Influenza vaccine composition

Seasonal influenza vaccination remains the most effective tool to protect the general population from influenza infection. There are currently two seasonal influenza vaccines that are approved for use in the U.S., trivalent inactivated vaccine (TIV) and live attenuated influenza vaccine (LAIV or FluMist; also trivalent). Both of these vaccines express 3 pairs of HA and NA genes: two pairs from influenza A (H1N1 and H3N2 subtypes) and a pair from an influenza B strain. The WHO Global Influenza Surveillance Network annually gathers global influenza sequencing data and performs antigenic analysis to determine the influenza A and B strains that will be represented in seasonal influenza vaccines.

This decision is based on anticipating which A and B strains are expected to circulate during the coming influenza season and choosing appropriately matched strains to blend into vaccines for maximum efficacy. TIV, which is FDA approved for all ages, is produced by recombining predicted seasonal HA and NA genes with internal genes derived from A/PR/08/34. LAIV is an attenuated, cold-adapted, and temperature sensitive live vaccine composed of three 6:2 genetic reassortants containing HA and NA genes of circulating seasonal influenza strains and internal genes from A/AA/06/60 (H2N2) and B/AA/01/66 (83). Like TIV, LAIV is currently produced in embryonated chicken eggs, but since it is a live vector, it is not subjected to inactivation or chemical disruption. LAIV also differs from TIV in that it is administered intranasally and is approved for an age range of 2 to 49.

1.9 Humoral immunity to influenza

Decades of research studying immune responses and protection in human and mouse models has led to the established canon that protection from recurrent influenza infection is mediated primarily by antibodies against HA and to a lesser extent, NA (84,85). The protective capacity of antibodies is easily demonstrated in mice. HA and NA-specific antibodies generated via vaccination provide protection against influenza challenge in the absence of CD4 or CD8 T cells (86,87). Additionally, passive immunization of mice with anti-HA antibodies has been shown to result in sterilizing immunity following influenza challenge, and this effect was also demonstrated in the absence of T cells (88). Direct antibody-mediated protection is quite difficult to confirm in humans, although

there is evidence that transplacentally transferred maternal antibodies may help prevent influenza A infection in infants (89).

In humans, post-vaccination serum levels of HA and NA antibodies correlate strongly with protection, restriction of viral replication upon infection, and amelioration of disease (47-49). These antibodies are primarily produced by immunoglobulin G (IgG) and IgA antibody-secreting plasma cells (ASCs; also called effector B cells) that mature from the stimulation of influenza-specific naïve or memory B cells in the draining lymph node (90,91). Influenza-specific IgG ASC populations in the blood peak approximately 7 days post-vaccination, composing up to 16% of all circulating B cells (92), and expansion is quite transient, often returning to baseline levels 2 to 3 weeks following vaccination (93,94). Vaccine-associated IgG memory B cell responses peak 2 to 6 weeks post vaccination. Peak post-vaccination frequencies of circulating IgG memory B cells is approximately 1% of total B cells, which is considerably lower than those observed for IgG ASCs; however, these responses are long-lived, remaining above baseline for months following vaccination (92).

Recently, a few groups have compared the efficacy of TIV and LAIV in eliciting antibody responses in humans. In one study, IgA ASC responses elicited by TIV or LAIV were similar in adults and children; however, in adults, TIV was a superior inducer of IgG ASCs (95). Memory B cell responses (both IgG and IgA) were found to be more robust in the TIV group for both adults and children. In

additional reports involving the elderly and military recruits, donors immunized with TIV displayed higher serum antibody levels and reported reduced illness compared to LAIV recipients (96-98).

1.10 Murine T cell responses to influenza

When compared to the wealth of data detailing B cell responses to influenza, our knowledge and understanding of human T cell responses to the virus is quite modest. The characterization of human CD4 and CD8 T cell responses to influenza was in its infancy as late as the mid 1980s, by which time influenza-specific B cell responses had been heavily researched for decades (99,100). Most of what is known regarding T cell responses to influenza is the result of relatively recent studies conducted in mice, and the exact role of T cells in influenza infection and vaccination remains difficult to interpret. There are ample murine studies that support a protective role for CD4 and CD8 T cells in influenza infection. In multiple studies, CD8-deficient mice have been shown to clear virus at a decreased rate following infection (101,102). Consistent with this observation are experiments that demonstrate the ability of adoptively-transferred influenza-specific CD8 T cells to ameliorate disease and promote clearance in challenged mice lacking B cells (103,104). Additionally, transfer of CD8 T cells specific for NP into recipient animals prior to viral challenge confers protection by limiting viral replication (105).

T cell responses to influenza in mice have been chiefly characterized by studying responses to HKx31 (H3N2) and A/PR/08/34 (H1N1). HKx31 shares

internal genes with A/PR/08/34, but it expresses distinct external proteins from different subtypes, H3 and N2. Priming mice with one of the viruses elicits neutralizing antibody responses that do not recognize and eliminate the other strain upon secondary challenge, and as a result, heterologous prime/boost regimens employing HKx31 and A/PR/08/34 are useful for investigating secondary influenza-specific T cell responses. CD8 T cell responses specific for HKx31 and A/PR/08/34 are dominated by two conserved antigenic determinants in NP ($NP_{366-374}$) and PA ($PA_{224-233}$; 106). By transferring $NP_{366-374}$- specific CD8 T cells derived from HKx31 infection into naïve recipients, mice are partially protected upon challenge with A/PR/08/34, demonstrating the potential of CTL-mediated cross-protection against influenza infection in a heterosubtypic manner (107,108). The role of CD8 T cells in protection against heterosubtypic viruses has been confirmed in various other murine studies, leading to speculation that "universal" influenza vaccines designed to generate cross-reactive CTL responses against a range of subtypes may be candidates worthy of investigation (107,109,110,111).

Despite evidence of CD8 T cell-mediated protection in influenza infection, they do not always appear to be beneficial. For example, naïve mice that were immunized with dendritic cells pulsed with peptides containing $NP_{366-374}$ and $PA_{224-233}$ generated CD8 T cell responses specific for NP and PA, as expected, yet only $NP_{366-374}$ vaccination resulted in accelerated clearance following challenge. In fact, $PA_{224-233}$-vaccinated animals cleared virus more slowly than

uninfected controls (112,113). Similar studies have demonstrated that immunization with $PA_{224-233}$ or CD8 epitopes from HA and M1 ($HA_{332–340}$, and $M1_{128–135}$) leads to robust production of influenza-specific CD8 T cells but subsequent delayed viral clearance upon challenge infection (114). The reasons for this seemingly detrimental vaccine outcome are unclear, and there are multiple possibilities. $PA_{224-233}$, $HA_{332–340}$, and $M1_{128–135}$ are expressed at a high level on murine dendritic cells, but their presentation on non-dendritic cell populations is considerably lower or absent (112,113,115). If the expression of these peptides is markedly reduced on lung epithelial cells, one may speculate that the observed delay in viral clearance is due in part to the overproduction of CTLs specific for epitopes that are not expressed on infected lung cells. A second possibility for delayed clearance could be attributed to an effect similar to antigenic sin in B cells, whereby populations of non-protective CD8 T cells resulting from primary immunization massively expand during viral challenge and populate the T cell compartment to an extent that the generation of potentially protective CD8 populations is suppressed. In either case, this data suggests that influenza CD8 epitopes differ in their efficacy with regards to controlling infection, and this dynamic warrants consideration when designing CTL-based vaccines (114).

CD4 T cells are traditionally known to assist in the activation of CD8 T cells and provide help in antibody production, and murine studies suggest that they perform these functions following influenza infection (116). Previous studies

have shown that MHC class $II^{-/-}$ and CD40-deficient mice are severely impaired in their ability to generate protective antibody responses upon influenza infection compared to control animals (117,118). Additionally, B cell-deficient μMT mice, which generate protective CD8 T cell responses in the presence of CD4 T cells upon A/PR/08/34 infection, fail to control the virus if CD4 T cells are depleted (119-121). The contribution of CD4 T cells in influenza immunity has been further demonstrated in T cell-deficient nude mice. These animals typically succumb to A/PR/08/34 infection, but the adoptive transfer of HA- or NA-specific CD4 T cells on the day following A/PR/08/34 infection leads to robust antibody production and subsequent recovery (88).

Helper-independent roles have also been observed in influenza-specific CD4 T cells. Influenza-primed effector CD4 T cells lysed infected targets in cytotoxicity assays via perforin-mediated cytolysis, and A/PR/08/34 infection of mice with perforin-deficient CD4 T cells resulted in decreased survival (122). Additionally, in antibody-depleted, $RAG2^{-/-}$ mice passively immunized with influenza NP-specific CD4 T cells, infection with A/PR/08/34 resulted in reduced viral loads compared to unimmunized animals (123). This result was found to be IFNγ-dependent and probably involved the recruitment and mobilization of innate immune mediators into infected tissues.

Mouse studies suggest that CD4 T cells are not absolutely required to protect against influenza infection. CD4 T cell-depleted mice infected with HKx31

readily clear virus from the lungs and generate only slightly diminished CD8 T cell responses (124). Similar results were reported in MHC class $II^{-/-}$ mice, although influenza-specific CD8 T cell responses were delayed early in infection (125). Decreased expression of several inflammatory cytokines, such as IFNγ, IL-2, and IL-10, was observed in MHC class $II^{-/-}$ mice, and it has been suggested in these studies that the primary protective function of CD4 T cell help in influenza infection may be the production of necessary immune mediators (126).

1.11 Human T cell responses to influenza.

The obvious limitation of influenza-specific T cell studies performed in mice is that these models may not accurately reflect human T cell responses. First, humans are subjected to a vast array of bacterial and viral infections that generate numerous T cell specificities, and it is difficult to determine any effect that these populations have on influenza-specific T cell responses. Additionally, influenza research is performed in donors that have already established some level of influenza-specific T cell immunity due to previous exposure or vaccination. Finally, influenza research in animals is commonly carried out at artificially high infectious doses and via routes of infection that may yield physiologically inaccurate results.

For obvious reasons, it is impossible to study all of the relevant anatomical compartments when conducting human T cell research. Typically, human T cell investigations are performed with peripheral blood mononuclear cells (PBMC). Influenza-specific T cell populations are maintained at low frequency in PBMC,

making their detection difficult. Very sensitive techniques are required to measure influenza-specific T cell responses in human samples, and the lack of suitable tools to study these responses has been a major hindrance to investigating influenza immunity. During the past decade, however, the continued refinement of MHC tetramer staining technologies, ELIspot, and intracellular cytokine staining (ICS) assays has provided methods for studying T cell responses. MHC tetramers provide rapid, direct *ex vivo* detection of T cells at the epitope level and are valuable tools for determining physical phenotypes of antigen-specific T cells. ELISpot and ICS assays are commonly used to detect antigen-specific T cell function, most commonly by measuring cytokine production. Common antigens for ELISpot and ICS assays include whole protein, viral lysates, plasmid DNA, recombinant vaccinia, and overlapping peptide pools (127-131). Despite the utility of these antigens, each has one or more significant limitations, which are discussed in Chapter 2. Pools of overlapping peptides representing entire proteins or even small viruses are widely regarded as the "gold standard" antigen for T cell assays (127). The primary limitation of overlapping peptide pools is that the cost of synthesis could potentially make their use economically prohibitive in some studies, such as vaccine trials that commonly generate massive numbers of samples to be processed, mapping immunodominant epitopes in moderately large genomes, or detecting T cell responses to viruses that undergo high levels of antigenic variation, such as influenza. Chapter 2 describes the development of a novel T cell quantitation assay that employs recombinant vesicular stomatitis virus (rVSV) as a source of

antigen. This method has been optimized for use in human subjects and represents a valid approach to measuring influenza-specific T cell responses.

Despite the difficulties associated with human T cell research, our understanding of influenza-specific T cell immunity has advanced considerably in recent years. Numerous reports have shown that humans possess pre-existing levels of influenza-specific CD4 and CD8 T cells gained through infection or seasonal vaccination that have the potential to cross-react with antigenically variant strains, such as 2009 H1N1 and H5N1 avian influenza virus. CD4 T cells in individuals previously uninfected with 2009 H1N1 react with peptides (HA, NA, M1, and NP) derived from 2009 H1N1 in [^{3}H]thymidine incorporation assays, and CD4 T cells from healthy adult donors produce IFNγ when stimulated with autologous antigen-presenting cells (APCs) infected with 2009 H1N1 (132,133). Additionally, CD4 T cells that are cross-reactive with H5 have been described in heathy adults (134,135). There is also an abundance of human data describing the presence CD8 T cells in healthy adults that recognize various internal and external and internal proteins from 2009 H1N1 and H5N1 (136-139). Some degree of pre-existing T cell immunity to novel influenza viruses is expected, as immunotypic sequence analysis of various strains, including 2009 H1N1, has confirmed the invariant nature of several MHC class I and class II epitopes (139,140). The potential contribution of cross-reactive influenza-specific T cell immunity was recently demonstrated. During the 2009 H1N1 pandemic, it was discovered that pre-existing humoral immunity against 2009 H1N1 in the general

adult population was minimal (141); however, the ensuing pandemic was relatively mild, leading to speculation that the severity of the pandemic was diminished by 2009 H1N1 cross-reactive T cells previously generated via natural exposure or seasonal vaccination. Even though it would be challenging, if not impossible, to properly evaluate this possibility in humans, it has created considerable interest in developing influenza vaccines that establish broadly cross-protective T cell immunity.

The capability of conventional seasonal influenza vaccine formulations to induce T cell responses in humans has only been partially explored. Currently, there have been only a few studies of human T cell responses to influenza vaccination and some of these investigations have drawn different conclusions. For example, in a trial of 30 adult donors receiving TIV or LAIV, both vaccines were show to induce robust T cell responses specific for HA from the influenza A strains contained in the vaccines (96, 142); however, in a separate trial, both vaccines failed to generate significant T cell responses in adults (143). This discrepancy is possibly a result of different methods for assaying influenza-specific T cells, as the first study was conducted following *in vitro* expansion of donor PBMC populations while second involved direct *ex vivo* analysis of peripheral blood samples. Additionally, since these studies were performed in different years, it is possible that there was variability in the antigenicity of the influenza strains that were blended into seasonal vaccines on those years. In children, both TIV and LAIV appear to be effective in inducing influenza-specific

T cell responses (83,143,144). The efficacy of both vaccines in children is not surprising, because unlike adults, young populations have limited pre-existing influenza immunity. Adults possess influenza-specific antibodies and influenza-reactive T cells that could significantly reduce the efficacy of seasonal vaccines. This complication is less of an issue in children. One concern with the above studies is that they focus on T cell responses specific for seasonal vaccine antigens. Achieving T cell immunity following influenza vaccination will require the establishment of broadly cross-reactive T cell populations. To our knowledge, the generation of cross-reactive T cells following TIV and LAIV vaccination in humans has not yet been characterized.

It is possible that enhancing influenza-specific CD4 helper T cell immunity may result in superior antibody responses following vaccination. Interactions between antigen-specific B cells and cognate CD4 helper T cells have been linked to B cell expansion, somatic hypermutation, and class-switching in multiple model systems (145-147). Since it is generally accepted that protection from influenza is correlated strongly to post-vaccination serum antibody titers against HA and NA, it is reasonable to conduct studies comparing HA-specific CD4 T cells and B cell responses resulting from seasonal influenza vaccination. In a recent human trial, CD4 T cells specific for HA from H1N1 and H3N2 viruses were induced by both TIV and LAIV; however, there was no evidence of any correlation in donors who displayed seroprotective HAI titers (96,142). Similar results were reported in a separate trial involving human donors that were

administered seasonal influenza vaccine followed by a 2009 H1N1 monovalent subunit vaccine 6 weeks later (148). Although most donors produced antibodies specific for 2009 H1N1, corresponding vaccine-associated T cell responses were highly variable. Additional studies will be necessary to conclusively determine whether a correlation exists between vaccine-induced CD4 T cell responses and humoral immunity in order to exploit this relationship in vaccine design.

Chapter 2:

Use of Replication Restricted Recombinant Vesicular Stomatitis Virus Vectors for Detection of Antigen-Specific T Cells

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Running Title: Antigen delivery via VSV vectors for T cell assays

Abstract

Detection of antigen-specific T cells at the single-cell level by ELISpot or flow cytometry techniques employing intracellular cytokine staining (ICS) is now an indispensable tool in many areas of immunology. When precisely mapped, optimal MHC-binding peptide epitopes are unknown, these assays use antigen in a variety of forms, including recombinant proteins, overlapping peptide sets representing one or more target protein sequences, microbial lysates, lysates of microbially-infected cells, or gene delivery vectors such as DNA expression plasmids or recombinant vaccinia or adenoviruses expressing a target protein of interest. Here we introduce replication-restricted, recombinant vesicular stomatitis virus (VSV) vectors as a safe, easy to produce, simple to use, and highly effective vector for genetic antigen delivery for the detection of human antigen-specific helper and cytotoxic T cells. To demonstrate the broad applicability of this approach, we have used these vectors to detect human T cell responses to the immunodominant pp65 antigen of human cytomegalovirus, individual segments of the yellow fever virus polyprotein, and to influenza A and B hemagglutinin (HA).

Introduction

The development and application of techniques such as MHC tetramer staining, ELISpot, and intracellular cytokine staining has transformed the study of T cell immune responses to microbes, tumors, auto-antigens, and vaccines. These assays—which permit detection of antigen-specific cells at the single cell level—do not require difficult to reproduce *in vitro* expansion protocols, and are widely regarded as the new "gold standards" for the characterization of T cell responses. When optimal peptide epitopes and their MHC restriction elements are mapped, MHC tetramers provide the most rapid method for detection of antigen-specific T cells and give direct access to physical phenotypes, but they do not detect function. In contrast, the ELISpot and ICS assays detect a specific function—the ability to produce one or more cytokines upon short-term stimulation with antigen—and they are considerably more flexible with respect to the form and range of antigens that can be used in the assays. Neither the ELISpot nor the ICS assay requires mapping of MHC restriction elements or optimal MHC-binding peptide epitopes, and both assays are often performed with "complex" antigens that might contain multiple distinct epitopes that are recognized by the T cell population of interest.

Although a wide range of antigens may be used for ELISpot or ICS assays, each has one or more significant limitations. Antigens delivered in the form of recombinant proteins, microbial lysates, and lysates of infected cells are largely restricted to exogenous antigen-processing pathways, and are therefore effective

for stimulation of $CD4^+$ T cells but ill-suited to efficiently stimulate $CD8^+$ T cells. Plasmid DNA has been used to deliver antigens for T cell assays (130), but since primary cells have low transfection efficiencies, its use requires highly transfectable cultured cell lines with an additional requirement for co-transfection with expression plasmids for one or more MHC alleles. Recombinant vaccinia viruses have been extensively used to deliver antigens for detection of antigen specific $CD4^+$ and $CD8^+$ T cells (128-129), but their production requires lengthy protocols involving homologous recombination and multiple rounds of plaque purification. Furthermore, pre-existing immunity prevents their use in vaccinia-exposed individuals, including those who have received experimental vaccinia-based vaccines. Finally, pools of overlapping peptides representing entire proteins or even small viruses are now commonly used to stimulate both $CD4^+$ and $CD8^+$ T cells, but it is not economically feasible to routinely purchase all of the peptides that are required to contain all possible epitopes for large viruses, such as those in the pox and herpesviridae families, or for broad coverage for highly variable viruses, such as HIV or HCV. Under defined and common circumstances, each of these forms of antigen has significant theoretical and/or practical limitations, and new options for antigen delivery are needed.

Our search for a suitable antigen-delivery system began with the following criteria. (1) The antigen-delivery system had to be capable of stimulating both CD4 and CD8 T cells. (2) It had to do so using only fresh, ex vivo cell populations such as PBMC, without recourse to cultured stimulator cells such as B lymphoblastoid cell lines (B-LCL). In practice, this narrowed the search to viral

vectors. (3) Once the search was narrowed to recombinant viral vectors, they had to be easy to produce from plasmid DNA, without recourse to systems that require homologous recombination (effectively ruling out poxviruses). (4) The viral vector had to have reasonably broad tropism, both at the cellular and species level (it would be desirable if we could use the same constructs in mice, non-human primates, and humans). (5) There had to be little-to-no pre-existing immunity to vector epitopes; this made us leery of adenoviral vectors, because although many of them express negligible amounts of vector antigens upon infection, it is possible that the input adenovirus structural proteins could stimulate cells, especially CD4 T cells. (6) The vector should have the capacity to accommodate reasonably large insert sizes (up to 4 kb). (7) The vector had to give high levels of antigen expression early after infection. (8) The vector should have relatively low cytopathicity. (9) The vector should be inherently safe, at least at the BSL2 level. One virus that seems to meet all of these criteria is vesicular stomatitis virus (VSV).

Vesicular stomatitis virus is a member of the Rhabdoviridae family. It has an extremely compact, nonsegemented negative strand RNA genome with five non-overlapping genes coding for viral proteins. Reverse genetics systems for efficient production of recombinant VSV from plasmid DNA were developed in the mid-1990s by the laboratories of Rose and Wertz (149,150). These techniques were originally designed to genetically manipulate the VSV genome for RNA virus assembly and replication studies. Further development of these methods soon made it possible to construct recombinant VSV encoding a foreign

protein of interest in place of the viral glycoprotein (VSV-G) in the VSV genome (VSV-ΔG). VSV-ΔG vectors are safe for routine laboratory use, because even though the recombinant virions are coated with VSV-G, they lack VSV-G in the recombinant genome and are thus capable of only one round of replication. Additionally, VSV-ΔG has a relatively large insert capacity (≥4 kb). For these reasons, we have used VSV-ΔG as a vector to introduce antigen to T cells. We generated a panel of VSV-ΔG constructs encoding various viral antigens and have developed a method to employ these constructs for antigen delivery in T cell ICS and ELISpot assays. Our results demonstrate that VSV-ΔG vectors represent an alternative and efficient antigen source for detecting antigen-specific T cells.

Materials and Methods

2.1. ELISpot

The IFN-γ ELISPOT assay was performed as previously described (129,151). $2x10^5$ to $5x10^5$ human PBMC from a CMV-seropositive donor were infected with a recombinant VSV expressing the pp65 gene of human cytomegalovirus (VSV-ΔG.CMVpp65). PBMC from the same donor were also infected with a recombinant VSV encoding the nucleoprotein gene from lymphocytic choriomeningitis virus (VSV-ΔG.LCMV-NP). Since LCMV infection is uncommon in human populations, VSV-ΔG.LCMV-NP infection was not expected to stimulate cytokine production, and this vector was used as a negative control virus for our studies in human subjects.

2.2. Plasmid Construction

Plasmid pVSV-ΔG is a Bluescript-based plasmid that encodes the anti-genome RNA of VSV. In this plasmid, the coding region for VSV-G has been removed and replaced with a polylinker (152). Genes of interest were inserted into the polylinker region of pVSV-ΔG using *Kpn I*, *Sph I*, and *Nhe I* restriction enzyme sites. For some constructs, a gene of interest was inserted into pVSV-ΔG using a ligation-independent cloning (LIC) method (153). Briefly, the polylinker region of pVSV-ΔG was replaced with a LIC sequence that upon linearization with *Sma I* and subsequent treatment with T4 DNA polymerase and dATPs yields specific overhangs. In the absence of ligase, these overhangs anneal with complimentary sequences flanking genes of interest that have been amplified

with LIC primers and treated with T4 DNA polymerase and dTTPs. This method requires very few manipulations and is ideal when introducing various different inserts into the same vector.

2.3. Recovery of Recombinant VSV-ΔG

Recombinant VSV-ΔG was produced as previously described (152,154,155). Briefly, baby hamster kidney cells (BHK-21; American Type Culture collection) were maintained in Dulbecco's modified Eagle's medium (DMEM)/5% fetal bovine serum (FBS) at 37ºC. BHK-21 cells on 6-well plates (~95% confluent) were infected with recombinant modified vaccinia virus Ankara (MVA) expressing T7 polymerase (MVA-T7) at a multiplicity of infection (MOI) of approximately 1. Following a 90 min. incubation, plasmids encoding VSV-N, P, L, and G proteins and pVSV-ΔG containing a gene of interest (VSV-ΔG) were transfected at a ratio of 3:5:1:8:5, respectively, into the cells by using a liposome suspension of dimethyldioctadecyl ammonium bromide and L-a-dioleoylphosphatidylethanolamine for 4 hours. 48 hours after transfection, supernatants were 0.22μm syringe-filtered (to remove MVA-T7) on to BHK-21 cells previously transfected with pCAGGS-VSV-G (~85% confluent) using Lipofectamine reagent (Invitrogen). VSV-G must be introduced in trans, because recombinant VSV-ΔG does not produce the viral glycoprotein. Following 24 to 48 hour incubation, successful recoveries were indicated by visualization of extensive cell rounding/cytopathic effect. Following recovery, virus was amplified on VSV-G-transfected BHK-21 cells, and viral titers were determined via plaque assay.

2.4 Cryopreservation and Thawing of PBMC

Following PBMC isolation, cells were washed with RPMI-1640/10% FBS (R10) and resuspended at a final concentration of 5-10^7/ml in R10 plus 10% DMSO (Sigma) in freezer vials. The cells were placed in a freezing container (Nalgene) and placed in a -80°C freezer overnight before transfer to liquid nitrogen. Cells were thawed in a 37°C water bath and washed with R10. After washing, cells were resuspended in R10 and incubated overnight in a 37°C incubator. On the following day, cells were washed with R10 prior to assay.

2.5. Specimen Preparation and Assay Setup

Donor blood samples were collected in sodium citrate cell preparation tubes (Vacutainer, BD) and centrifuged at 1500g for 30 min. PBMC were then processed as directed by manufacturer and resuspended at 10^7/ml in R10. In a 5ml polypropylene tube, 10^6 human PBMC were infected with VSV-ΔG viral supernatant in a total volume of 200µl at an MOI of 10 unless otherwise noted. Costimulatory antibodies CD28/CD49d (FastImmune, BD) were added to each sample at a final concentration of 1µg/ml. A cytokine blocking reagent, GolgiPlug (Brefeldin A, BfA; BD), was added 4 hr post-infection, unless otherwise specified. Samples were then incubated overnight at 37ºC/5%CO_2 and were processed for flow cytometry analysis on the following day. In some experiments, pools of overlapping CMVpp65 peptides (BD) were used for PBMC stimulation at a final concentration of 1.75µg/ml. For these samples, BfA was added at the time of stimulation, unless otherwise stated.

2.6. Surface Staining and Intracellular Cytokine Staining

Monocyte and dendritic cell (DC) tropism: PBMC infected with a recombinant VSV expressing GFP (VSV-ΔG-GFP) for 6 hr were washed and assayed via flow cytometry. CD14-APC (IO Test) was used to visualize monocytes. DCs were defined as staining positive for CD11c-APC (BD) and HLA-DR PerCP (BD) and negative for a lineage cocktail composed of (CD20, CD3, CD56, CD16, CD14)-PE (all BD). For monocyte viability studies, propidium iodide (BD) was included for live/dead discrimination.

T cell cytokine assays: Following VSV-ΔG infection and incubation, human PBMC were washed with PBS and permeabilized with FACS Permeabilizing Solution 2 (BD). Following permeabilization, the cells were again washed with PBS and stained with CD4-FITC (Coulter), CD3-PE (Coulter), CD8-PerCP (Coulter), and IFNγ-APC (BD). Samples were acquired on a FACSCalibur or LSRII flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.7 Sucrose Purification of Recombinant VSV-ΔG Viral Supernatant

A 30% sucrose in PBS solution was added to 38.5ml Ultra-Clear™ tubes (Beckman). Viral supernatant was slowly added to the tubes in order to prevent disruption of the sucrose layer. The tubes were then centrifuged for 90 min at 60,000 x *g* in an Optima™ L-70K ultracentrifuge (Beckman) using an SW-28 rotor (Beckman). After centrifugation, the supernatant was removed, and purified virus was resuspended in 500µl R10.

2.8. *In Vitro* T Cell Proliferation (CFSE)

A solution of 0.6μM CFSE in PBS was prepared. Human PBMC were washed and resuspended in PBS at a concentration of 0.5 to $1x10^7$ /ml. Equal volumes of PBMC and 0.6μM CFSE were mixed and incubated for 7 min at room temperature in the dark, vortexing every other minute. The CFSE-labeled PBMC were then washed twice with R10 to block CFSE and resuspended in R10 at a final concentration of $1x10^6$ /ml. 1ml CFSE-labeled PBMC was added to 5ml polypropylene tubes and infected with VSV-ΔG at an MOI of approximately 10 or stimulated with staphylococcal enterotoxin B (SEB, Sigma) as a positive control. Samples were then incubated at 37ºC/5%CO_2 for 6 days. Following incubation, the cells were washed and stained with a live/dead discriminatory dye as well as CD8-PE (BD), CD4-APC (BD), and CD3 PacBlue (BD). Samples were acquired on a LSRII flow cytometer and analyzed using FlowJo software.

Results

3.1. VSV is Monocyte and Dendritic Cell Tropic

To determine the cell types in human PBMC that express an antigen of interest delivered by recombinant VSV-ΔG and are thus potentially capable of presenting antigen to T cells, we infected human PBMC with a recombinant VSV vector expressing GFP (VSV-ΔG-GFP; Figure 2.1a). Following 6 hr incubation, infected PBMC were stained for analysis by flow cytometry. GFP expression was found predominantly in $CD14^{+}$, $CD4^{low}$ cells, indicating a tropism for monocytes. Dendritic cells (DCs), defined as CD11c+, Lin-, HLA-DR+, but not T, B, or NK cells, also expressed GFP. In short term culture (6 hr) GFP expression was not accompanied by significant cell death (data not shown). Kinetics studies were performed to determine the peak of GFP expression in monocytes and DCs infected with VSV-ΔG-GFP. In monocytes, the peak of GFP expression occurred earlier (0-6 hr) than in DCs (6-20 hr; Figures 2.1b and 2.1c), suggesting that monocytes are the chief presenters of antigen in early *ex vivo* infection with VSV. Furthermore, DCs are a significantly more rare population in PBMC compared to monocytes.

We infected fresh PBMC with VSV-ΔG-GFP at various MOI (0.4-25) to maximize the percentage of monocytes expressing GFP and determine the effect on monocyte viability. As expected, GFP expression was proportional to MOI, but importantly, monocyte viability was similar at each MOI, indicating that VSV infection does not induce extensive cytopathology after 6 hr in *in vitro* cultures

(data not shown). In an attempt to increase infectivity, experiments were conducted in the presence of polybrene, a cationic polymer that may serve to cluster virus and increase the number of particles that come into contact with infectable cells. The percentage of GFP positive monocytes increased only slightly when infected PBMC were incubated in the presence of polybrene (data not shown), and as a result, polybrene was not used in future experiments.

We tested the infectivity and viability of monocytes in fresh vs. cryopreserved PBMC for 3 donors, and we determined that monocyte viability following cryopreservation was similar to that of fresh PBMC (data not shown). When compared to fresh PBMC, infection of cryopreserved PBMC with VSV-ΔG-GFP resulted in decreased but easily detectable percentages of monocytes expressing GFP (Figure 2.1d). These results indicate that while fresh PBMC are presumably optimal in VSV-ΔG assays, cryopreserved PBMC are also appropriate for use.

3.2. IFNg ELISpot Assay

As an initial test of whether antigen delivered by VSV-ΔG could stimulate the production of cytokines by antigen-specific T cells, we employed an IFNg ELISpot. Human PBMC from a CMV-seropositive donor were infected with several dilutions of VSV-ΔG.CMVpp65. The CMVpp65 gene codes for a tegument protein that has been previously characterized as highly immunodominant (156). PBMC from the same donor were also infected with a negative control construct, VSV-ΔG.LCMV-NP. In PBMC infected with VSV-

ΔG.CMVpp65, an assayable number of spots representing individual IFNg-producing T cells was observed. Samples infected with VSV-ΔG.LCMV-NP displayed only background levels of cytokine production equivalent to that of uninfected controls, demonstrating that the observed cytokine production was entirely dependent upon the CMVpp65 insert (data not shown).

3.3. Optimization of Time of Brefeldin A Addition

A limitation of the ELISpot assay is that it does not permit the direct discrimination between responding CD4 and CD8 antigen-specific T cells. In light of this, a flow cytometry-based intracellular cytokine staining (ICS) protocol was developed. 10^6 PBMC from a CMV-seropositive donor were infected with VSV-ΔG.CMVpp65 at an MOI of approximately 10. In parallel, PBMC from the same donor were stimulated with an overlapping 15-mer CMVpp65 peptide pool. Infection and CMVpp65 peptide pool stimulation were followed by BfA addition at various time points. BfA is commonly used in ICS assays as a means to block cytokine secretion (157), but it has also been shown to inhibit cell surface presentation of class I MHC molecules (158). Thus, it was essential to determine the optimal time post-infection to add BfA in order to generate maximum IFNγ secretion by antigen-specific T cells. Following BfA addition and overnight incubation, IFNγ production was assayed via ICS. In the VSV-ΔG.CMVpp65-infected samples, optimal IFNγ responses were observed at slightly different BfA addition time points for CD4 and CD8 T cells (3 hr vs. 4 hr post-infection, respectively; Figure 2.2a). Similar results were obtained after screening two additional donors (data not shown), and in future experiments, BFA was added 4

hr post-infection. In the CMVpp65 peptide pool-stimulated samples, optimal CD4 and CD8 T cell cytokine responses were attained when BfA was added at the time of stimulation (0 hr), consistent with previously published reports (127). For the VSV-ΔG.CMVpp65-infected samples, the percentages of IFNγ-producing CD4 and CD8 T cells at the 4 hr time point was 0.5% (CD4) and 0.61% (CD8). These frequencies are somewhat smaller than those of the CMVpp65 peptide pool-stimulated samples at the optimal 0 hr time point (0.86%, CD4; 0.74%, CD8) when compared directly, and similar results were found with samples from 3 additional donors (data not shown). It should be noted that the levels of CD4 and CD8 down-regulation were greater in the samples that were stimulated with the CMVpp65 peptide pool. This could be an indication that the CMVpp65 peptide pool is a stronger T cell stimulator than VSV-ΔG.CMVpp65, which may reflect previously published reports that potent inducers of T cell responses promote TCR down-regulation (159).

Cytokine production resultant from VSV-ΔG.CMVpp65 infection was compared in fresh vs. cryopreserved PBMC for 4 donors. Our results demonstrated that the frequencies of IFNγ-producing CD4 and CD8 T cells were only slightly diminished following infection of cryopreserved samples (Figure 2.2b), a desirable result given the interest in assessing T cell responses in cryopreserved samples, such as from a vaccine clinical trial.

3.4. Effects of Carryover Antigen

For samples infected with VSV-ΔG.CMVpp65, IFNγ production by CD4 T cells was detected when BfA was added at the time of infection, suggesting that IFNγ production at this time point might be the result of carryover CMVpp65 antigen in our viral preparations. This was not unexpected as amplification of VSV vectors on BHK-21 cells presumably leads to some cell lysis as well as recombinant virus production, resulting in the subsequent release of antigen into the viral supernatant. Western blot analysis verified the presence of CMVpp65 protein antigen in viral supernatant stocks (data not shown). To determine the contribution of carryover CMVpp65 antigen, we infected human PBMC from CMV seropositive donors with VSV-ΔG.CMVpp65 viral supernatant at an approximate MOI of 5 under the following conditions: unpurified viral supernatant, unpurified/UV-inactivated, sucrose-purified supernatant, and sucrose-purified/UV-inactivated supernatant. BfA was added at 0, 2, and 4 hr post-infection, followed by overnight incubation. Whole protein antigen is known to be a very weak inducer of CD8 T cell responses, and as expected, UV inactivation of viral supernatant eliminated CD8 T cell responses (Figure 2.3), indicating that these responses were driven by newly synthesized antigen resulting from VSV-ΔG.CMVpp65 infection. Carryover CMVpp65 antigen does play a significant role in generating CD4 T cell responses, as indicated by the production of IFNγ by CD4 T cells infected with UV-inactivated VSV-ΔG.CMVpp65. However, by comparing the frequencies of IFNγ-producing CD4 T cells in PBMC that were infected with sucrose-purified virus stock versus PBMC infected with sucrose-

purified/UV-inactivated virus stock, it is demonstrated that VSV-ΔG.CMVpp65 infection is capable of generating detectable CD4 T cell responses.

3.5. MOI Optimization

Infection of human PBMC with recombinant VSV at an MOI of 25 results in optimal APC infection (data not shown). To determine if this MOI leads to maximum IFNγ production by responding T cells, we infected human PBMC from 2 CMV-seropositive donors with VSV-ΔG.CMVpp65 at an MOI range from 0.4-25. BfA was added 4 hr post-infection. For each donor tested, an MOI of 12.5-25 generated the most robust IFNγ responses by antigen-specific CD4 and CD8 T cells (Figures 2.4a and 2.4b), although cytokine production was detectable at an MOI of less than 1. Based on these results, future assays were performed at a high MOI (≥10).

3.6. Range of Antigens

We have used recombinant VSV vectors to detect T cell responses resulting from vaccination with the yellow fever virus vaccine strain 17D (YFV-17D). YFV-NS3 and YFV-NS4 are nonstructural YFV proteins, one of which (NS3) has been shown to contain a dominant CD8 T cell epitope in YFV 17D-immunized mice (160). PBMC from a YFV-17D-vaccinated individual (day 60 post-vaccination) were infected with VSV-ΔG constructs expressing YFV-NS3 (VSV-ΔG.YFV-NS3) and YFV-NS4 (VSV-ΔG.YFV-NS4). We were able to detect IFNγ-producing CD4 and CD8 T cells specific for YFV-NS4 as well as CD8 T cell responses to YFV-NS3 (Figure 2.5a).

We also generated VSV-ΔG constructs encoding various influenza genes, including HA from A/New Caledonia/20/99 and B/Florida/04/06. PBMC from a healthy adult donor were infected with VSV-ΔG-A/New Caledonia/20/99-HA, and PBMC from a donor vaccinated with the 2008-2009 seasonal influenza vaccine were infected with VSV-ΔG-B/Florida/4/06-HA (day 14 post-vaccination). ICS analysis demonstrated that both vectors were capable of stimulating IFNγ production by influenza-specific CD4 T cells (Figure 2.5b). We rarely detected CD8 T cell responses to HA-expressing VSV-ΔG vectors (data not shown). This result was not unexpected, as recently published data indicates that CD8 T cell responses do not typically target influenza HA (173).

3.7. VSV-ΔG-induced Proliferation of Antigen-specific T cells

We determined that VSV-ΔG constructs are capable of effectively stimulating antigen-specific T cells in ICS assays. As an alternative to an ICS-based assay in elucidating antigen-specific T cells, we tested the ability of VSV-ΔG vectors to induce T cell proliferation *in vitro*. PBMC from a CMV-seropositive donor were labeled with CFSE and infected with VSV-ΔG.CMVpp65 or VSV-ΔG.LCMV-NP at an MOI of approximately 10. At 6 days post-infection, the percentage of $CFSE^{low}$ CD4 and CD8 T cells in samples infected with VSV-ΔG.LCMV-NP was equivalent to baseline levels observed in uninfected controls (Figure 2.6a). In the samples infected with VSV-ΔG.CMVpp65, approximately 6% and 26% of CD4 and CD8 T cells, respectively, were $CFSE^{low}$, demonstrating that VSV-ΔG vectors are capable of driving the proliferation of antigen-specific T cells. Additionally, we have used VSV-ΔG vectors to induce the proliferation of low

frequency influenza-specific CD8 T cell populations (Figure 2.6b). PBMC were prepared from a donor that was previously shown to possess CD4 and CD8 T cell populations specific for various A/California/04/09 (H1N1) gene products, including polymerase subunit B1 (PB1) and the viral nucleoprotein (NP; data not shown). These PBMC were infected with VSV-ΔG constructs encoding NP and PB1 from A/California/04/09 and cultured for 6 days. While both VSV-ΔG-influenza constructs were able to drive the proliferation of influenza-specific CD8 T cells, we were unable to detect the proliferation of CD4 T cells above background levels. It is possible that various culture manipulations, such as CD8 T cell depletion and the addition of cytokines, may promote a more favorable environment for the proliferation of CD4 T cells, but we have yet to explore these conditions.

Discussion

ELISpot and ICS assays are widely regarded as the most powerful tools for detecting antigen-specific T cells and studying their function. Traditional antigens for ELISpot and ICS assays include whole protein, viral lysates (131), plasmid DNA (130), recombinant vaccinia (128-129), and overlapping peptide pools (Maecker et al. 2001), but these reagents each have significant limitations. Our aim was to develop an antigen delivery method that addressed these limitations while satisfying the previously discussed set of criteria established to ensure maximum assay utility. The most practical method for stimulating both CD4 and CD8 T cell responses in PBMC would employ the use of antigen delivery by viral vectors, but recombinant vaccinia and adenovirus are less than ideal vectors due to the high frequency of the general population with pre-existing immunity. All things taken into consideration, the most promising candidate was recombinant VSV-ΔG.

VSV has been reported as capable of infecting a broad range of cell types (161). We determined that following infection of PBMC with VSV-ΔG-GFP, the predominant GFP+ cell type was monocytes while GFP expression in DCs was apparent but delayed by several hours. GFP expression was not seen in T cells, B cells, and NK cells, and though it is entirely possible that T cells were infected with VSV-ΔG, it was beyond the scope of our studies to differentiate between viral entry and neo-antigen expression. For our purposes, it was significant that T cells were not eliminated to a large extent in early infection and that antigen

expression was limited to antigen-presenting cells. ICS assays were performed to measure IFNg production followed overnight infection with recombinant VSV-ΔG, and in all of our studies, T cell viability remained high, consistently providing ample cells for the detection of antigen-specific populations (data not shown).

Optimization of recombinant VSV-ΔG vectors for use in ICS assays was dependent upon two key parameters: the time of BfA addition to infected PBMC and optimal MOI. Maximum IFNg responses in CD4 and CD8 T cells were observed when BfA was added 3-4 hr following infection at an MOI of 10, a time point and MOI that is common in assays that employ recombinant vaccinia viruses for antigen delivery (128). Kinetic studies of monocyte and DC infection demonstrated that while a large percentage of monocytes express antigen delivered by VSV-ΔG 4 hr post-infection, few, if any, DCs expressed antigen at this time point. Given that addition of BfA 4 hr post-infection would be expected to largely inhibit surface presentation of MHC, this result is suggestive that monocytes are primarily responsible for activating antigen-specific T cell populations in overnight VSV-ΔG T cell assays.

Overlapping peptide pools are among the most commonly used and effective reagents for stimulating both CD4 and CD8 T cells in *ex vivo* cell populations. Recombinant VSV-ΔG vectors performed comparably to overlapping peptide pools in ICS assays, a highly desirable result when considering the well-established effectiveness of overlapping peptide pools in detecting antigen-specific T cell populations. The primary limitation of peptide pools is that the cost of synthesis could potentially make their use economically prohibitive in some

studies, such as vaccine trials that commonly generate massive numbers of samples to be processed or when mapping immunodominant epitopes in moderately large pathogens. VSV-ΔG replicates to a high titer in culture (10^8 to 10^9 infectious units/ml, typically), and expansion in a single 35mL tissue culture flask typically yields sufficient virus stock to perform hundreds of assays. This represents a significant advantage of recombinant VSV-ΔG in T cell assays – the potential to quickly and easily generate a large panel of reliable and effective reagents at little financial expense.

In addition to detecting T cell responses to immunodominant viral epitopes from HCMV in humans, VSV-ΔG vectors were effective in detecting T cell responses to YFV and influenza vaccination. Indeed, we believe that VSV-ΔG is an ideal tool for evaluating essentially any candidate vaccine designed to elicit T cell responses. Recombinant VSV-ΔG vectors are easy to produce, inexpensive, and potent stimulators of *in vitro* recall responses by antigen-specific T cells across multiple species. Our results clearly demonstrate that these vectors represent a powerful alternative to traditional methods for stimulating antigen-specific T cells in ELISpot and ICS assays.

Acknowledgements

This work was supported by the NIH grant U19-AI057266 and the NIH/NIAID HHSN266200700006C Center of Excellence for Influenza Research and Surveillance. We thank Holden Maecker at BD Biosciences for supplying CMV.pp65 overlapping peptide pools, and Ed Mocarski for supplying human cytomegalovirus pp65 cDNA. We also acknowledge the Emory Center for AIDS Research (P30 AI050409) for flow cytometry support.

Figure Legends

Figure 2.1. VSV is monocyte tropic. (A) GFP expression in monocytes following 6 hr infection of human PBMC with VSV-ΔG-GFP. Gated populations represent the percentages of GFP-expressing $CD11c^+$, $CD14^+$, and $CD4^+$ cells. (B) Kinetics of GFP expression in DCs (top) and monocytes (bottom) following VSV-ΔG-GFP infection. The percentages of GFP-expressing DCs and monocytes, respectively, are shown as gated populations with the frequencies of DCs and monocytes in total PBMC populations shown in the lower right corner of each plot. DCs are defined as $CD11c^+$, lin^- (CD20, CD3, CD56, CD16, CD14), $HLA\text{-}DR^+$ events, and monocytes are visualized by CD14 staining. (C) Graphical representation of GFP expression in VSV-ΔG-GFP-infected DCs (squares) and monocytes (triangles). (D) GFP expression in monocytes from fresh vs. cryopreserved PBMC for three donors following VSV-ΔG-GFP infection. The histograms shown are gated on live, $CD14^+$ events, and the percentage of GFP-expressing monocytes is shown.

Figure 2.2. Optimization of assay parameters for flow cytometric detection of T cell responses by VSV-ΔG antigen delivery. (A) Optimization of time of BfA addition. PBMC from a CMV-seropositive donor were stimulated with a CMVpp65 peptide pool (top) or infected with VSV-ΔG-CMVpp65 (bottom). BfA was added at various time points post-stimulation/infection. CD3+ lymphocytes are shown. IFNγ-producing cells (black) are overlaid on bulk CD4 and CD8 T cell populations, and statistics represent the percentages of IFNγ-producing CD4 and

CD8 T cells. (B) Comparison of IFNγ production in fresh vs. cryopreserved PBMC for two CMV-seropositive donors following VSV-ΔG-CMVpp65 infection**.** Shown are CD3+ lymphocytes. IFNγ-producing cells (black) are overlaid on bulk CD4 and CD8 T cell populations, and statistics represent the percentages of IFNγ-producing CD4 and CD8 T cells.

Figure 2.3. Effects of carryover antigen. PBMC from a CMV-seropositive donor were infected with unpurified VSV-ΔG-CMVpp65, unpurified/UV inactivated virus, sucrose-purified virus, and sucrose-purified/UV inactivated virus. BfA was added at 0, 2, and 4 hr post-infection. Shown are CD3-gated lymphocytes. IFNγ-producing cells (black) are overlaid on bulk CD4 and CD8 populations, and statistics given represent the percentages of IFNγ-producing CD4 and CD8 T cells.

Figure 2.4. Optimization of MOI. PBMC from two CMV-seropositive donors were infected with VSV-ΔG-CMVpp65 at an MOI range of 0.4-25. CD3-gated lymphocytes are shown. IFNγ-producing cells (black) are overlayed on bulk CD4 and CD8 populations, and statistics given represent the percentages of IFNγ-producing CD4 and CD8 T cells. (B) The graph represents the fraction of the peak IFNγ response for CD4 (squares) and CD8 (triangles) T cells as a function of MOI for the donors in (A).

Figure 2.5. Evaluation of T cell responses to a range of antigens encoded by VSV-ΔG vectors. A. PBMC from a YFV-17D-vaccinated donor (day 60 post-vaccination) were infected with VSV-ΔG-YFV-NS4A4B (top) and VSV-ΔG-YFV-

NS3 (bottom); B. PBMC from a healthy donor were infected with VSV-ΔG-A/New Caledonia/20/99-HA (top), and PBMC from a donor vaccinated with the 2008-2009 seasonal influenza vaccine were infected with VSV-ΔG-B/Florida/4/06-HA (day 14 post-vaccination; bottom). For all samples, CD3-gated lymphocytes are shown, and IFNγ-producing cells (black) are overlayed on bulk CD4 and CD8 populations. Statistics represent the percentages of IFNγ-producing CD4 and CD8 T cells.

Figure 2.6. VSV-ΔG vectors drive the proliferation of antigen-specific T cells *in vitro*. (A) PBMC from a CMV-seropositive donor were CFSE-labeled, infected with VSV-ΔG-CMVpp65; (B) PBMC from a donor that has previously been demonstrated to possess influenza-specific T cell populations were CFSE-labeled and infected with VSV-ΔG vectors encoding PB1 or NP from influenza A (H1N1). Samples from (A) and (B) were cultured for 6 days prior to flow cytometry analysis, and the plots are gated on live, CD3+ events. As a positive control, PBMC were stimulated with staphylococcal enterotoxin B (SEB). The statistics shown represent the percentages of $CFSE^{low}$ CD4+ or CD8+ T cells.

Figure 2.1a

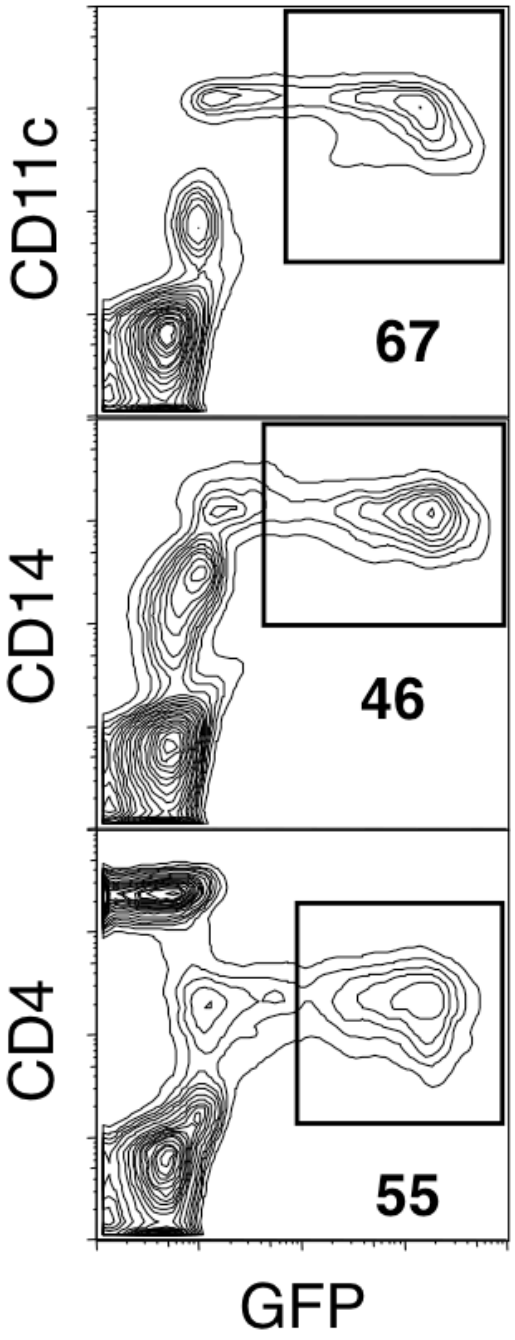


Figure 2.1b

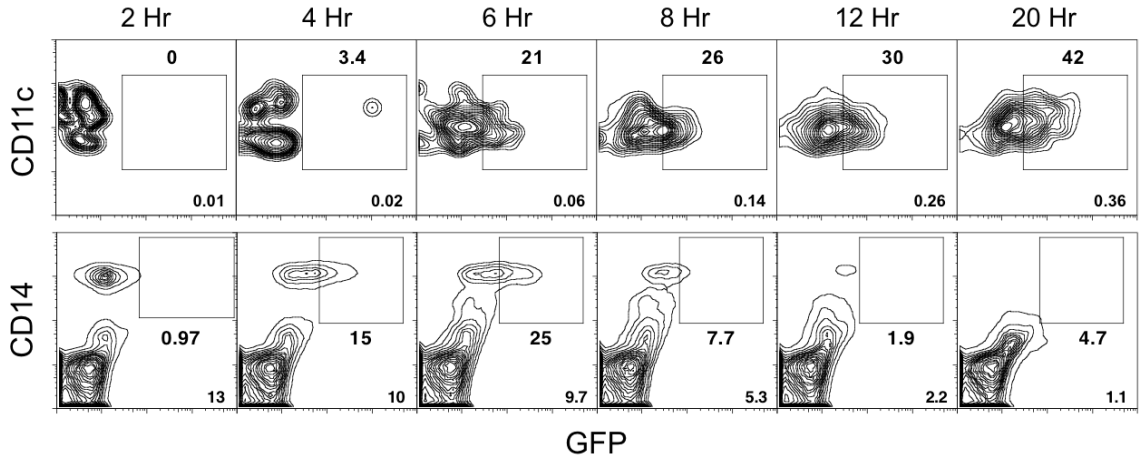

2 Hr
4 Hr
6 Hr
8 Hr
12 Hr
20 Hr
CD11c
CD14
0
3.4
21
26
30
42
0.01
0.02
0.06
0.14
0.26
0.36
0.97
15
25
7.7
1.9
4.7
13
10
9.7
5.3
2.2
1.1
GFP

Figure 2.1c

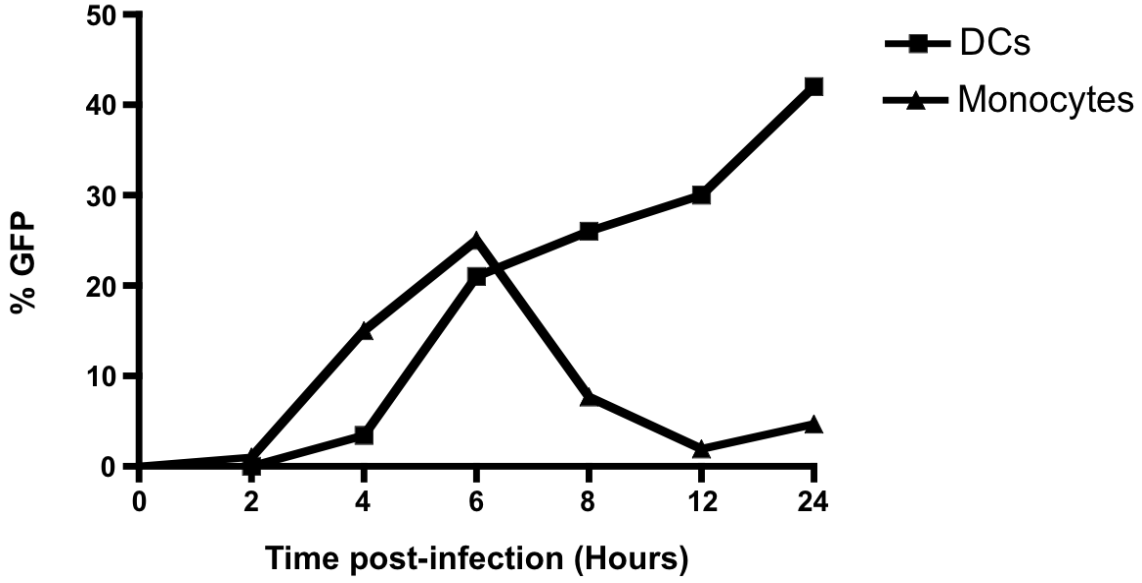

50
40
30
20
10
0
% GFP
0
2
4
6
8
12
24
Time post-infection (Hours)
DCs
Monocytes

Figure 2.1d

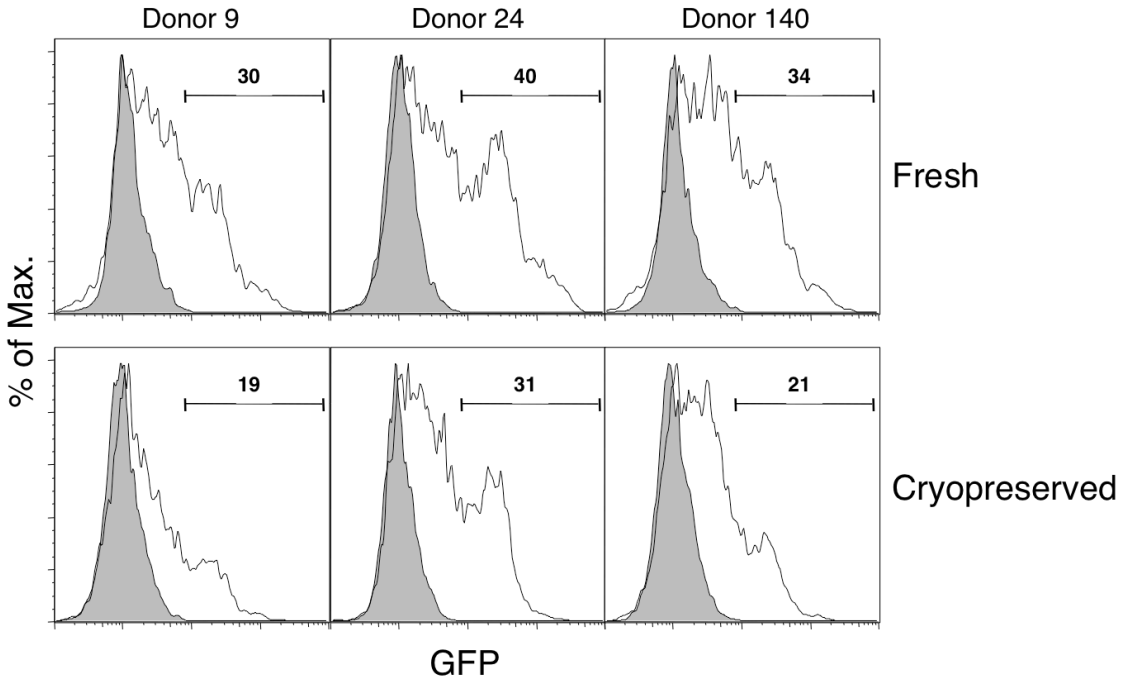

Donor 9
Donor 24
Donor 140
30
40
34
19
31
21
Fresh
Cryopreserved
% of Max.
GFP

Figure 2.2a

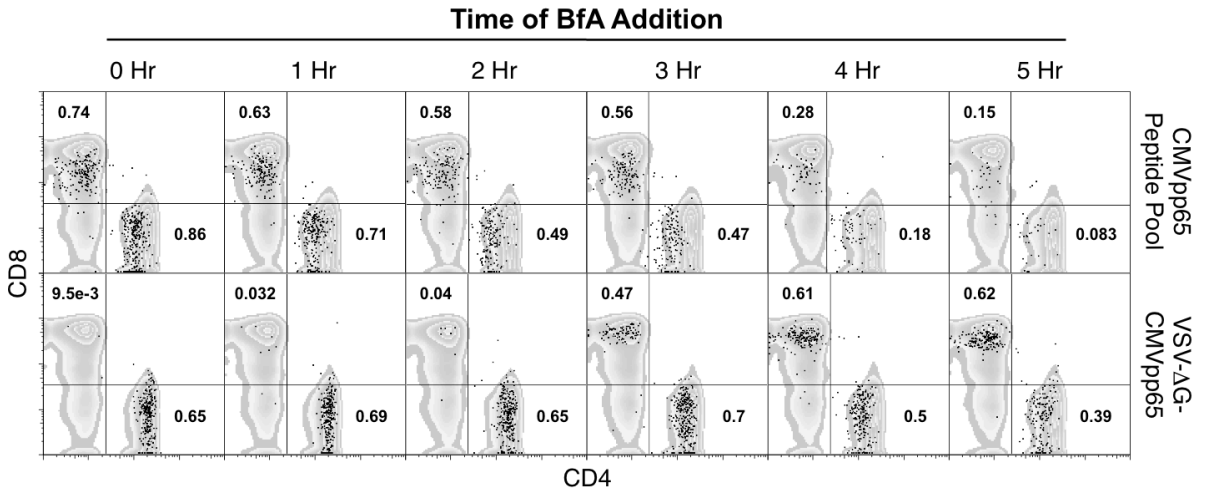

Time of BfA Addition
0 Hr
1 Hr
2 Hr
3 Hr
4 Hr
5 Hr
0.74
0.86
0.63
0.71
0.58
0.49
0.56
0.47
0.28
0.18
0.15
0.083
9.5e-3
0.65
0.032
0.69
0.04
0.65
0.47
0.7
0.61
0.5
0.62
0.39
CD8
CD4
CMVpp65 Peptide Pool
VSV-ΔG-CMVpp65

Figure 2.2b

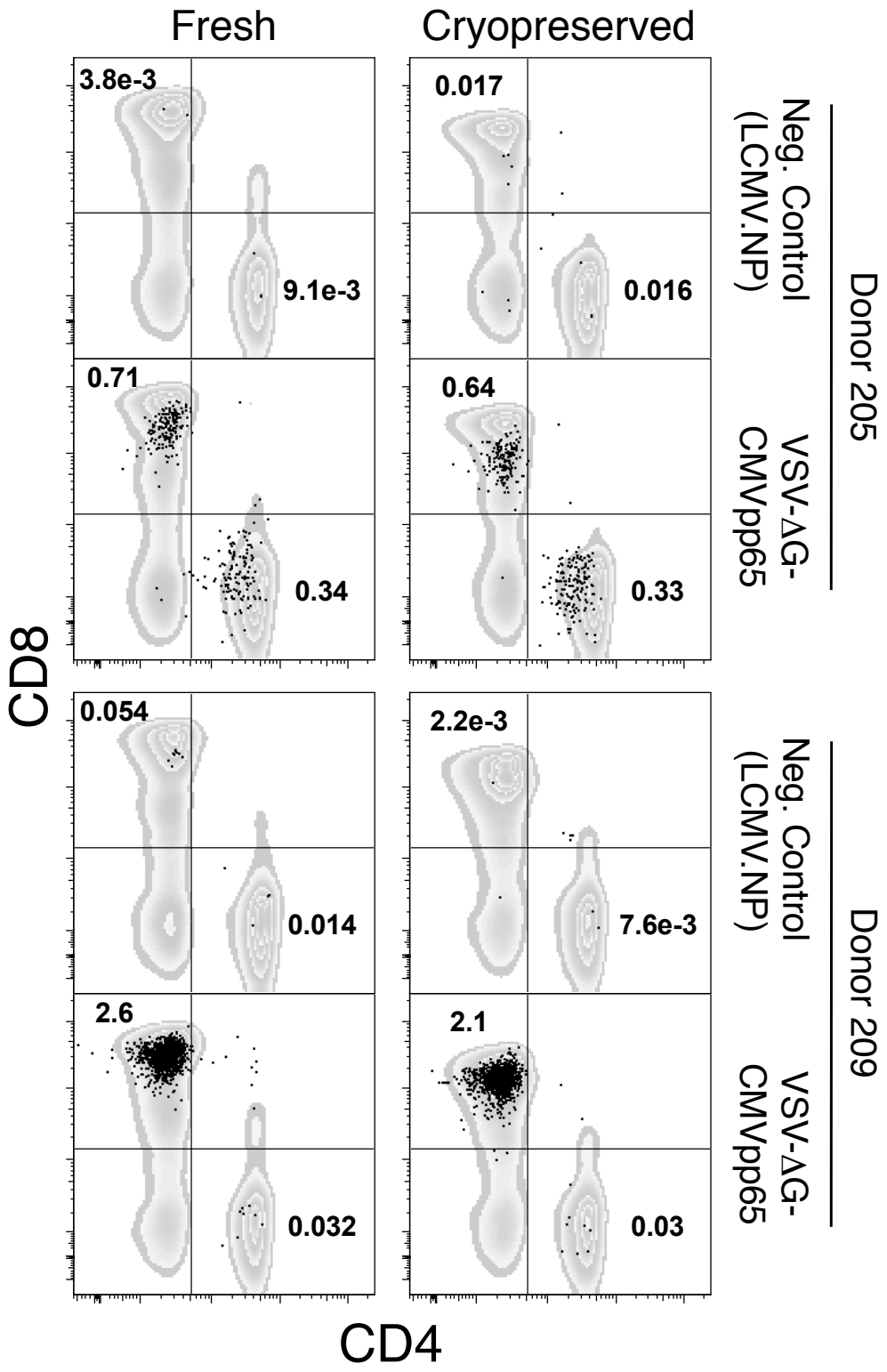

Fresh
Cryopreserved
3.8e-3
9.1e-3
0.71
0.34
0.017
0.016
0.64
0.33
0.054
0.014
2.6
0.032
2.2e-3
7.6e-3
2.1
0.03
Neg. Control (LCMV.NP)
VSV-ΔG-CMVpp65
Donor 205
Donor 209
CD8
CD4

Figure 2.3

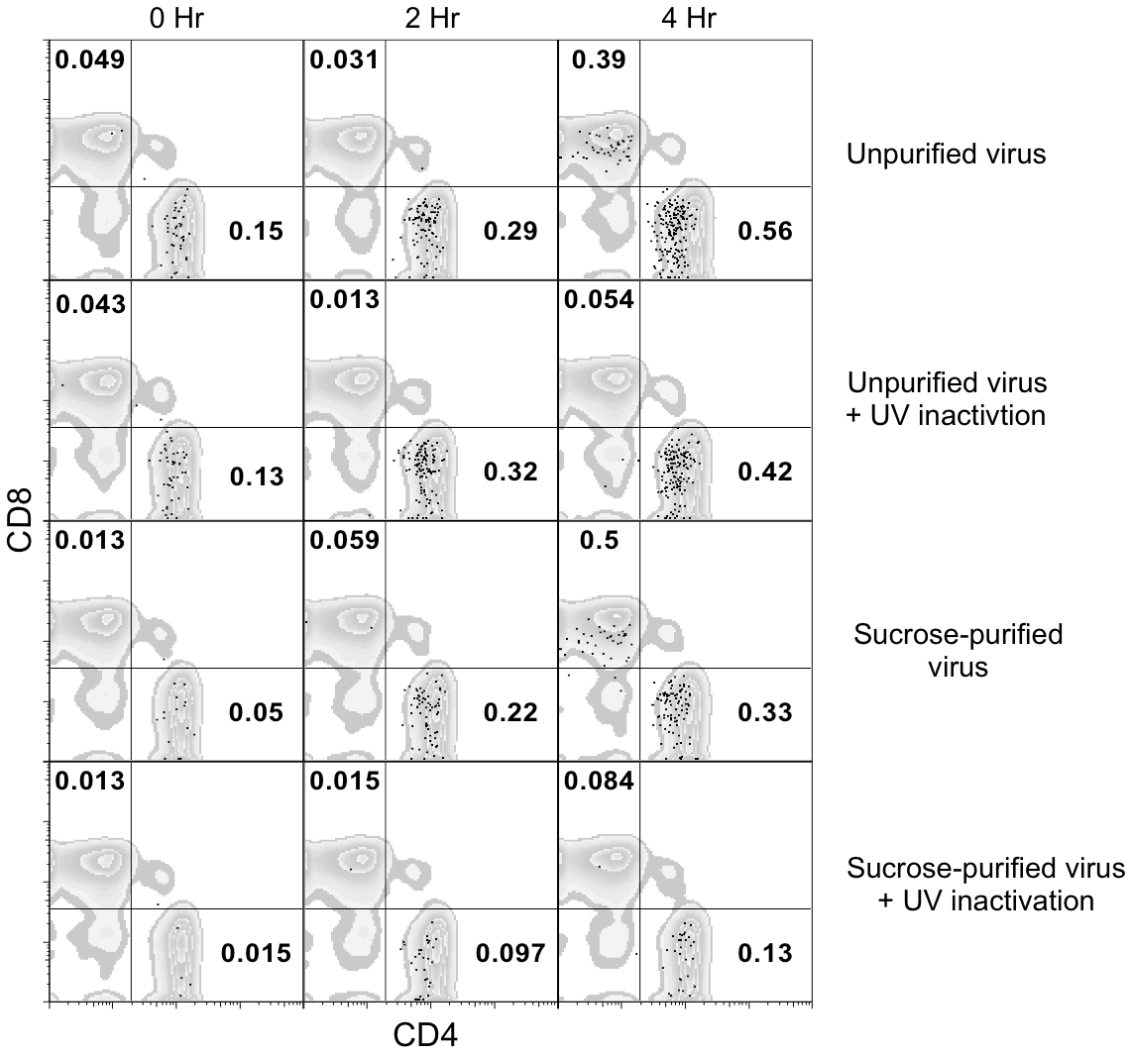

0 Hr
2 Hr
4 Hr
0.049
0.15
0.031
0.29
0.39
0.56
Unpurified virus
0.043
0.13
0.013
0.32
0.054
0.42
Unpurified virus
+ UV inactivtion
0.013
0.05
0.059
0.22
0.5
0.33
Sucrose-purified
virus
0.013
0.015
0.015
0.097
0.084
0.13
Sucrose-purified virus
+ UV inactivation
CD8
CD4

Figure 2.4a

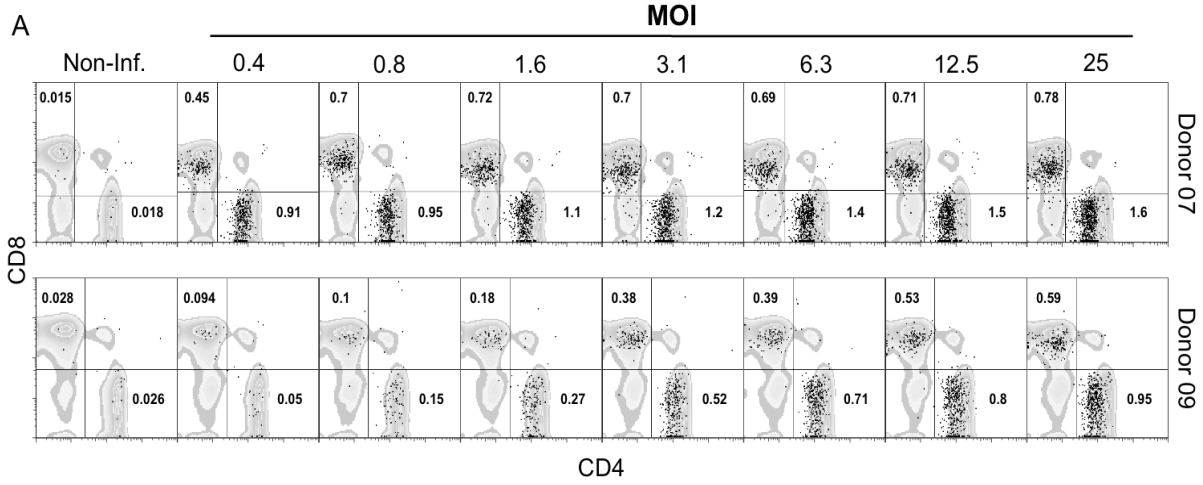

A
MOI
Non-Inf.
0.4
0.8
1.6
3.1
6.3
12.5
25
0.015
0.018
0.45
0.91
0.7
0.95
0.72
1.1
0.7
1.2
0.69
1.4
0.71
1.5
0.78
1.6
Donor 07
CD8
0.028
0.026
0.094
0.05
0.1
0.15
0.18
0.27
0.38
0.52
0.39
0.71
0.53
0.8
0.59
0.95
Donor 09
CD4

Figure 2.4b

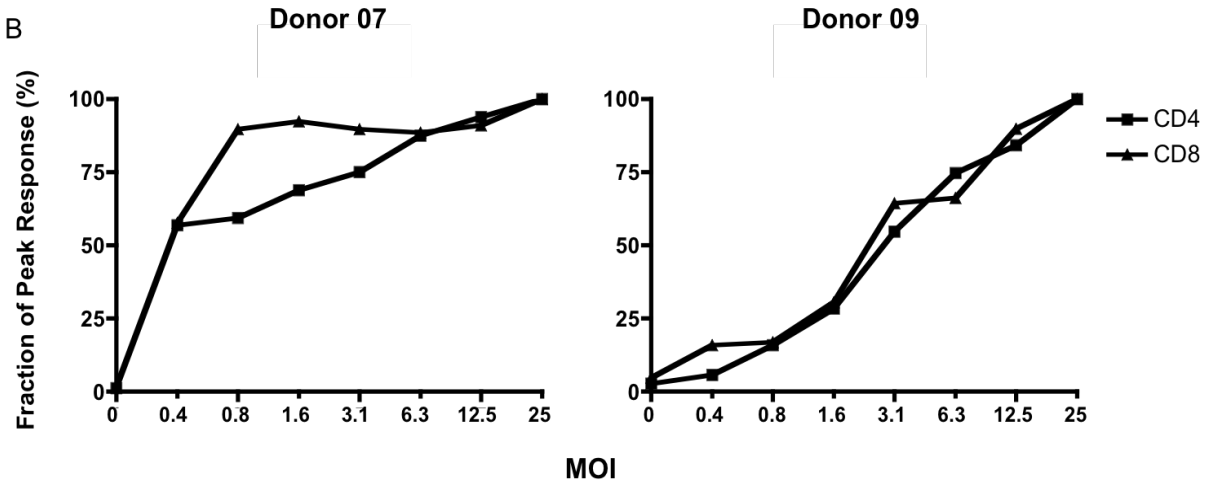

B
Donor 07
Donor 09
Fraction of Peak Response (%)
100
75
50
25
0
0
0.4
0.8
1.6
3.1
6.3
12.5
25
MOI
CD4
CD8

Figure 2.5a,b

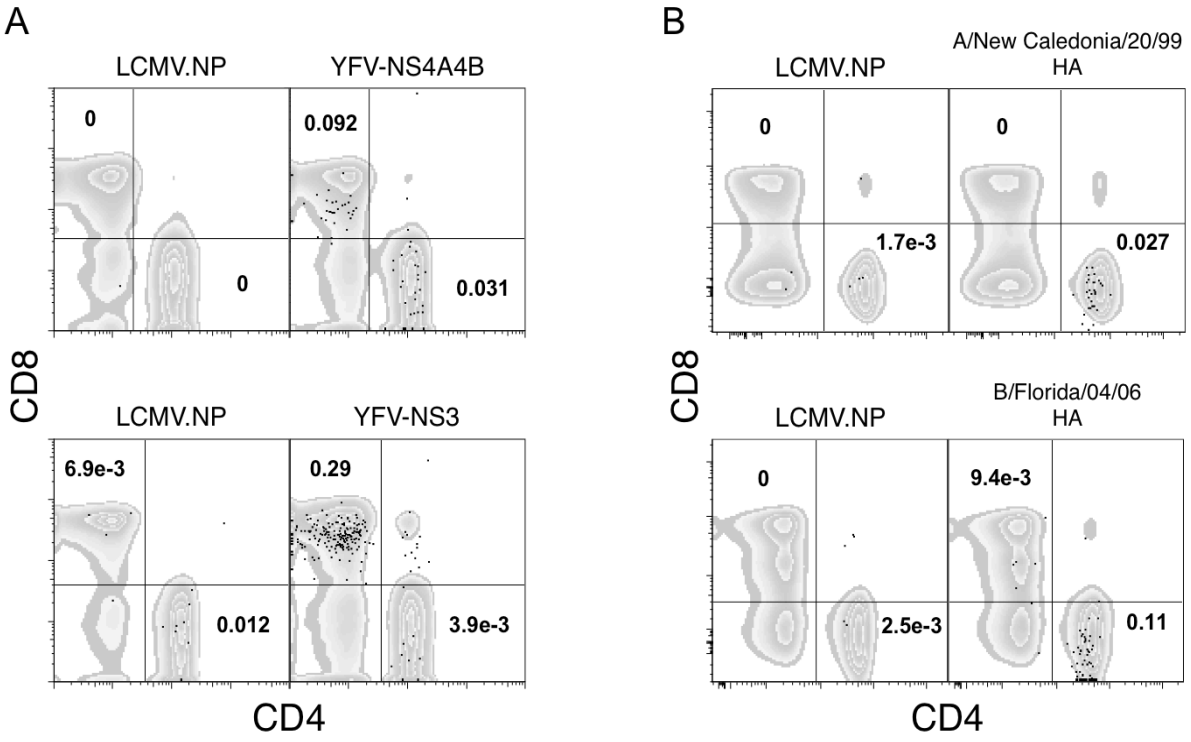

A
LCMV.NP
YFV-NS4A4B
0
0.092
0
0.031
CD8
LCMV.NP
YFV-NS3
6.9e-3
0.29
0.012
3.9e-3
CD4
B
A/New Caledonia/20/99
HA
LCMV.NP
0
0
1.7e-3
0.027
CD8
B/Florida/04/06
HA
LCMV.NP
0
9.4e-3
2.5e-3
0.11
CD4

Figure 2.6a

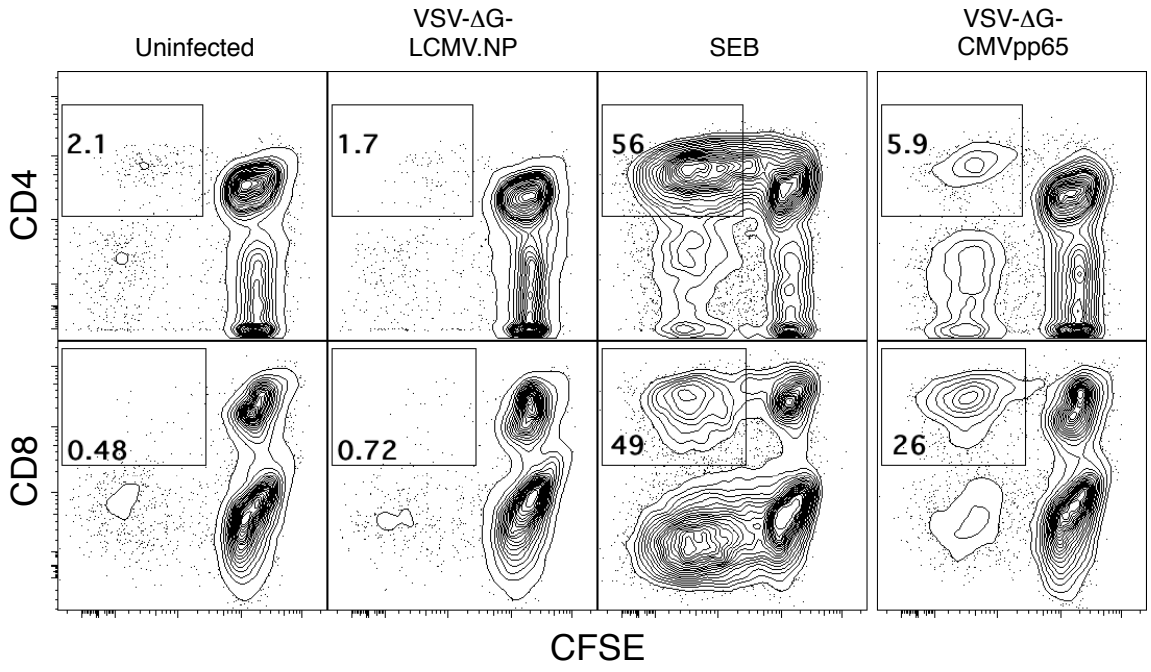
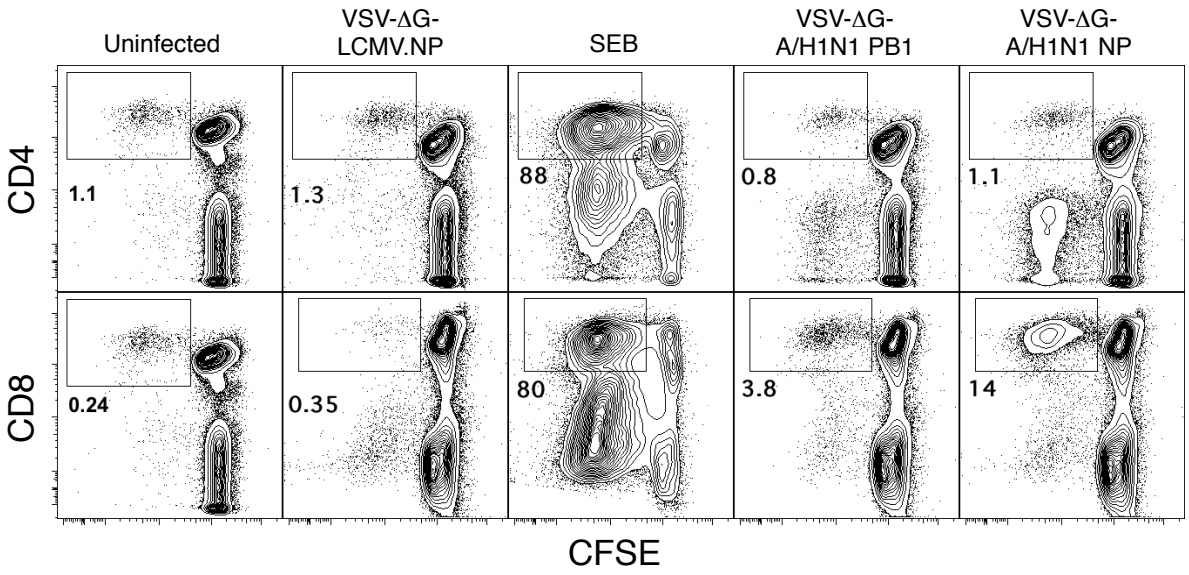

Uninfected
VSV-ΔG-
LCMV.NP
SEB
VSV-ΔG-
CMVpp65
CD4
CD8
2.1
1.7
56
5.9
0.48
0.72
49
26
CFSE

Figure 2.6b


Uninfected
VSV-ΔG-LCMV.NP
SEB
VSV-ΔG-A/H1N1 PB1
VSV-ΔG-A/H1N1 NP
CD4
CD8
1.1
1.3
88
0.8
1.1
0.24
0.35
80
3.8
14
CFSE

Chapter 3:

Characterization of human T cell responses to 2010-2011 seasonal influenza vaccines

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Abbreviations: recombinant vesicular stomatitis virus (rVSV)

Abstract

There is considerable interest in enhancing the efficacy of seasonal influenza vaccination by incorporating conserved T cell epitopes to establish broadly cross-reactive T cell populations. The suitability of this approach is difficult to assess, because human T cell responses to influenza vaccination are poorly characterized. To address this issue, we measured vaccine-induced CD4 and CD8 T cell responses in adult donors following the administration of trivalent inactivated influenza vaccine (TIV) or live, attenuated influenza vaccine (LAIV). Our investigation included external proteins, hemagglutinin (HA) and neuraminidase (NA), and internal proteins, matrix protein 1 (M1) and nucleoprotein (NP), across multiple influenza A strains. Pre-existing T cell populations specific for external and internal influenza proteins were readily detectable in several donors. Although selected individuals generated modest vaccine-associated CD4 T cell responses specific for external proteins, trial participants as a group demonstrated poor boosting of baseline CD4 T cell responses specific for external proteins. LAIV was more effective than TIV in generating and maintaining CD4 T cell responses specific for internal proteins while both vaccines were ineffective in boosting external or internal protein-specific CD8 T cell responses. Overall, our data suggests that current seasonal influenza vaccine approaches may be not be suitable for boosting and maintaining protective T cell immunity.

Keywords: influenza, trivalent inactivated influenza vaccine (TIV), live attenuated influenza vaccine (LAIV), CD4 T cell, CD8 T cell, cross-reactive

Introduction

Influenza is a virus that causes acute, febrile disease of the respiratory tract and represents a major health burden for humans of all ages, especially infant and elderly populations. Seasonal vaccination remains the most effective tool for protecting individuals from disease, and there are currently two FDA-approved seasonal vaccines available in the United States, trivalent inactivated influenza vaccine (TIV) and live, attenuated influenza vaccine (LAIV). TIV is administered via intramuscular injection and is approved for use in individuals older than 6 months. LAIV is delivered to the lungs via inhalation of an intranasal mist and is currently approved for individuals between the ages of 2 and 49. TIV and LAIV also differ in the identity of their internal genomic backbones into which circulating, seasonal HA and NA genes are inserted. Internal genes for TIV are derived from an H1N1 strain, A/Puerto/Rico/08/34, and LAIV contains internal genes from A/Ann Arbor/06/60 (H2N2) and B/Ann Arbor/01/66. TIV is currently administered in the United States in split-virion form, in which inactivated viral particles are treated with detergent, such as Triton X-100, resulting in the enrichment of HA and NA in vaccine preparations.

It is generally accepted that in humans, the primary correlate of protection against seasonal influenza is the generation of antibodies specific for HA and to a lesser extent, NA. No T cell correlate of protection has been established in humans, even though mouse studies have demonstrated the protective potential of influenza-specific CD4 and CD8 T cells (103-105). Very little systematic

research of T cell responses to influenza vaccination has been performed in human subjects, and these studies have produced conflicting results. In trials comparing TIV and LAIV in children and adults, LAIV induced significant CD4 and CD8 T cell responses in children, but no vaccine-associated increase in influenza-reactive T cells was noted in adults for either vaccine group (143). T cell responses were highly variable in adults, and baseline levels of influenza-specific CD4 and CD8 T cells were identified as significant negative correlates of vaccine-induced responses (162). Conversely, in a recent trial of 30 adult vaccinees, it was reported that the H1N1 and H3N2 components of both TIV and LAIV elicited significant H1 and H3-specific T cell responses in a majority of trial members, and this effect was observed in donors with both high and low influenza-specific T cell baseline levels (96,142). This discrepancy could be due to the different methods employed in each study. In the first, IFNγ production in donor PBMC was measured by intracellular cytokine staining (ICS) following a 17hr infection with live influenza virus. In the second, influenza-specific T cells were detected via an IFNγ ELISpot assay after expansion for seven days with overlapping HA peptide pools in the presence of IL-2. These results call attention to the need for more suitable and standardized methods of detecting influenza-specific T cell populations.

Following the 2009 H1N1 pandemic, it was found that less than 5% of individuals under the age of 30 possessed antibodies that were cross-reactive with 2009 H1N1, demonstrating that prior seasonal vaccination or exposure

provided no protective antibody response against the highly divergent pandemic strain (141). Due to a high prevalence of conserved epitopes, influenza-specific T cells are thought to be less sensitive to high levels of antigenic variation. Numerous reports have shown that adults possess pre-existing levels of CD4 and CD8 T cells that cross-react with various proteins from H1N1 2009 (133,163). Additionally, it has recently been reported that both CD4 and CD8 T cells in healthy adults cross-react with internal proteins from H5N1 avian influenza virus (135,164,165), demonstrating that T cell populations established by seasonal vaccination or exposure are capable of recognizing epitopes present in novel subtypes. These and similar results have led to proposals that universal T cell influenza epitopes may be incorporated into seasonal vaccines to broaden protection and mitigate the severity of antigenically variant strains (166-168).

We recently developed a novel T cell quantitation assay that has been optimized for direct *ex vivo* detection of influenza-specific populations in human PBMC. This flow cytometry-based method employs recombinant vesicular stomatitis virus (rVSV) vectors to deliver antigen and represents a practical, economical, and efficacious alternative to overlapping peptide pools covering multiple influenza genes and strains. We have generated a panel of rVSV vectors that express various external (HA and NA) and internal (M1 and NP) influenza genes across multiple strains and subtypes, and we have used these reagents to evaluate direct *ex vivo* CD4 and CD8 T cell responses to 2010-2011 TIV and LAIV in adult donor PBMC. Our primary goals were (1) to investigate the

specificity and kinetics of T cell responses following TIV and LAIV vaccination, (2) to determine the effect of pre-existing influenza-specific T cells levels on responses to vaccination, and (3) to examine the potential of influenza vaccines to elicit broadly cross-reactive T cell responses. This study characterizes T cell responses to 2010-2011 seasonal influenza vaccines and provides insight into the suitability of employing conventional vaccine strategies in generating broadly cross-reactive T cell immunity.

Materials and Methods

2.1. Plasmid Construction

Plasmid pVSV-ΔG is a Bluescript-based plasmid that encodes the anti-genome RNA of VSV. In this plasmid, the coding region for VSV-G has been removed and replaced with a polylinker (152). Various internal and external influenza genes were inserted into the polylinker region of pVSV-ΔG using *Kpn I*, *Sph I*, and *Nhe I* restriction enzyme sites. For some constructs, a gene of interest was inserted into pVSV-ΔG using a ligation-independent cloning (LIC) method (153). Briefly, the polylinker region of pVSV-ΔG was replaced with a LIC sequence that upon linearization with *SmaI* and subsequent treatment with T4 DNA polymerase and dATPs yields specific overhangs. In the absence of ligase, these overhangs anneal with complimentary sequences flanking genes of interest that have been amplified with LIC primers and treated with T4 DNA polymerase and dTTPs. This method requires very few manipulations and is ideal when introducing various different inserts into the same vector.

2.2. Recovery of Recombinant Vesicular Stomatitis Virus

Recombinant vesicular stomatitis virus (rVSV) was produced as previously described (152-155). Briefly, baby hamster kidney cells (BHK-21; American Type Culture collection) were maintained in Dulbecco's modified Eagle's medium (DMEM)/5% fetal bovine serum (FBS) at 37ºC. BHK-21 cells on 6-well plates (~95% confluent) were infected with recombinant modified vaccinia virus Ankara (MVA) expressing T7 polymerase (MVA-T7) at a multiplicity of infection (MOI) of

approximately 1. Following a 90 minute incubation, plasmids encoding VSV-N, P, L, and G proteins and pVSV-ΔG containing a gene of interest (VSV-ΔG) were transfected at a ratio of 3:5:1:8:5, respectively, into the cells by using a liposome suspension of dimethyldioctadecyl ammonium bromide and L-a-dioleoylphosphatidylethanolamine for 4 hours. 48 hours after transfection, supernatants were 0.22μm syringe-filtered (to remove MVA-T7) on to BHK-21 cells previously transfected with pCAGGS-VSV-G (~85% confluent) using Lipofectamine reagent (Invitrogen). VSV-G must be introduced in trans, because recombinant VSV-ΔG does not produce the viral glycoprotein. Following 24 to 48 hour incubation, successful recoveries were indicated by visualization of extensive cell rounding/cytopathic effect. Following recovery, virus was amplified on VSV-G-transfected BHK-21 cells, and viral titers were determined via plaque assay.

2.3. Specimen Preparation and Assay Setup

Donor blood samples were collected in sodium citrate cell preparation tubes (Vacutainer, BD) and centrifuged at 1500g for 30 minutes. PBMC were then processed as directed by the manufacturer and resuspended at 10^7/ml in RPMI-1640/10% FBS (R10). In a 5ml polypropylene tube, 10^6 human PBMC were infected with rVSV viral supernatant at an MOI of approximately 10 in a total volume of 200μl. Costimulatory antibodies CD28/CD49d (FastImmune, BD) were added to each sample at a final concentration of 1μg/ml. A cytokine blocking reagent, GolgiPlug (Brefeldin A, BD), was added 4 hr post-infection.

Samples were then incubated overnight at 37ºC/5%CO_2 and were processed for flow cytometry analysis on the following day.

2.4. Intracellular Cytokine Staining

Following rVSV infection and incubation, human PBMC were washed with PBS and permeabilized with FACS Permeabilizing Solution 2 (BD). After permeabilization, the cells were again washed with PBS and stained with CD4-FITC (BD), CD3-PacBlue (BD), CD8-ECD (Coulter), CD14-APC/Cy7 (BioLegend), CD20-APC/Cy7 (BioLegend), CD69-PerCP (BD), IFNγ-APC (BD), TNFα-PE/Cy7 (eBioscience), and IL-2-PE (R&D Systems). All samples were acquired on an LSRII flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.5. IgG antibody-secreting cell (ASC) ELISpot Assay

The IgG ASC ELISpot assay was performed as previously described (92). The antigen used in the assay was a 2010-2011 inactivated seasonal influenza vaccine. Following stimulation of donor PBMC, influenza-specific IgG ASCs were detected via incubation with biotinylated anti-human IgG antibodies (Caltag) followed by development with AEC substrate (Sigma). Analysis of developed ELISpot plates was performed with an automated ELISpot counter (Cellular Technologies Ltd., Shaker Heights, OH).

2.6. Statistical Analysis

The two-tailed Mann Whitney test was used to compare baseline T cell

responses specific for influenza proteins with negative controls. Influenza-specific T cell responses within the same vaccine group were compared with the two-tailed Wilcoxon matched pairs test. *P* values were designated as follows: $P < 0.05$ (*), 0.01 (**), and 0.005 (***). These tests were performed with Prism software (GraphPad Software, Inc., La Jolla, CA). Linear regression analysis was also conducted with Prism software. A mixed linear model was performed to generate response estimates, in number of $CD69^+$ $IFN\gamma^+$ cells / 10^6 T cells, for days 0, 7, and 14 for all influenza strains in the TIV and LAIV groups. The differences in CD4 T cell responses between day 0-7 and day 0-14 for both the TIV and LAIV group were tested, and significance was defined as $P < 0.05$. In this study, we conducted repeated measurements on test subjects over time. Repeated measurements on the same subject usually correlated and often exhibited heterogeneous variability. A SAS Proc Mixed procedure was used to conduct the repeated measures analysis that account for within-subject covariability. This analysis was conducted using SAS 9.2 software (SAS Institute Inc., Cary, NC).

Results

3.1. Detection of influenza-specific T cells using rVSV vectors

We recently developed and optimized an ICS-based T cell quantitation assay that employs recombinant vesicular stomatitis viruses (rVSV) as a source of antigen (submitted for publication). This assay permits direct *ex vivo* visualization of influenza-specific CD4 and CD8 T cell populations in human PBMC, as demonstrated in Figure 3.1. PBMC from two donors who were administered TIV (Donor 083) or LAIV (Donor 093) were isolated at multiple time points post-vaccination and infected with rVSV vectors expressing M1 or NP from multiple influenza A strains: A/Puerto Rico/08/34 (A/PR/08/34; H1N1), A/Ann Ann Arbor/06/60 (A/AA/06/60, H2N2), and A/California/04/09 (A/Cal/04/09; H1N1). As a negative control, cells were infected with an rVSV construct that expresses NP from lymphocytic choriomeningitis virus (rVSV.LCMV-NP). LCMV does not typically infect humans, so most donors would not be expected to respond to rVSV.LCMV-NP in a short-term T cell assay. Following a 16 h incubation, the cells were analyzed for IFNγ production via intracellular cytokine staining (ICS). Robust CD4 T cell responses to M1 from A/PR/08/34 and A/AA/06/60 were observed following vaccination in Donor 093 (Figure 3.1a). These responses peaked at day 7 post-vaccination and remained above baseline levels through day 30. CD4 T cells specific for M1 from A/Cal/04/09 were not detected in this donor, although responses to $M1_{A/Cal/04/09}$ were observed in additional subjects (Figure 3.5b). An example of a CD8 T cell response to NP is demonstrated in

Figure 3.1b. Donor 083 possessed detectable baseline levels of CD8 T cells specific for NP from all three strains tested. Following a slight decrease of NP-specific CD8 T cell levels on day 7, responding populations rose above baseline levels by day 14. For this donor, on days 0-14, a greater number of CD8 T cells responded to $NP_{A/PR/08/34}$ and $NP_{A/Cal/04/09}$ than $NP_{A/AA/06/60}$ although by day 30, responses specific for NP from each strain were essentially identical. Importantly, background levels of responding cells in the negative controls were low, less than 0.005% of total CD4 T cells for Donor 093 and undetectable for Donor 083. This is a key requirement when attempting to visualize low frequency populations.

It has been previously reported that upon stimulation with influenza viral lysate, influenza-specific CD4 T cells commonly co-express multiple cytokines, such as IFNγ, TNFα, and IL-2, (220), and we observed this using the rVSV antigen delivery system as well (Figure 3.1c). PBMC from one donor were infected with rVSV vectors expressing NP from A/PR/08/34 or LCMV. This donor demonstrated a clear CD4 T cell response to NP from A/PR/08/34, which peaked at day 7 and remained above baseline through day 30. At each time point, IFNγ production was accompanied by the production of both TNFα and IL-2. Triple cytokine production was common in essentially all donors that possessed influenza-specific CD4 T cells (data not shown). Overall, these results demonstrate that utilizing rVSV vectors as an antigen source in T cell assays

represents a valid method for studying human T cell responses to influenza vaccination.

3.2. Study parameters

To investigate human T cell responses to 2010-11 influenza vaccines we analyzed vaccine-induced CD4 and CD8 T cell responses specific for two external proteins (HA and NA) and two internal proteins (M1 and NP) across multiple influenza A strains following vaccination with TIV or LAIV (FluMist®, Medimmune LLC). All study samples were collected during the 2010-11 influenza vaccine trial conducted by the Emory Influenza Pathogenesis and Immunology Research Center (Emory IPIRC). The demographics of study participants are provided in Table 1. All trial donors were adults ranging from 23 to 53 years of age. The previous vaccination and infection history of the trial members was unknown. A few donors demonstrated baseline levels of CD4 or CD8 T cells that responded the rVSV.LCMV-NP negative control construct (data not shown). It is possible that these donors were exposed to LCMV, VSV, or another virus that generated T cell cross-reactivity to rVSV.LCMV-NP, and these donors were excluded from the remainder of our study. Our investigation of vaccine-associated T cell responses to external proteins included 2009 H1N1, A/Cal/04/09, and all of the H1N1 representatives of seasonal influenza vaccines since 2000: A/New Caledonia/20/99 (A/NC/20/99), A/Solomon Islands/03/06 (A/SI/03/06), and A/Brisbane/59/07 (A/Br/59/07; Table 2). A/California/07/09 is an A/Cal/04/09-like strain that was chosen as the H1N1 component of 2010-2011 seasonal vaccines. External proteins from this strain are genetically similar to HA

from A/Cal/04/09 (1 amino acid substitution) and identical to A/Cal/04/09 NA (169). The strains included in our studies of T cell responses to internal proteins included A/Cal/04/09, as well as A/PR/08/34 and A/AA/06/60, which serve as the genetic backbones of TIV and LAIV, respectively. Donors were split into two groups to be assayed for either external or internal influenza protein-specific T cell responses at days 0, 7, 14, 28, and 90 relative to vaccination. This division was necessary, because we did not have an adequate number of cells to assay for both external and internal responses in individual donors. For this reason, we were also unable to investigate H3N2-specific T cell responses.

3.3. T cell responses to external influenza proteins following vaccination

PBMC from 18 vaccinated donors (8 TIV and 10 LAIV) were assayed for CD4 and CD8 T cell responses specific for external influenza proteins using rVSV vectors encoding HA and NA from the influenza strains listed in Table 2. Baseline CD4 T cell responses specific for HA and NA from each strain were significantly higher than negative controls (Mann Whitney test; Figure 3.2a). Day 0 HA- and NA-specific CD4 T cell responses were of similar magnitude, all within a range of 75 to 175 $CD69^+$ $IFN\gamma^+$ cells / 10^6 CD4s, with baseline responses to NA from A/Cal/04/09 and A/NC/20/99 being the highest. Baseline CD4 T cell responses to $HA_{A/Cal/04/09}$ were noticeably higher in the LAIV group, with 5 out of 10 LAIV-vaccinated donors demonstrating day 0 $HA_{A/Cal/04/09}$-specific CD4 T cell responses greater than 100 $CD69^+$ $IFN\gamma^+$ cells / 10^6 CD4s (Figure 3.2b). This is almost certainly attributable to the random placement of donors with higher baseline $HA_{A/Cal/04/09}$-specific CD4 T cell levels into the LAIV group. Day 0 CD4 T

cell responses to HA from the vaccine strain, A/Cal/04/09, did not significantly change at any time point for either vaccine group. Weak, yet statistically significant vaccine-associated cross-reactive CD4 T cell responses to $HA_{A/NC/20/99}$ and $HA_{A/Br/59/07}$ were observed at day 14 in the TIV group (Wilcoxon matched pairs test). We also noted a small, significant vaccine-associated day 14 CD4 T cell response specific for $NA_{A/Cal/04/09}$ in the TIV group, but there was no indication of vaccine induced cross-reactive responses to NA (Figure 3.2c).

According a mixed linear model, significant CD4 T cell responses specific for $HA_{A/NC/20/99}$ and $NA_{A/Cal/04/09}$ were detected at day 14 in the TIV group (Table 3), although the meagerness of these responses would suggest that any vaccine-induced response observable in the peripheral blood is minimal. No vaccine-associated CD4 T cell boost was noted in the LAIV group. Taken together, the results from our studies of human CD4 T cell responses to external influenza proteins indicates that while baseline populations are easily detected in many donors, their levels are only weakly boosted, if at all, by seasonal influenza vaccination.

We did not detect significant baseline levels of CD8 T cells specific for HA or NA from any of the influenza strains tested, indicating that these proteins may not be major targets for CD8 T cell responses (data not shown). Additionally, we did not detect significant vaccine-associated increases in CD8 T cell responses to HA or NA from either the TIV or LAIV group (data not shown).

3.4. Vaccine-associated CD4 T cell responses to external influenza proteins are cross-reactive

Although adults as a group display few significant increases in CD4 T cell responses to external influenza proteins following TIV or LAIV administration, a number of individual donors generated CD4 T cell responses to external H1N1 vaccine components (Figure 3.3). For these donors, populations of circulating CD4 T cells specific for HA or NA from A/Cal/04/09 typically peaked at day 7 or 14 and returned to baseline by day 30. 5 out of 18 (28%; 3 TIV/2 LAIV) donors tested responded to HA, and 6 out of 18 (33%; 4 TIV/2 LAIV) subjects responded to NA (Figure 3.3a).

Due to a presumed presence of shared epitopes, we anticipate that donors who responded to the vaccine strain, A/Cal/04/09, should demonstrate cross-reactivity to external proteins derived from more conventional H1N1 strains. As expected, we found that CD4 T cell cross-reactivity was quite common in responding donors. An example of vaccine-induced cross-reactive CD4 T cell responses is shown in Figure 3.3b. For this donor, cross-reactive responses were comparable in magnitude and followed nearly identical kinetics to those specific for A/Cal/04/09. Overall, these results indicate that while influenza vaccination does not generally induce potent CD4 T cell responses in the general adult population, many individual vaccine recipients generate detectable responses that are cross-reactive across multiple strains within the H1N1 subtype.

3.5. Comparison of post-vaccination IgG-secreting ASC levels and CD4 T cell responses to external influenza proteins at day 7

Figure 3.4 shows peak IgG ASC responses for 18 donors, 8 TIV and 10 LAIV, involved in our study of T cell responses to external influenza proteins. It has been previously reported in adults that TIV induces IgG ASC responses superior to those following LAIV administration (95), and this observation was reproduced in our vaccine trial, as only 1 out of 10 donors in the LAIV group exhibited appreciable IgG ASC responses at day 7 compared to 6 out of 8 in the TIV group (Figure 3.4). Since interactions between antigen-specific B cells and cognate CD4 T cells have been linked to B cell help in multiple systems (90,145,146), we compared day 7 levels of IgG ASCs with corresponding CD4 T cell responses specific for external proteins from the vaccine strain, A/Cal/04/09. The sum of day 7 A/Cal/04/09 HA- and NA-specific CD4 T cell responses for TIV and LAIV group members is shown. Although linear regression analysis of TIV group members revealed no significant relationship between vaccine-induced CD4 T cell responses and ASC levels, we did not have a sufficient number of trial members in this group to properly investigate any correlation. Due to the paucity of IgG responders in the LAIV group, we did not perform statistical analysis on these donors.

3.5. CD4 T cell responses to internal influenza proteins following vaccination

Our investigation of CD4 and CD8 T cell responses to internal influenza proteins included the strains listed in Table 2. PBMC from 22 vaccinated donors

(12 TIV and 10 LAIV) were assayed for CD4 and CD8 T cell responses specific for internal influenza proteins using rVSV vectors expressing M1 and NP from each strain. We did not have adequate donor PBMC to screen more than two internal proteins, so we chose the internals that appear most prominently in the literature. Baseline CD4 T cell responses specific for M1 and NP from each strain were significantly higher than negative controls (Mann Whitney test; Figure 3.5a). Pre-existing A/Cal/04/09 M1-specific CD4 populations were lower than that of A/PR/08/34 and A/AA/06/60, possibly because the donors tested had not been exposed to A/Cal/04/09 M1, which is derived from a Eurasian swine lineage that had not been reported in North America prior to the 2009 H1N1 pandemic (62). Sequence analysis of the three strains reveals that A/Cal/04/09 differs from both A/PR/08/34 and A/AA/0/60 by 9 amino acids that are contained within 7 previously published class II epitopes (Supplementary Data 1). TIV recipients did not display a vaccine-associated M1-specific CD4 T cell response for any of the strains tested (Figure 3.5b). This result was not unexpected, as TIV is administered in split-virion form in which internal influenza proteins have been removed from vaccine preparations by detergent treatment and purification. The extent of internal protein removal has, to our knowledge, not been explored fully; however, it has been shown that detectable concentrations of internal proteins are present in TIV (170-172). LAIV significantly boosted pre-existing CD4 T cell populations specific for A/AA/06/60 and also induced expansion of CD4 T cells specific for M1 from A/PR/08/34, and to a lesser extent, A/Cal/04/09, in a cross-reactive manner (Wilcoxon matched pairs test). Responses were transient,

peaking at day 7, and although we observed statistically significant responses beyond this time point for each strain in the LAIV group, expanded populations returned nearly to baseline levels by day 14. CD4 T cell responses to NP followed similar kinetics to that of M1 but were noticeably less robust (Figure 3.5c). Members from both the TIV and LAIV group responded significantly to NP from their respective genetic backbones, A/PR/08/34 and A/AA/06/60, at day 7. These responses are comparable, except for two donors in the LAIV group (donors 062 and 117). These donors generated robust NP-specific CD4 T cell responses and were largely responsible for the increased statistical significance attributed to the LAIV group. Both TIV and LAIV induced low level but statistically significant expansion of cross-reactive CD4 T cell responses to A/Cal/04/09 NP at day 7, and this response was more effectively maintained for donors in the LAIV group.

According to a mixed linear model, LAIV was the more potent inducer of internal protein-specific CD4 T cell responses (Table 4). Significant day 7 responses specific for M1 and NP were detected for all strains tested in the LAIV group, and with the exception of M1 from A/Cal/04/09, the estimated differences between baseline and day 7 were greater than 150 $CD69^+$ $IFN\gamma^+$ cells / 10^6 CD4s. Taken together, these results suggest that LAIV is more effective than TIV in boosting and maintaining CD4 T cell responses to internal influenza proteins.

3.6. CD8 T cell responses to internal influenza proteins following vaccination

Baseline CD8 T cell responses specific for M1 and NP from each strain were significantly higher than negative controls (Mann Whitney test; Figure 3.6a). Day 0 internal protein-specific CD8 T cell responses were generally higher than those observed for CD4 T cells, with selected individual donors in each vaccine group possessing pre-existing M1- and NP- specific populations greater than 2000 $CD69^+$ $IFN\gamma^+$ CD8 T cells / 10^6 total CD8s. Despite the presence of baseline M1- and NP-specific CD8 T cell populations, both TIV and LAIV were shown to be ineffective at boosting these cells following vaccination (Figure 3.6b,c). We noted a significant day 7 response to NP from A/Cal/04/09 in the LAIV group, but this vaccine-induced effect was quite weak. The significant NP-specific CD8 T cell responses at days 30 and 90 in the LAIV group were skewed by a few donors who tested higher at these time points, possibly due to seasonal influenza exposure. Since any vaccine-associated boosting typically occurred at days 7 or 14, it is probable that these responses were unrelated to vaccination. These results were consistent with the findings of a mixed linear model (Table 5). Overall, we conclude that while CD8 T cells specific for internal influenza proteins are readily detectable in the general population, both TIV and LAIV fail to appreciably boost these populations.

Discussion

The role of T cells in the immune response to influenza infection in humans is poorly understood. Properly defining any T cell-mediated contribution has been greatly hindered by numerous factors, including a paucity of reagents that permit the accurate measurement of influenza-specific CD4 and CD8 T cell populations in blood samples. Circulating influenza-specific T cells are known to be quite scarce, and visualizing them requires the use of very sensitive techniques. Currently, the most common and effective method for detecting antigen-specific T cells is the use of overlapping peptide pools as a source of antigen followed by ELISpot or ICS assay. These reagents are expensive to synthesize, however, and may be economically inappropriate for studies involving multiple antigens or large numbers of samples to be screened. We propose that influenza protein-expressing rVSV vectors represent an appropriate alternative to overlapping peptide pools in influenza-specific T cell assays for the following reasons. First, we observed that when donors generated CD4 or CD8 T cell responses specific for an internal influenza protein from one of the strains tested, they typically reacted to one or both of the other strains, both in terms of magnitude and kinetics (Figure 3.1). This was expected, because numerous MHC class I and class II epitopes are conserved across multiple strains and subtypes. This gives us confidence that the use of influenza protein-expressing rVSV vectors in T cell assays yields physiologically sound results. Second, a number of groups that have published influenza-specific T cell studies employing overlapping peptides as an antigen source report T cell frequencies that are

comparable to those observed in our study (132, 133, 139). Third, like overlapping peptide pools, rVSV vectors perform well in short-term overnight assays, averting the need for *in vitro* culture manipulations. Fourth, rVSV vectors have the added advantage of expressing proteins that are naturally processed and presented by antigen-presenting cells. Finally, rVSV vectors are inexpensive and can quickly be produced and expanded.

We report significant levels of pre-existing CD4 T cell populations specific for external influenza proteins from multiple influenza A strains. However, we observed minimal boosting above baseline following vaccination. In responding donors, we noted CD4 T cell cross-reactivity against various other H1N1 viruses, demonstrating that previous seasonal influenza vaccination or exposure induces broadly cross-reactive CD4 T cell immunity. Since post-vaccination serum antibody titers against HA and NA are strongly correlated to protection in humans, multiple groups have compared cognate HA-specific CD4 T cell and B cell responses resulting from seasonal influenza vaccination. Recent comparisons of pre-existing H1N1 HA-specific T cell responses and seasonal H1N1 hemagglutination inhibition assay (HAI) titers failed to reveal a correlation (218). Similar results were reported in a separate trial comparing post-vaccination H3N2 HA-specific T cell and vaccine-associated B cell responses (96). We observed no obvious correlation between influenza-specific CD4 T cell responses and influenza-specific IgG ASC levels following vaccination; however our results were not conclusive, as we did not have sufficient donors in our study

to perform a comprehensive comparison. Additional studies will be necessary to conclusively determine whether any correlation exists between influenza-specific CD4 T cells and humoral responses in order to exploit this relationship for the purpose of designing more efficacious seasonal influenza vaccines.

We were unable to detect significant levels of pre-existing CD8 T cells specific for external influenza proteins or any appreciable vaccine-induced responses to these proteins. There currently exists no data in humans that would suggest a role for CD8 T cell responses to external influenza proteins in humans. Recent studies comparing predicted MHC class I epitopes with previously published class I-restricted peptides failed to identify a single CD8 T cell epitope for HA and few for NA (173). Additionally, donor PBMC stimulated with peptides derived from observed predicted epitopes from external proteins exhibited weak *in vitro* recall CD8 T cell responses (168). Our conclusion is that in humans, influenza HA and NA are not major targets for CD8 T cell responses.

It has been recently reported that while CD8 T cell responses to an immunodominant seasonal influenza NP peptide (NP_{418} LPFDKSTIM) are present in individuals who were previously unexposed to A/Cal/04/09, corresponding responses to variant NP_{418} sequences from A/Cal/04/09 and 1918 H1N1 were absent, demonstrating the capability of influenza viruses to escape immunodominant CD8 T cell responses (138,174). However, based on sequence analysis of internal proteins from A/Cal/04/09, we expect some degree of cross-

reaction between conserved epitopes from A/Cal/04/09 and seasonal strains due to the invariant nature of these proteins (175). We showed that baseline CD8 T cell populations specific for M1 and NP from A/PR/08/34, A/AA/06/60, and A/Cal/04/09 were readily detectable in several trial participants. Furthermore, baseline responses to M1 and NP from A/Cal/04/09 were comparable to those of A/PR/08/34 and A/AA/06/60. These results are corroborated by recent reports of pre-existing immunity to A/Cal/04/09 in the general population (139,176). Our data suggests that in the presumed absence of exposure to highly variant influenza strains, such as A/Cal/04/09, seasonal vaccination and exposure to seasonal strains is sufficient to produce CD8 T cell cross-reactivity.

There is currently considerable interest in blending “universal” CD8 T cell epitopes that are broadly cross-reactive with various strains of internal influenza proteins into seasonal influenza vaccines in an effort to provide protection against highly variant strains, such as A/Cal04/09. This interest is largely driven by an abundance of data in mouse models that exhibit cross-protective vaccine-associated CD8 T cell responses to internal influenza proteins (177-181). Our results indicate that in human donors, both TIV and LAIV fail to appreciably boost CD8 T cell responses specific for internal influenza proteins from A/Cal/04/09, despite detectable baseline populations. We are aware of only one report of robust CD8 T cell responses to internal A/Cal/04/09 proteins, and this observation was made in patients who were clinically diagnosed with A/Cal/04/09 infection (174). This raises the question about whether inactivated and live,

attenuated vectors are sufficiently immunogenic to boost pre-existing CD8 T cell responses to an efficacious level.

Our characterization of human T cell responses to 2010-2011 seasonal influenza vaccines revealed two main findings. First, rVSV vectors are a practical and accurate means of antigen delivery for influenza-specific quantitative T cell assays in humans. Second, by using the rVSV system, we determined that 2010-2011 seasonal influenza vaccines were generally inefficacious in inducing robust post-vaccination T cell responses in human populations. It should be noted that T cell analysis in PBMC might not precisely predict immune responses that occur at major sites of viral replication, such as the lungs or draining lymph nodes, so we must be careful when interpreting data derived from peripheral blood samples. Despite this consideration, we conclude that new technologies, such as adjuvants and alternative vaccine delivery systems, will be essential to induce protective T cell immunity in influenza vaccination.

Acknowledgements

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Figure Legends

Figure 3.1. Visualization of influenza-specific T cells using rVSV vectors. Following influenza vaccination, PBMC from Donor 093 (LAIV-vaccinated; A) and Donor 083 (TIV-vaccinated; B) were were isolated at multiple time points and infected with M1- or NP-expressing rVSV vectors. Following overnight incubation, the cells were assayed for IFNγ production via ICS. PBMC from Donor 062 (LAIV-vaccinated) were infected with rVSV vectors encoding NP from A/PR/08/34 were tested for IFNγ, TNFα, and IL-2 production at various time points (C). CD3-gated lymphocytes are shown, and statistics represent the percentages of $CD69^+$ cytokine-producing CD4 or CD8 T cells.

Figure 3.2. CD4 T cell responses to external influenza proteins. Pre-existing CD4 T cell responses specific for HA and NA from A/NC/20/99, A/SI/03/06, and A/Br/59/07, and A/Cal/04/09 for all external group participants (*n* = 18) were compared with the Mann Whitney test. CD4 T cell responses specific for HA (B) and NA (C) from the above strains were measured at multiple post-infection time points in the TIV (*n* = 8) and LAIV (*n* = 10) groups. Statistical analysis was performed with the Wilcoxon matched pairs test. For all samples, responses are reported as number of $CD69^+$ $IFN\gamma^+$ cells / 10^6 CD4 T cells. Asterisks show $P < 0.05$ (*), 0.01 (**), and 0.005 (***).

Figure 3.3. Vaccine-induced CD4 T cell responses specific for external influenza proteins are cross-reactive. 5 out of 20 donors, 3 TIV (closed symbols) and 2 LAIV (open symbols), responded at days 7 or 14 post-

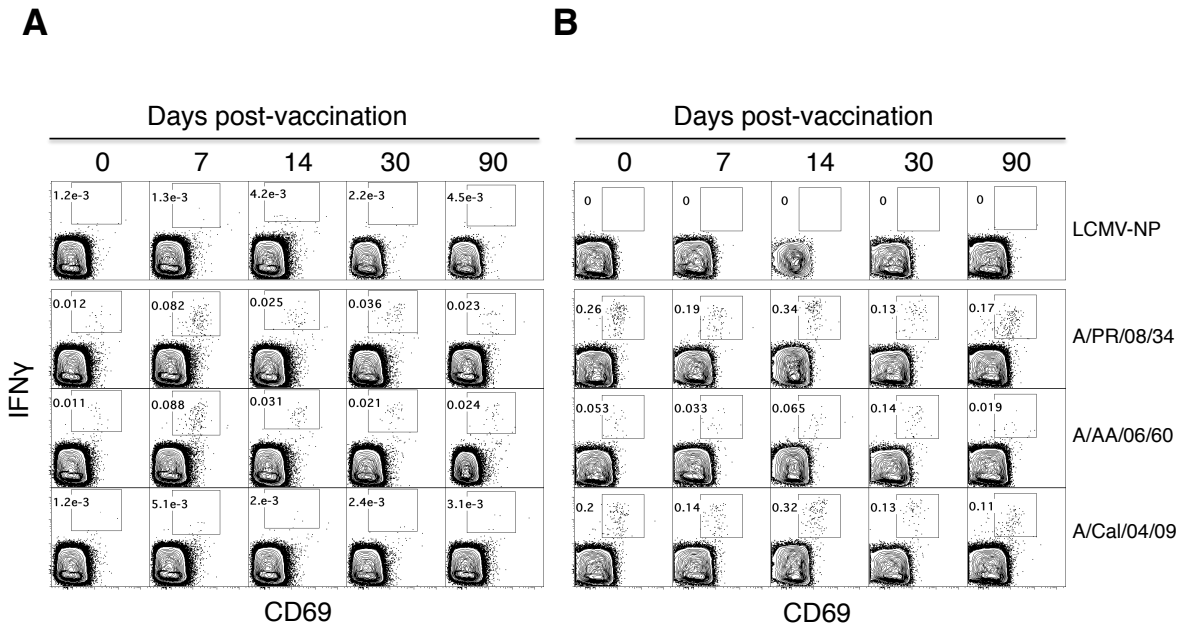
vaccination to HA from the vaccine strain, A/Cal/04/09, and 6 out of 20 donors, 4 TIV and 2 LAIV, responded to NA from A/Cal/04/09 (A). An example of vaccine-associated cross-reactive CD4 T cell responses is demonstrated in (B). CD4 T cell responses specific for HA and NA from A/Cal/04/09 (closed diamonds) were comparable in magnitude and kinetics to those for A/NC/20/99, A/SI/03/06, and A/Br/59/07. For all samples, responses are reported as number of $CD69^+$ $IFN\gamma^+$ cells / 10^6 CD4 T cells.

Figure 3.4. Comparison of post-vaccination IgG-secreting ASC levels and CD4 T cell responses to external influenza proteins at day 7. Day 7 IgG ASC responses for 18 vaccine trial participants (8 TIV, closed circles, and 10 LAIV, open circles) were measured by IgG ASC ELISpot assay. This data is compared to the sum of A/Cal/04/09 HA- and NA-specific CD4 T cell responses at day 7. Linear regression analysis was performed for members of the TIV group.

Figure 3.5. CD4 T cell responses to internal influenza proteins. Pre-existing CD4 T cell responses specific for M1 and NP from A/PR/08/34, A/AA/06/60, and A/Cal/04/09 for all internal group participants (n = 22) were compared with the Mann Whitney test. CD4 T cell responses specific for M1 (B) and NP (C) from the above strains were measured at multiple post-infection time points in the TIV (n = 12) and LAIV (n = 10) groups. Statistical analysis was performed with the Wilcoxon matched pairs test. For all samples, responses are reported as number of $CD69^+$ $IFN\gamma^+$ cells / 10^6 CD4 T cells. Asterisks show $P <$ 0.01 (**), and 0.005 (***).

Figure 3.6. CD8 T cell responses to internal influenza proteins. Pre-existing CD8 T cell responses specific for M1 and NP from A/PR/08/34, A/AA/06/60, and A/Cal/04/09 for all internal group participants (*n* = 22) were compared with the Mann Whitney test. CD8 T cell responses specific for M1 (B) and NP (C) from the above strains were measured at multiple post-infection time points in the TIV (*n* = 12) and LAIV (*n* = 10) groups. Statistical analysis was performed with the Wilcoxon matched pairs test. For all samples, responses are reported as number of $CD69^+$ $IFN\gamma^+$ cells / 10^6 CD8 T cells. Asterisks show $P <$ 0.01 (**), and 0.005 (***).

Figure 3.1a,b


A
B
Days post-vaccination
0
7
14
30
90
IFNγ
CD69
LCMV-NP
A/PR/08/34
A/AA/06/60
A/Cal/04/09
1.2e-3
1.3e-3
4.2e-3
2.2e-3
4.5e-3
0.012
0.082
0.025
0.036
0.023
0.011
0.088
0.031
0.021
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1.2e-3
5.1e-3
2.e-3
2.4e-3
3.1e-3
0
0
0
0
0
0.26
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0.34
0.13
0.17
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0.14
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Figure 3.1c

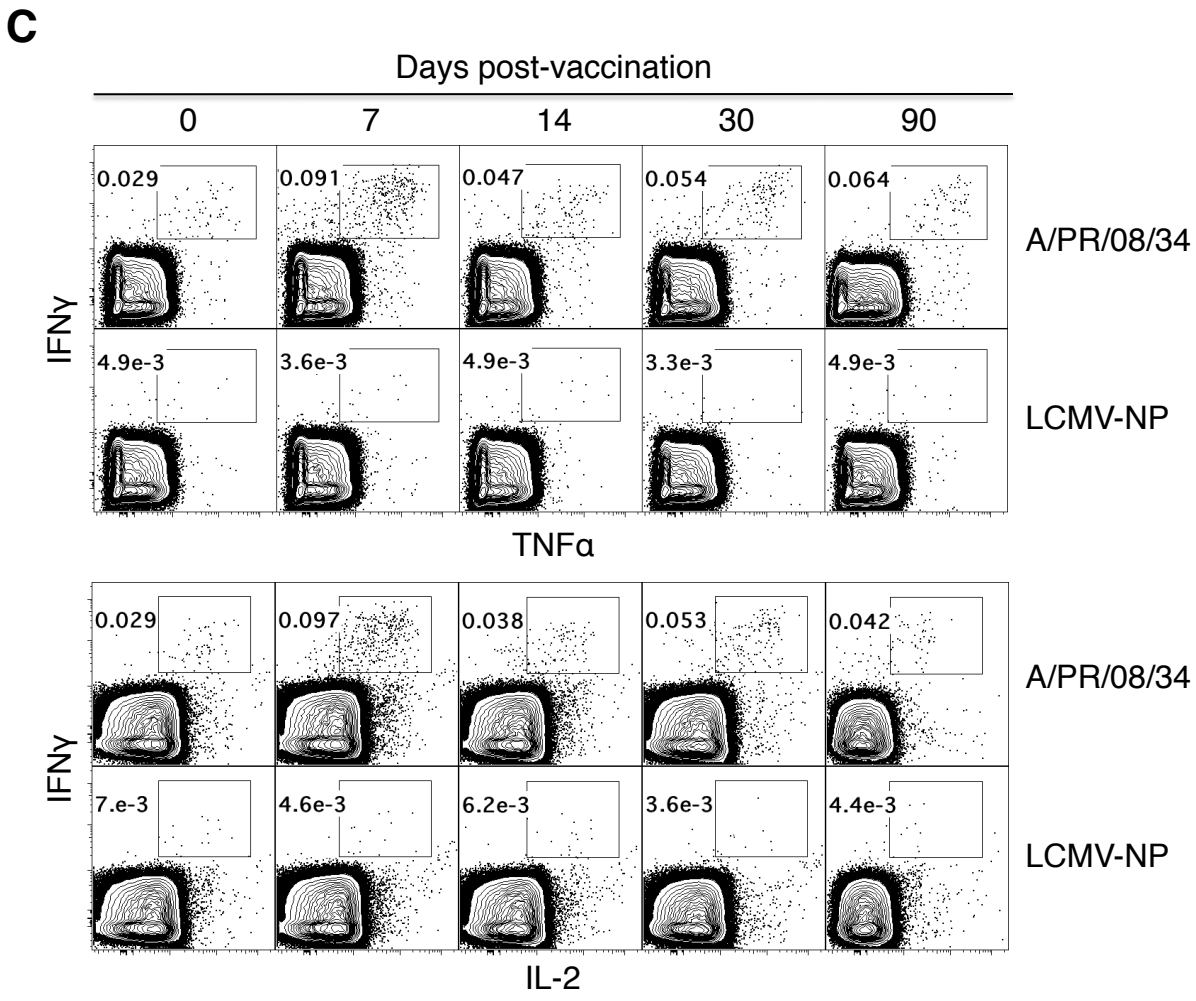

C
Days post-vaccination
0
7
14
30
90
0.029
0.091
0.047
0.054
0.064
A/PR/08/34
4.9e-3
3.6e-3
4.9e-3
3.3e-3
4.9e-3
LCMV-NP
IFNγ
TNFα
0.029
0.097
0.038
0.053
0.042
A/PR/08/34
7.e-3
4.6e-3
6.2e-3
3.6e-3
4.4e-3
LCMV-NP
IFNγ
IL-2

Figure 3.2a

A

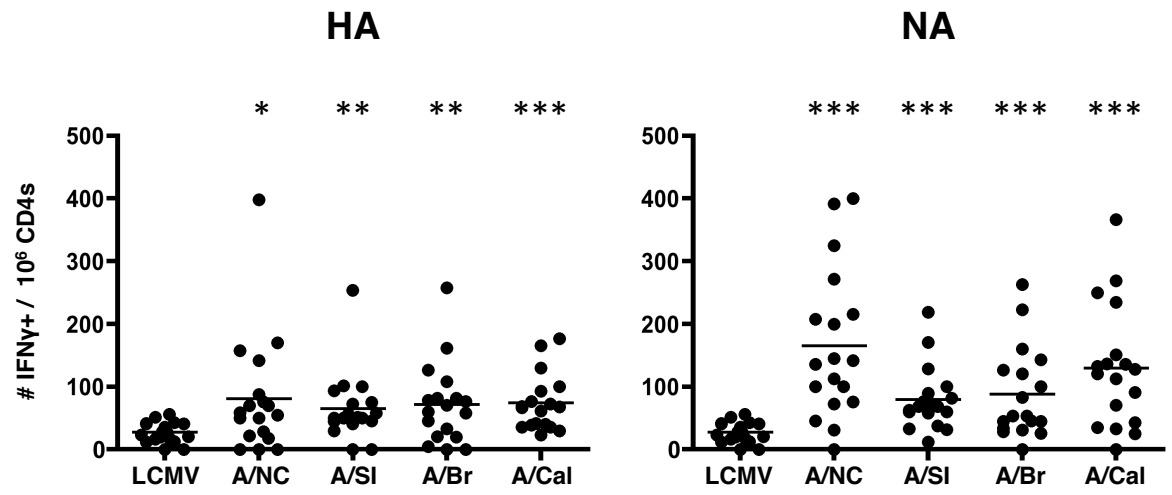


Figure 3.2b

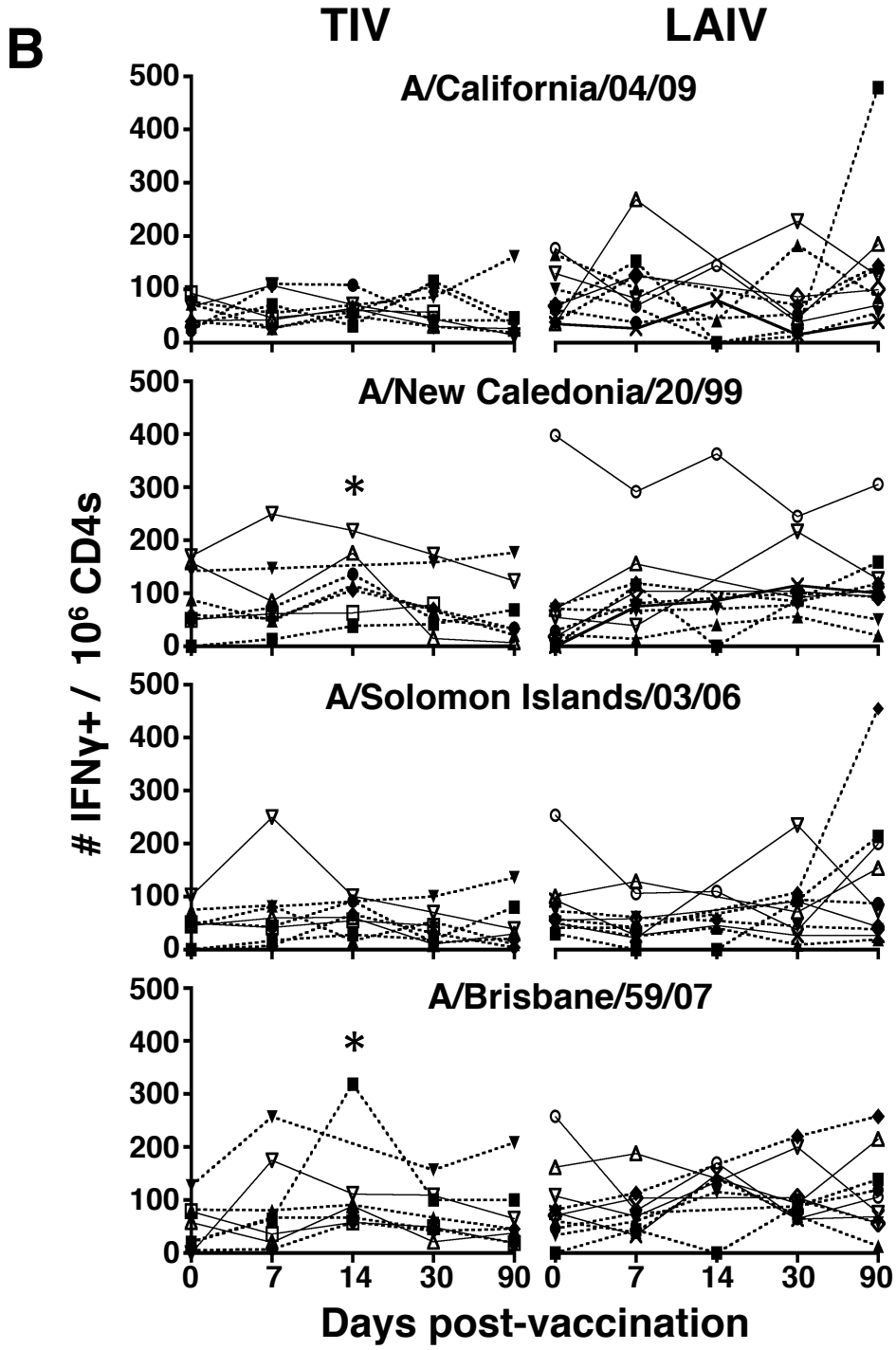

B
TIV
LAIV
A/California/04/09
A/New Caledonia/20/99
A/Solomon Islands/03/06
A/Brisbane/59/07
*
*
IFNγ+ / 10⁶ CD4s
0
100
200
300
400
500
0
7
14
30
90
Days post-vaccination

Figure 3.2c

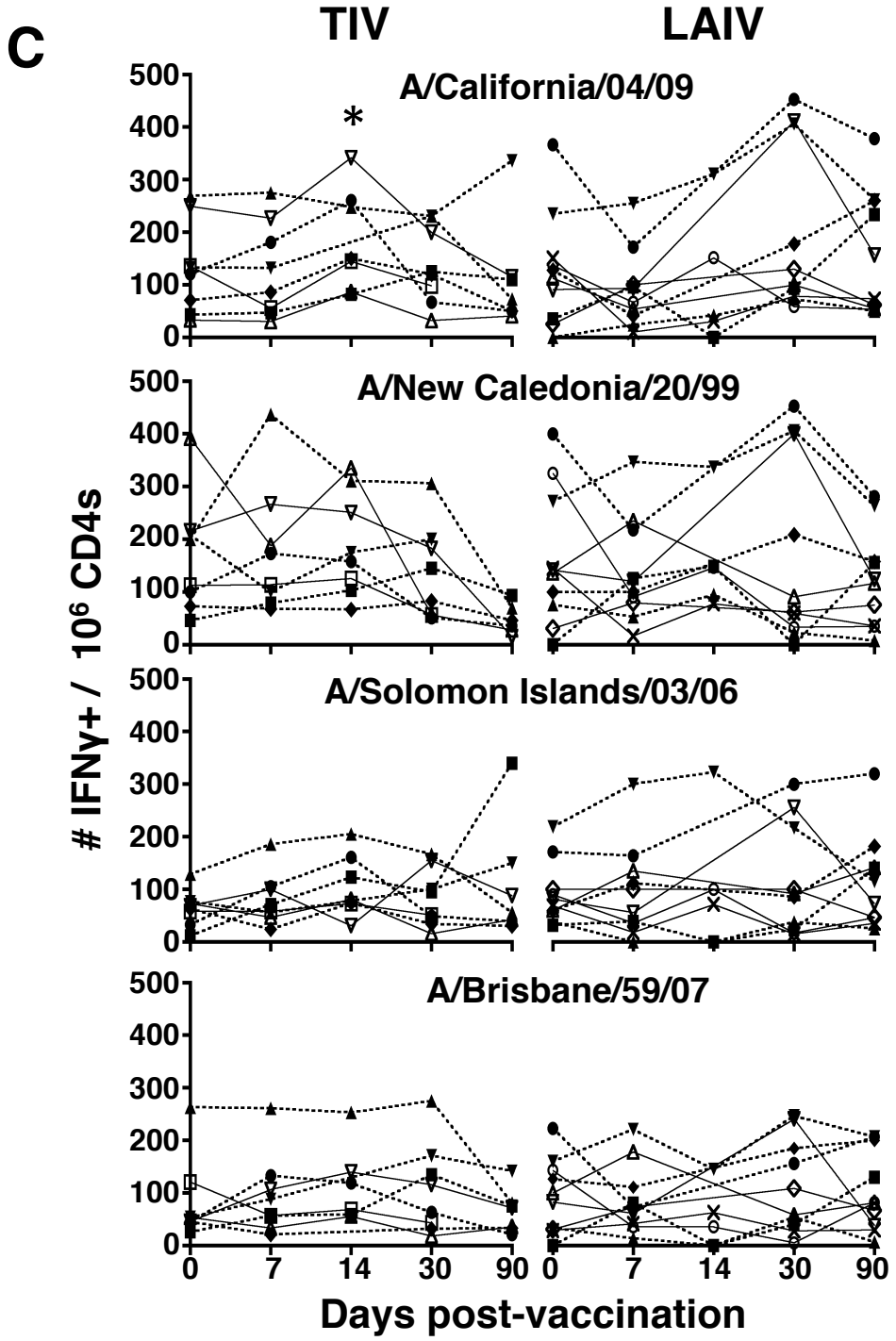
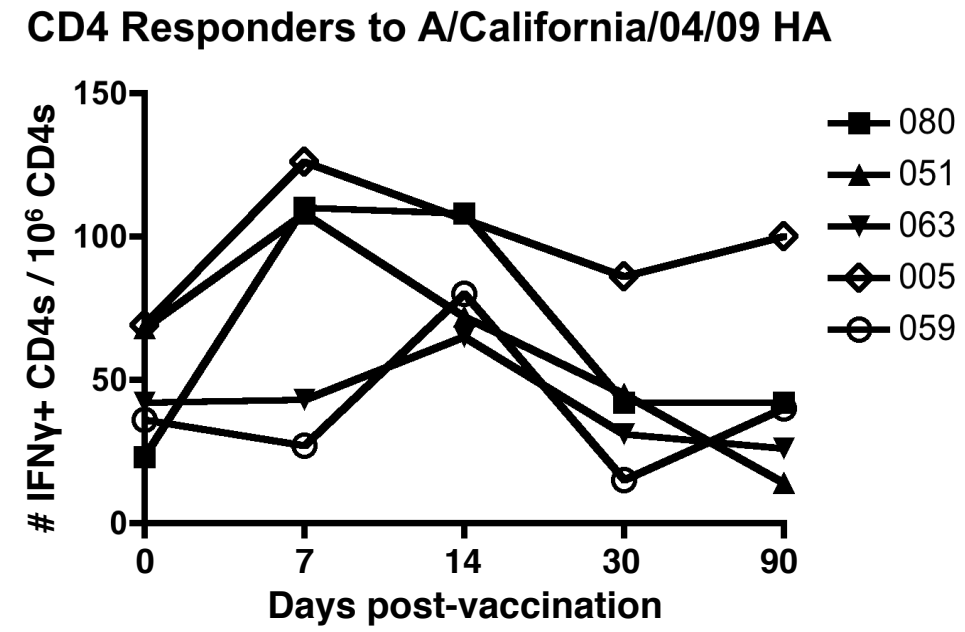

C
TIV
LAIV
A/California/04/09
A/New Caledonia/20/99
A/Solomon Islands/03/06
A/Brisbane/59/07
*
IFNγ+ / 10^6 CD4s
0
100
200
300
400
500
0
7
14
30
90
Days post-vaccination

Figure 3.3a

A


CD4 Responders to A/California/04/09 HA
IFNγ+ CD4s / 10^6 CD4s
150
100
50
0
0
7
14
30
90
Days post-vaccination
080
051
063
005
059

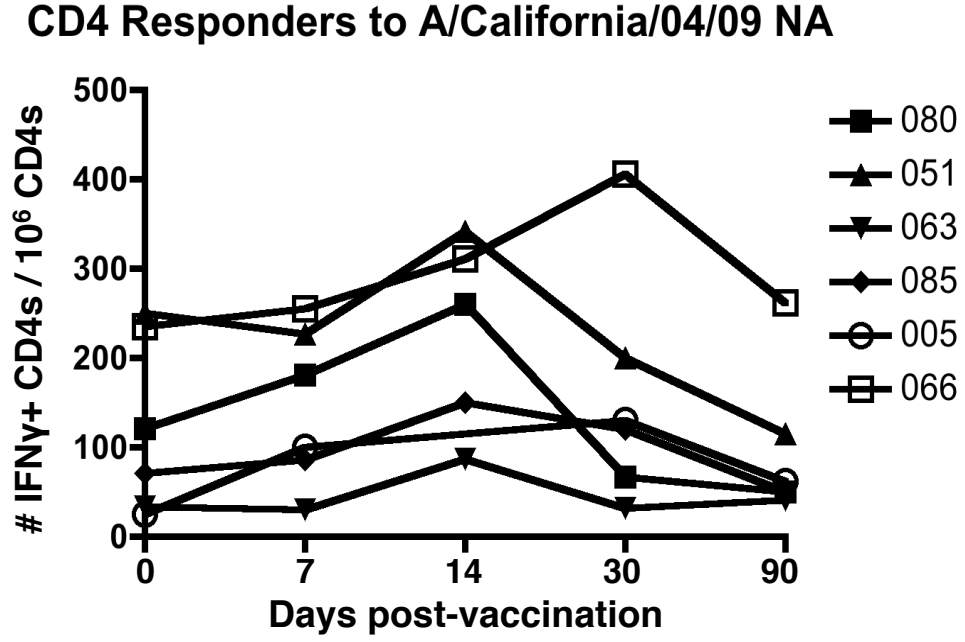

CD4 Responders to A/California/04/09 NA
IFNγ+ CD4s / 10^6 CD4s
500
400
300
200
100
0
0
7
14
30
90
Days post-vaccination
080
051
063
085
005
066

Figure 3.3b

B

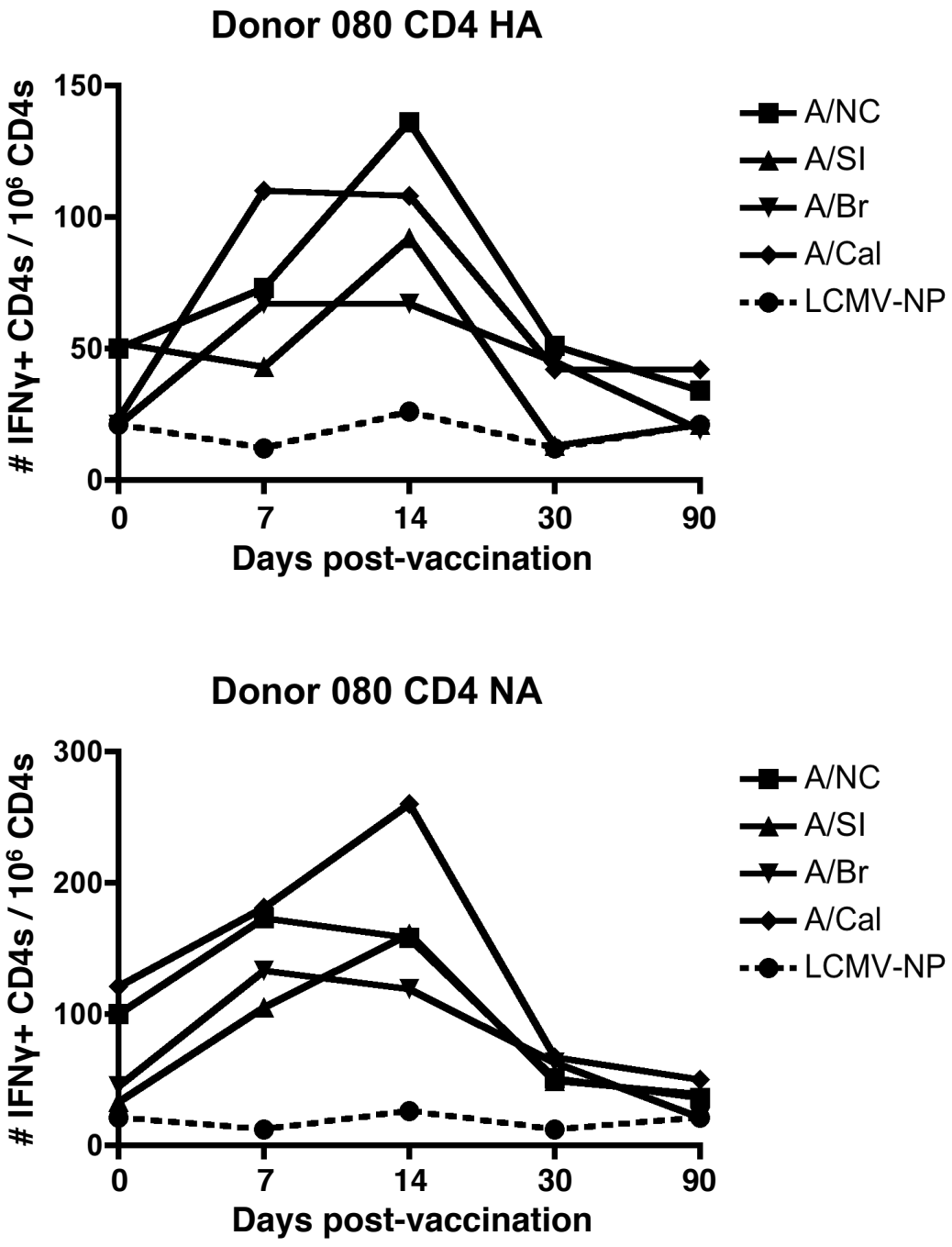

Donor 080 CD4 HA
IFNγ+ CD4s / 10^6 CD4s
150
100
50
0
0
7
14
30
90
Days post-vaccination
A/NC
A/SI
A/Br
A/Cal
LCMV-NP
Donor 080 CD4 NA
IFNγ+ CD4s / 10^6 CD4s
300
200
100
0
0
7
14
30
90
Days post-vaccination
A/NC
A/SI
A/Br
A/Cal
LCMV-NP

Figure 3.4

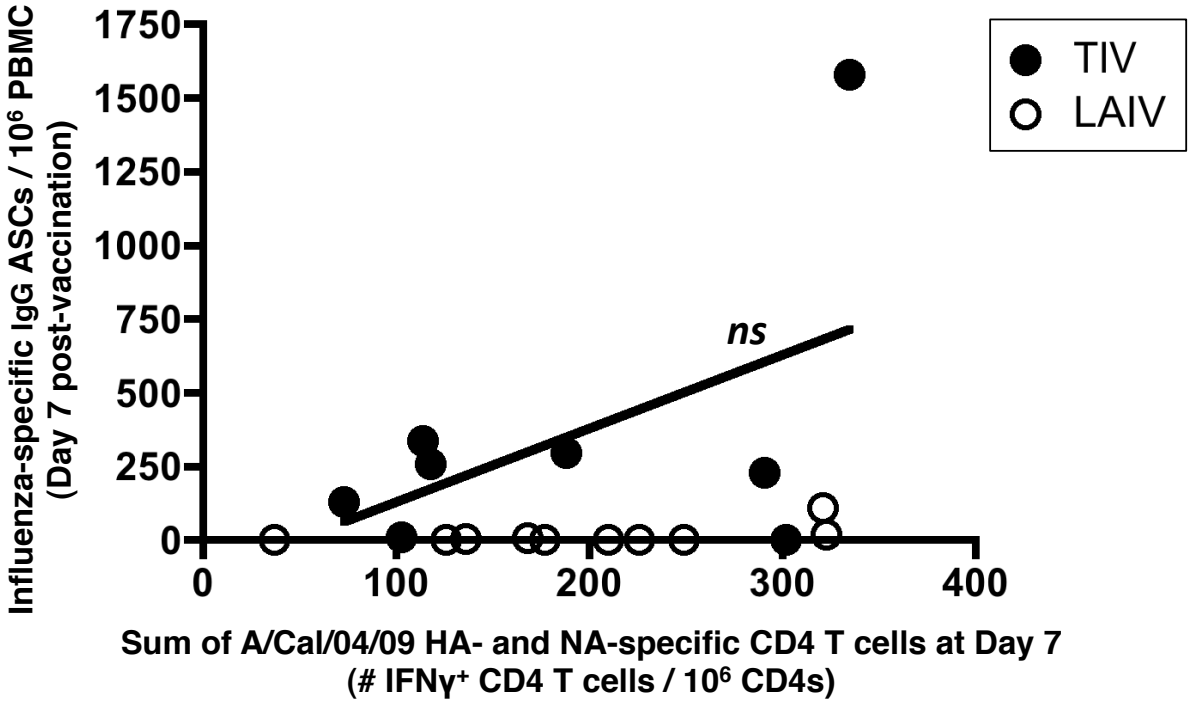

Influenza-specific IgG ASCs / 10^6 PBMC
(Day 7 post-vaccination)
1750
1500
1250
1000
750
500
250
0
0
100
200
300
400
ns
TIV
LAIV
Sum of A/Cal/04/09 HA- and NA-specific CD4 T cells at Day 7
(# IFNγ+ CD4 T cells / 10^6 CD4s)

Figure 3.5a

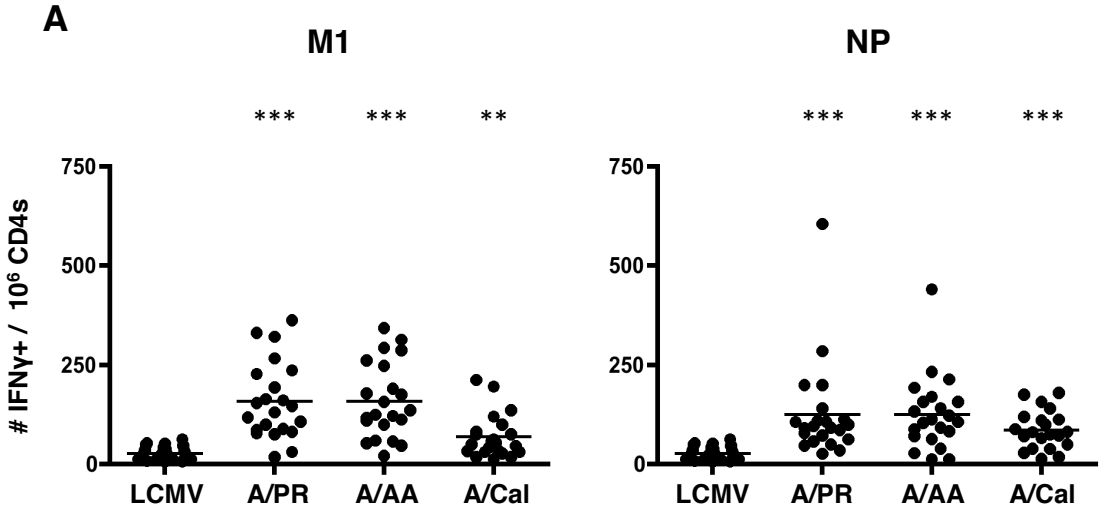


Figure 3.5b

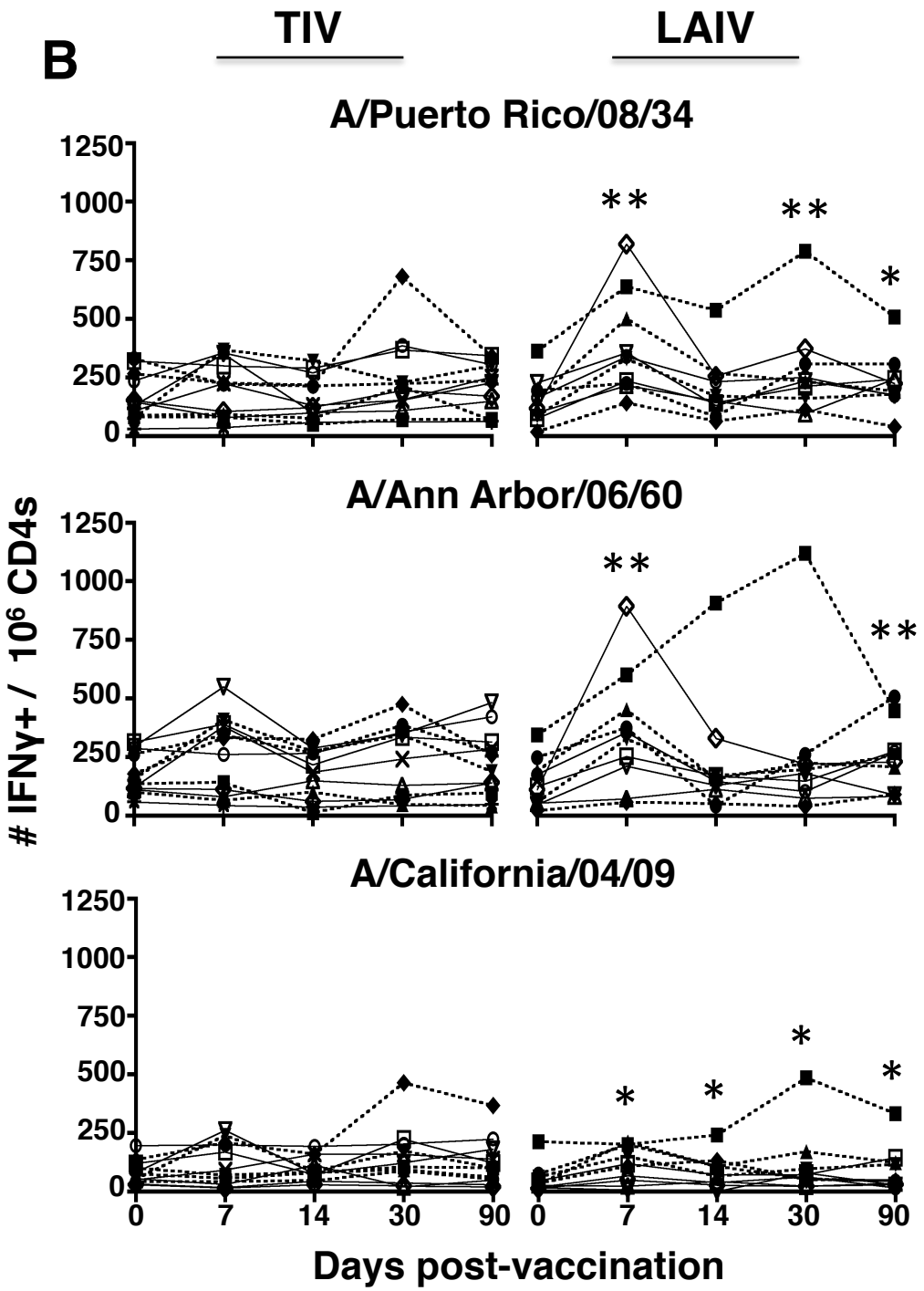

B
TIV
LAIV
A/Puerto Rico/08/34
A/Ann Arbor/06/60
A/California/04/09
IFNγ+ / 10^6 CD4s
1250
1000
750
500
250
0
**
**
*
**
**
*
*
*
0
7
14
30
90
Days post-vaccination

Figure 3.5c

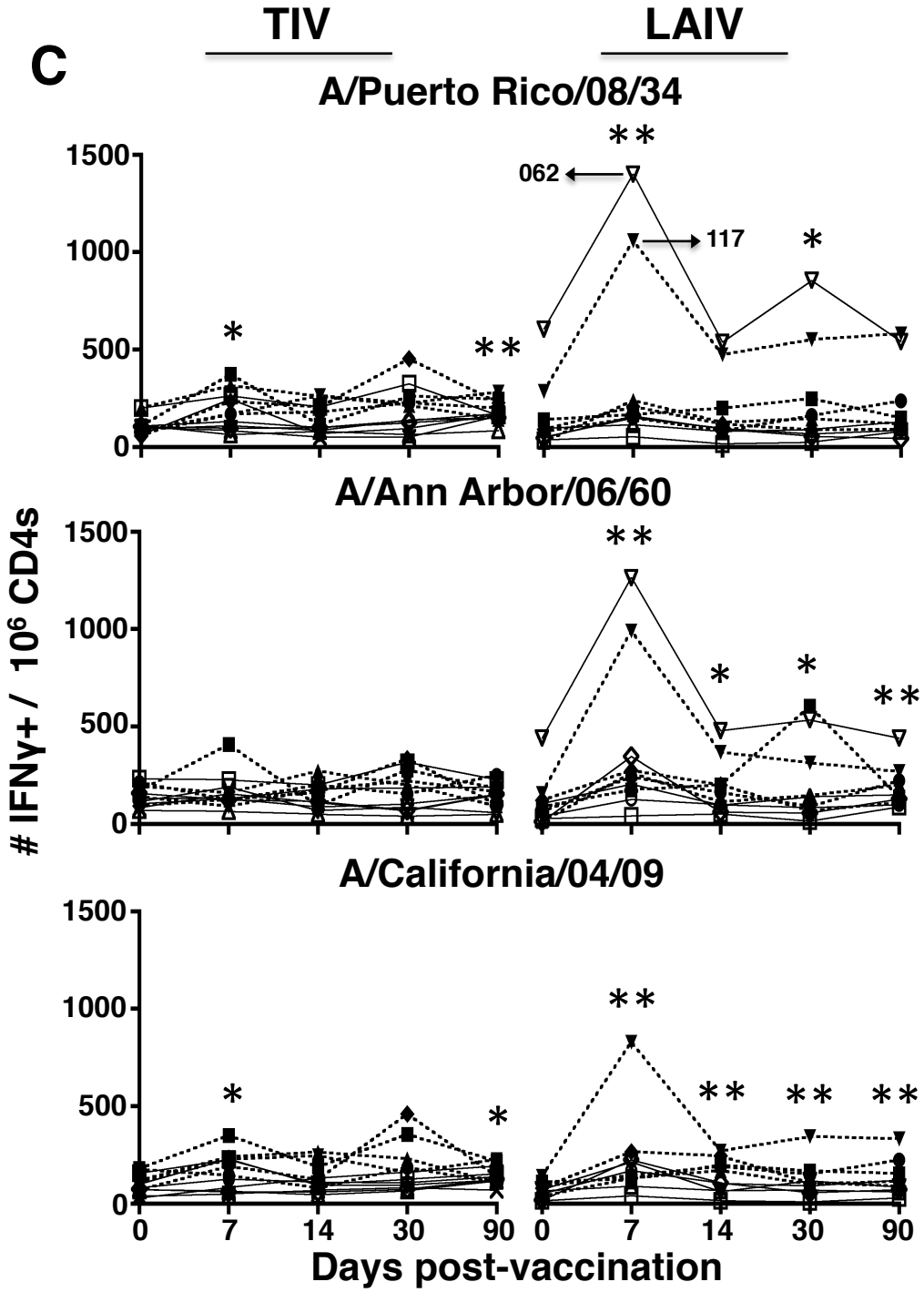

C
TIV
LAIV
A/Puerto Rico/08/34
A/Ann Arbor/06/60
A/California/04/09
062
117
IFNγ+ / 10^6 CD4s
1500
1000
500
0
0
7
14
30
90
Days post-vaccination

Figure 3.6a

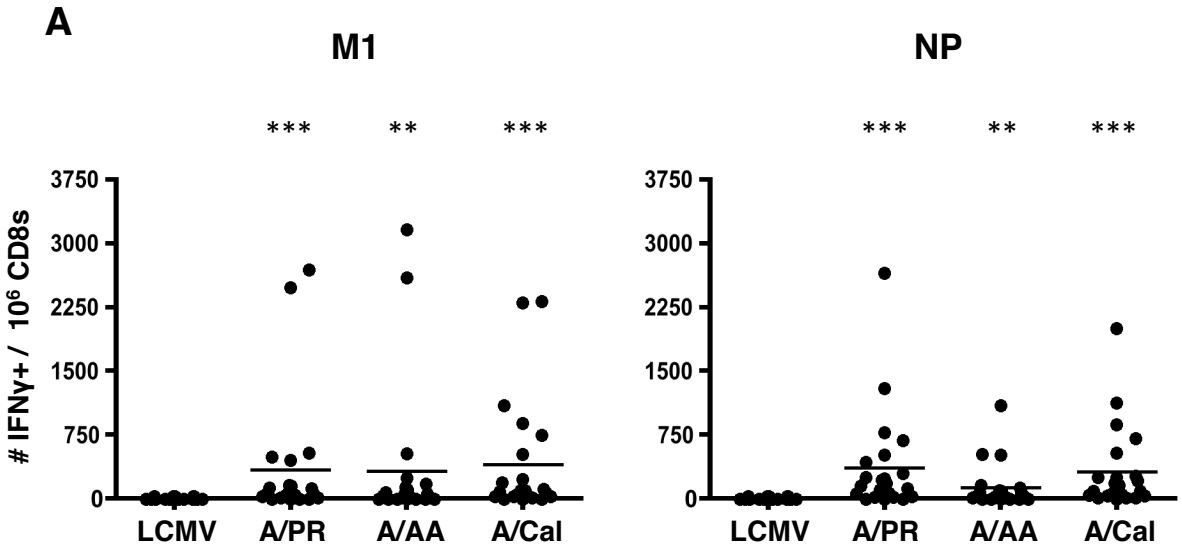

A
M1
NP
***
**
***
***
**
***
IFNγ+ / 10^6 CD8s
3750
3000
2250
1500
750
0
LCMV
A/PR
A/AA
A/Cal

Figure 3.6b

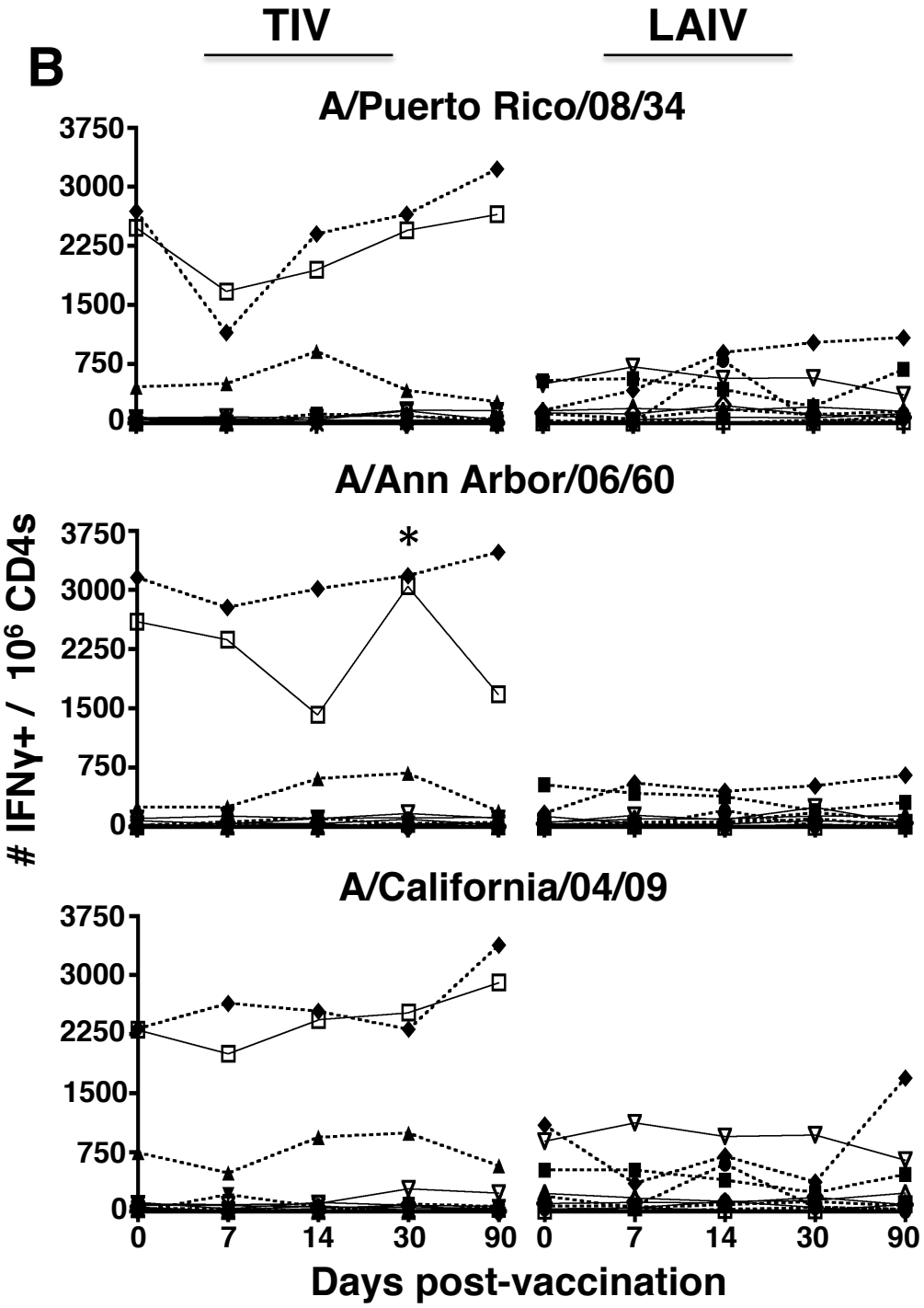

B
TIV
LAIV
A/Puerto Rico/08/34
A/Ann Arbor/06/60
A/California/04/09
*
IFNγ+ / 10^6 CD4s
3750
3000
2250
1500
750
0
0
7
14
30
90
Days post-vaccination

Figure 3.6c

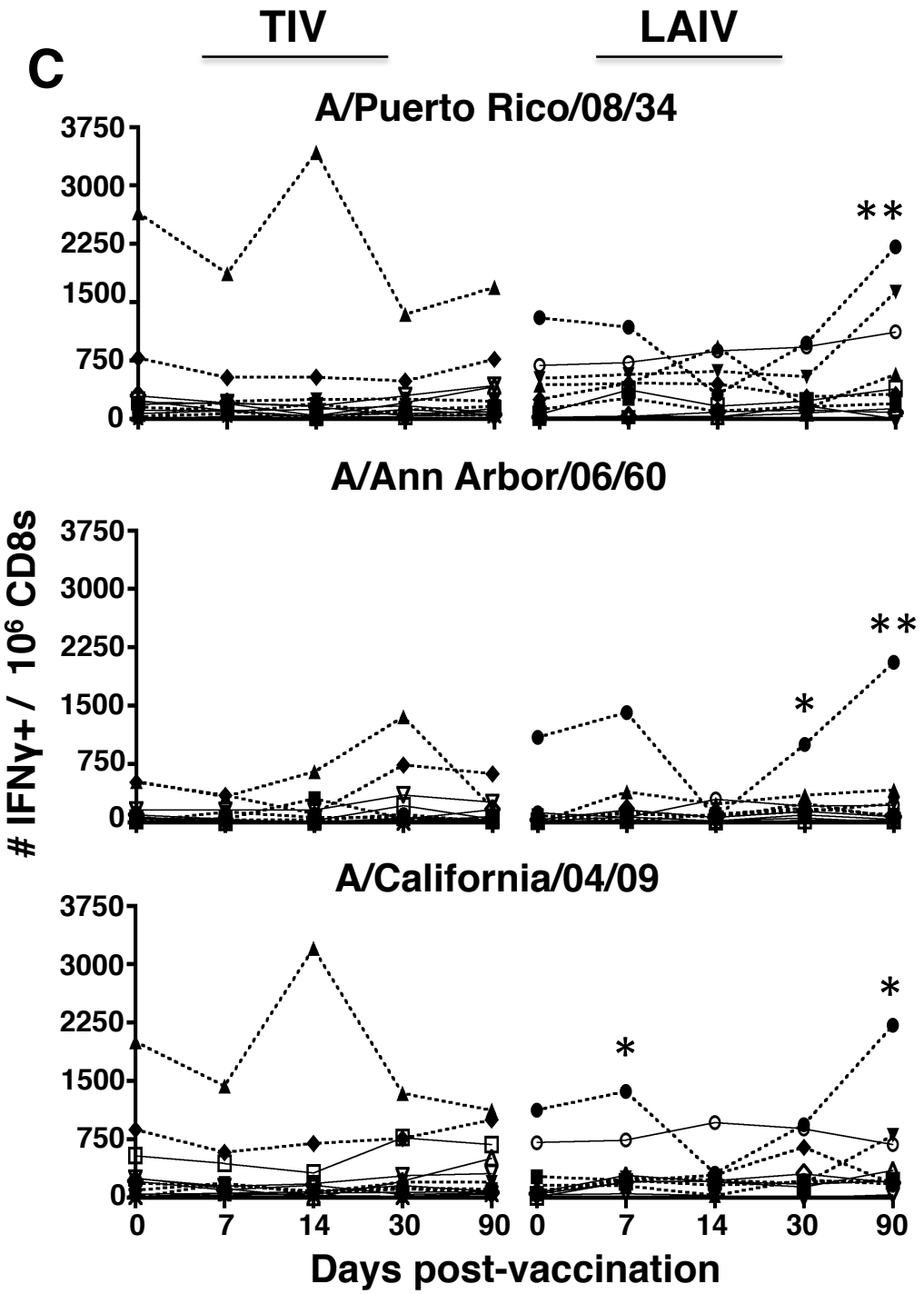

C
TIV
LAIV
A/Puerto Rico/08/34
A/Ann Arbor/06/60
A/California/04/09
IFNγ+ / 10^6 CD8s
3750
3000
2250
1500
750
0
**
*
0
7
14
30
90
Days post-vaccination

Table 1. Demographic information for influenza vaccine trial participants

Vaccine Group	Age Range	Age mean ± SD	N (male/Female)
TIV	23 - 53	37.8 ± 8.9	4 / 16
LAIV	23 - 46	34.6 ± 6.8	15 / 5

Table 2. Influenza proteins included in the our study of human T cell responses to 2010-2011 seasonal influenza vaccines

External Proteins (HA, NA)		Internal Proteins (M1, NP)		
Strain*	Vaccine Year	Strain	Subtype	Vaccine ID
A/New Caledonia/20/99	2000-07	A/Puerto Rico/08/34	H1N1	TIV
A/Solomon Islands/03/06	2007-08	A/Ann Arbor/06/60	H2N2	LAIV
A/Brisbane/59/07	2008-10	A/California/04/09	H1N1	-----
A/California/04/09	2010-11			

***** All external proteins are derived from the influenza A H1N1 subtype.

Table 3. Analysis of human vaccine-associated CD4 T cell responses to external influenza proteins via mixed linear model

	HA				NA				
	Day	Estimate of response*	(Time point - baseline)	*P* value	Day	Estimate of response	(Time point - baseline)	*P* value	
TIV	0	56			0	132			A/Cal/4/09
	7	61	5	0.7489	7	129	-3	0.8477	
	14	64	8	0.5615	14	188	56	0.0199	
LAIV	0	112			0	127			
	7	119	7	0.8708	7	88	-39	0.1517	
	14	28	-84	0.0258	14	115	-12	0.6531	
TIV	0	90			0	169			A/NC/20/99
	7	91	1	0.9213	7	178	9	0.8369	
	14	129	39	0.0024	14	191	22	0.2810	
LAIV	0	81			0	174			
	7	109	28	0.5423	7	142	-32	0.3889	
	14	95	14	0.7247	14	173	-1	0.9884	
TIV	0	46			0	64			A/SI/3/06
	7	73	27	0.1783	7	81	17	0.3122	
	14	60	14	0.3328	14	103	39	0.1202	
LAIV	0	107			0	119			
	7	62	-45	0.0562	7	101	-18	0.6208	
	14	63	-44	0.0538	14	128	9	0.8453	
TIV	0	49			0	82			A/Br/59/07
	7	88	39	0.1923	7	95	13	0.4856	
	14	111	62	0.1721	14	104	22	0.2347	
LAIV	0	102			0	111			
	7	87	-15	0.4580	7	103	-8	0.7371	
	14	69	-33	0.3713	14	71	-40	0.0659	

***** Response estimates are reported as the number of IFNγ^{+} cells / 10^{6} CD4 T cells.

Table 4. Analysis of human vaccine-associated CD4 T cell responses to internal influenza proteins via mixed linear model

	M1				NP			
	Day	Estimate of response*	(Time point - baseline)	*P* value	Day	Estimate of response	(Time point - baseline)	*P* value
TIV	0	164			0	109		
	7	204	40	0.2822	7	200	91	0.0142
	14	162	-2	0.9364	14	143	34	0.1407
LAIV	0	151			0	145		
	7	381	***230***	***0.0032***	7	370	***225***	***0.0394***
	14	203	52	0.1018	14	182	37	0.1312
TIV	0	178			0	142		
	7	259	81	0.0374	7	161	19	0.4590
	14	182	4	0.8799	14	143	14	0.9553
LAIV	0	137			0	107		
	7	360	***223***	***0.0083***	7	393	***286***	***0.0129***
	14	222	85	0.2136	14	183	76	0.0025
TIV	0	81			0	101		
	7	117	36	0.1316	7	167	66	0.0048
	14	95	14	0.2477	14	126	25	0.2565
LAIV	0	54			0	68		
	7	114	60	0.0126	7	224	***156***	***0.0318***
	14	84	30	0.0192	14	143	75	0.0031

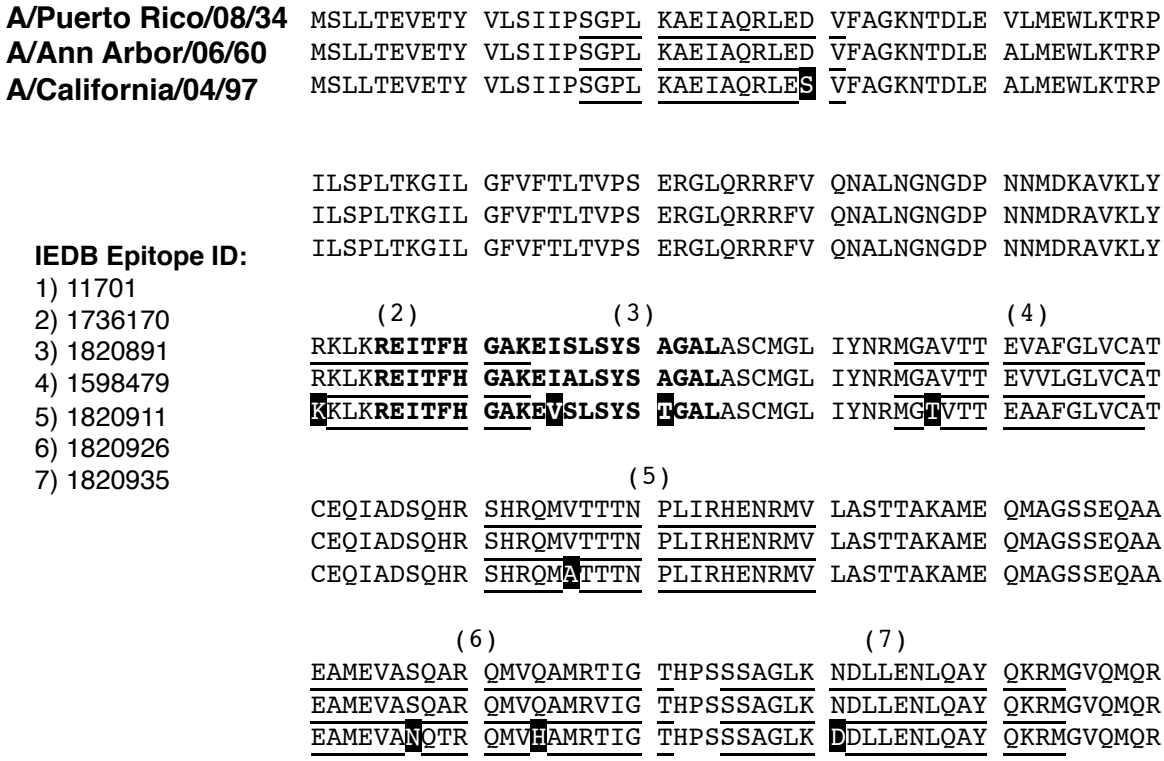
***** Response estimates are reported as the number of IFNγ^{+} cells / 10^{6} CD4 T cells

Table 5. Analysis of human vaccine-associated CD8 T cell responses to internal influenza proteins via mixed linear model

	M1				NP			
	Day	Estimate of response*	(Time point - baseline)	*P* value	Day	Estimate of response	(Time point - baseline)	*P* value
TIV	0	499			0	395		
	7	296	-203	0.1694	7	314	-81	0.2822
	14	471	-28	0.6834	14	417	22	0.7911
LAIV	0	166			0	342		
	7	217	51	0.1560	7	411	69	0.1100
	14	335	169	0.1165	14	358	16	0.8995
TIV	0	522			0	130		
	7	478	-44	0.2624	7	99	-31	0.2619
	14	455	-67	0.5385	14	113	-17	0.7083
LAIV	0	101			0	146		
	7	142	41	0.3526	7	241	95	0.0781
	14	138	37	0.3912	14	96	-50	0.6638
TIV	0	486			0	375		
	7	466	-20	0.6952	7	292	-83	0.1693
	14	531	45	0.0987	14	411	36	0.7487
LAIV	0	315			0	256		
	7	252	-63	0.4561	7	371	***115***	***0.0066***
	14	315	0	0.9986	14	265	9	0.9270

***** Response estimates are reported as the number of $IFN\gamma^+$ cells / 10^6 CD8 T cells.

Supplementary Data 3.1. Sequence alignments of M1 from A/PR/08/34, A/AA/06/60, and A/Cal/04/09



Chapter 4: Discussion

Investigations of human influenza-specific T cell responses, which are typically performed in PBMC, are complicated, due to low frequencies of circulating influenza-specific T cells. To address this issue, we developed an rVSV-based quantitative T cell assay that was used to characterize human T cell responses to 2010-2011 influenza vaccines. The rVSV assay was also effective in detecting T cell responses to yellow fever vaccines (YFV-17D) and immunodominant epitopes, such as CMVpp65. This system is ideal for the evaluation of essentially any vaccine candidate designed to elicit T cell responses, and it represents an effective method for mapping immunodominant T cell epitopes at the gene level. A primary advantage of rVSV vectors is that they are inexpensive, allowing investigators to map epitopes to large viruses and bacteria or conduct extensive vaccine trial analysis that may not be cost-effective using overlapping peptide pools as an antigen source. Additionally, our data suggest that APCs, particularly monocytes, are infected with rVSV vectors, which would result in natural processing and presentation of proteins expressed by rVSV constructs. Conversely, overlapping peptide pools bind directly to surface MHC molecules and are not subject to natural processing. This raises the possibility that T cell responses observed following stimulation with overlapping peptide pools are artificially increased, because they are partially driven by the recognition of epitopes that are not normally presented during viral infection. We also showed that rVSV vectors are capable of inducing T cell proliferation *in vitro*. Culturing CFSE-labeled PBMC with rVSV vectors induced the proliferation of

CD4 and CD8 T cells specific for CMVpp65 as well as CD8 T cells specific for influenza proteins. This application of the rVSV system provides an additional method for assaying antigen-specific T cells and may be particularly useful for detecting low-frequency populations.

Our studies of human T cell responses to 2010-2011 seasonal influenza vaccines reveal three main points: First, both TIV and LAIV were poor inducers of T cell responses specific for external H1N1 proteins. We did note the boosting of cross-reactive CD4 T cell populations across multiple strains, but these responses were typically weak and occurred in only selected donors. Second, LAIV was more effective than TIV in boosting pre-existing CD4 T cell responses specific for internal proteins, M1 and NP. These responses were short-lived, peaking at day 7 post-vaccination and essentially returning to baseline by day 14. Third, neither TIV nor LAIV effectively increased baseline CD8 T cell levels specific for external or internal proteins. These findings represent a significant advance in our understanding of human T cell responses to seasonal influenza vaccination and raise questions about whether conventional vaccine strategies may be suitable to realize our goal of generating long-lasting and effective T cell immunity to influenza.

Given that current seasonal influenza vaccine formulations do not induce robust T cell responses, it will likely be essential to incorporate new technologies into seasonal vaccination approaches in order to achieve T cell levels capable of

providing a greater contribution to protection. There are numerous putative T cell adjuvants and alternative vaccine delivery systems that have shown promise. In one report, priming of CD8 T cells with influenza NP peptide and a combination of aluminum salts and monophosphoryl lipid A established protective immunity in mice (181). This study is significant, because it suggests immunogenic potential for two adjuvants that are currently licensed for use in humans in the United States. Additional vaccine adjuvants, such as α-galactosylceramide analogs, TLR 3 or TLR 9 agonists complexed with cationic lipids, and sucrose fatty acid sulfate esters have demonstrated efficacy in enhancing T cell responses to vaccination in animal models (182-185). In humans, two candidate adjuvants have been the subject of recent vaccine trials. AS03 is a tocopherol oil-in-water emulsion that boosts vaccine-associated CD4 T cell responses in adults and children following 2009 H1N1 and H5N1 vaccination (186-188). Another oil-in-water emulsion, MF59, has been shown to enhance post-vaccination influenza-specific CD4 T cell levels in both adults and children (189,190). Alternative vaccine delivery methods have also been investigated in human trials. Vaccination of healthy adults with plasmid DNA encoding H5 HA generated influenza-specific T cell responses that persisted for more than 180 days following vaccination (191). Formulations of conventional influenza vaccines with immunostimulating complexes (ISCOMs) or liposomes have been tested in adult and elderly populations and were reported to improve CD8 T cell responses to vaccination (192,193). Taken together, these results indicate that improved influenza vaccine

methodologies may compensate for the minimal T cell boosting capability of current seasonal influenza vaccines.

Increasing influenza-specific T cell responses to vaccination may also be accomplished by viral vector-based approaches, such as modified vaccinia virus Ankara (MVA) or adenovirus (Ad). In human vaccine trials and preclinical studies, immunization of human donors with MVA vectors expressing various HIV proteins stimulated the expansion of antigen-specific CD4 and CD8 T cells (194-197). These responses were improved when MVA was part of a heterologous prime/boost regimen that included DNA vaccines or fowlpox vectors. Additionally, vaccine-associated T cell responses were observed following immunization with MVA vectors expressing malaria proteins, and effective boosting of TB-specific CD4 T cells was noted following administration of TB antigen-expressing MVA vectors (198-200). In one human trial, influenza M1 and NP-encoding MVA vaccines boosted influenza-specific CD4 and CD8 T cell responses (201). Reported post-vaccination influenza-specific CD4 T cell responses were comparable with our data from TIV- or LAIV-vaccinated individuals, but in contrast with our results, vaccine-associated influenza-specific CD8 T cell responses were seen in most trial participants. Ad vectors have also been shown to induce antigen-specific CD4 and CD8 T cell responses following vaccination. Most of these trials have been conducted with Ad vectors that encode HIV proteins (202-205), although Ad vectors have demonstrated efficacy in inducing ebola- and hepatitis C-specific T cell responses in humans and chimpanzees,

respectively (206,207). These viral vector-based vaccines are generally well-tolerated in humans and represent attractive candidates for the next generation of influenza vaccines.

No T cell correlate of protection has been established for influenza virus in humans, and this subject has been the focus of little investigation. In recent vaccine trials in the elderly, poor post-vaccination T cell-associated granzyme B (GrzB) responses were observed in participants who later developed clinically-diagnosed infection (170,208). Conversely, post-vaccination antibody responses could not be used to distinguish between uninfected and subsequently infected donors, demonstrating that vaccine-induced T cell responses may be more suitable than post-vaccination antibody levels as predictors of disease susceptibility in older populations. It would be informative to repeat these trials in adult populations with a more extensive analysis of GrzB production by influenza-specific T cells. If there is a protective T cell correlate associated with the production of GrzB, it may be possible to determine which T cell subsets and influenza protein specificities drive these responses. This could be accomplished by incorporating flow cytometry reagents designed to detect cytolytic molecules into the rVSV system. However, as the above studies point out, there are a couple of factors that may complicate this approach. First, commercially available flow cytometry reagents do not differentiate between active and inactive forms of GrzB in assayable cells. This issue can be addressed by adding reagents for detecting T cell activation markers into flow panels in order to exclude the

measurement of cytolytic molecules contained within non-specific T cells. Second, natural killer (NK) cells also contain cytolytic proteins that are identified by common flow cytometry antibodies, and reagents should be included to account for the innate production of these proteins by NK cells (208,209). Despite these considerations, the rVSV system and similar methods could potentially be used to identify influenza-specific T cell-based correlates of protection and provide new predictors of seasonal vaccine efficacy.

Studies of vaccine-induced influenza-specific T cell immunity in humans are difficult, because they are typically limited to peripheral blood lymphocyte populations. Circulating T cell populations may not accurately predict responses at major sites of viral replication or immune induction, such as the lungs or draining lymph nodes. For example, follicular helper cells (T_{FH} cells) are a subset of CD4 T cells that represent a large proportion of CD4 T cells found in human secondary lymphoid organs (210). These cells express high levels of CXCR5, a well-established chemokine receptor that is involved in B cell migration to lymphoid follicles prior to germinal center formation, and *in vitro* studies of human T_{FH} cells suggest that they provide increased B cell help, making T_{FH} cells an obvious area of interest to vaccine developers (211-213). Even though T_{FH} cells have been observed in peripheral blood samples, investigations human T cell responses in PBMC are not appropriate for exploring T_{FH} cells and B cell interactions that occur in lymphoid organs. Therefore, it is important to refrain from liberal extrapolation of T cell data derived from PBMC. We must also be

careful not to conclude that seasonal influenza vaccines provide no benefit to T cell immunity simply due to the observed paucity of circulating influenza-specific T cell populations following vaccination. However, when comparing the levels of circulating antigen-specific T cells following seasonal influenza vaccination with those induced by yellow fever virus (YFV-17D) administration or smallpox vaccination (Dryvax), a significant disparity is revealed (214-216). YFV-17D and Dryvax are live, attenuated vectors, like LAIV, and they are generally regarded as two of the most effective human vaccines. At the peak of the post-vaccination response, YFV-17D- and Dryvax-reactive CD8 T cells have been reported to account for up to 12.5% and 40% of total circulating CD8 T cells, respectively. Although these investigations were performed using overlapping peptide pools as opposed to viral vectors for antigen delivery, these frequencies are multiple orders of magnitude higher than CD8 T cell responses observed following seasonal influenza vaccination in our study. It should be noted that YFV-17D and Dryvax are commonly administered to individuals with no pre-existing immunity, and these vaccines have been shown to cause systemic infection with detectable viremia (216,217). These factors probably contribute to the increased immunogenicity of these vaccines. The extent to which robust T cell responses to yellow fever and smallpox vaccination contribute to overall protection is unknown, but the magnitude of these responses would imply that they are capable of some measure of viral control. These studies suggest that the T cell-boosting potential of seasonal influenza vaccines may need to be increased considerably in order to establish long-lasting, protective T cell immunity to influenza.

Augmenting CD4 T cell help for influenza-specific CD8 T cell populations and antibody-producing B cells in human seasonal vaccine recipients is an additional area of interest that is poorly understood. If mouse models are any indication, vaccine-induced influenza-specific CD4 T cell populations may play a significant role in the generation of robust antibody responses and deliver critical signals that drive the activation of effector CD8 T cell populations (120,121). Currently, the relationship between influenza-specific T cell responses and humoral immunity in seasonal vaccination has not been fully explored. Recent studies failed to identify a correlation between pre-existing H1N1 HA-specific T cell responses and seasonal H1N1 hemagglutination inhibition assay (HAI) titers (218). Furthermore, in a trial of 2007-2008 seasonal influenza vaccine recipients, vaccination resulted in the boosting of H3N2 HA-specific T cells in several donors; however, there was no observed statistical correlation between vaccine-associated B and T cell responses (96). To our knowledge, there are no reports comparing vaccine-induced influenza-specific T cell populations with ASC or memory B cell responses. In our analysis, we observed no obvious correlation between influenza-specific IgG ASC and CD4 T cell responses in 2010-2011 seasonal vaccine recipients, although it must be stated that we did not have enough donors to conduct a comprehensive comparison. Additional investigations are clearly needed to fully explore the relationship between B and T cell immunity in influenza infection.

Multiple investigations, including our own, have demonstrated that LAIV is more effective than TIV in stimulating influenza-specific CD4 T cell responses (96,142). The reasons for this observation are unknown, although it has been proposed that LAIV, which is delivered to the respiratory tract via intranasal administration, replicates in an environment that is more conducive to the initiation of influenza-specific T cell responses than in the lymph nodes that drain intramuscularly injected TIV antigen (143). Regardless of the reasons for enhanced boosting of influenza-specific T cell responses by LAIV, human studies suggest that this effect does not translate into superior B cell responses. For example, in vaccine trials comparing TIV and LAIV in adults and children, TIV induced greater IgG ASC responses than LAIV in adults. No significant difference in IgG ASC responses was noted in children vaccinated with either vaccine, possibly because low pre-existing influenza immunity in children enhances the replicative potential of LAIV (219). In both age groups, IgG and IgA memory B cell responses were found to be more robust following TIV vaccination (95). Additionally, in recent reports involving the elderly and military recruits, donors immunized with TIV displayed higher serum antibody levels than LAIV-vaccinated individuals (96-98). These studies raise the question of whether the generation of influenza-specific T cell immunity is beneficial to vaccinated individuals.

To conclude, the ultimate goal of this study was to conduct a comprehensive characterization of human T cell responses to seasonal influenza

vaccines. To accomplish this goal, we first developed an inexpensive, yet efficient, T cell assay that measures direct *ex vivo* CD4 and CD8 T cell responses specific for multiple influenza proteins across various strains. We applied this novel method to characterize human T cell responses to 2010-2011 seasonal influenza vaccines. Based on our findings, we propose that conventional vaccine approaches are potentially inadequate to induce protective T cell immunity in humans. Additionally, It is likely that incorporating “universal” cross-reactive T cell epitope approaches into current formulations will also be inefficacious. Our studies support this view, because although we observed a significant boost in cross-reactive T cell populations following influenza vaccination in many donors, vaccine-associated effects were often minimal, especially when compared to T cell responses induced by more successful human vaccines, such as YFV-17D and Dryvax. Our investigation points to the need for developing and implementing new methodologies into current influenza vaccine strategies if we are to realize the goal of enhancing influenza vaccination by improving T cell immunity.

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