University of Alberta

Investigations of Influenza Vaccination in Kidney & Lung Transplant Populations

by

Amber Dawne Bergeron

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master's of Science

in

Experimental Medicine

Department of Medicine

Spring 2010

Edmonton, Alberta

Examining Committee

Dr. Deepali Kumar, Medicine

Dr. Jutta Preiksaitis, Infectious Diseases

Dr. Atul Humar, Medicine

Dr. Patricia Campbell, Nephrology

Dr. Lori West, Pediatrics

Abstract

These two studies investigate the immune responses of lung and kidney transplant recipients to the influenza vaccine. The study involving kidney transplant recipients developed a novel flow cytometry assay to measure cell-mediated immunity in response to influenza vaccination. The activation of T-cells was assessed through the change in T-cell production of interferon gamma after vaccination. In lung transplant recipients, the study examined the formation of de novo anti-HLA antibodies following influenza vaccination. Anti-HLA antibodies were classified as donor-specific or not. The study in kidney transplant recipients found that the influenza vaccine is effective at stimulating the immune response and producing long-lived memory in these patients, as evidenced by high baseline T-cell activity. The study of lung transplant recipients found that receiving the influenza vaccine did not result in the production of anti-HLA antibodies. Both studies found vaccine to be safe for use in these populations.

Acknowledgements

First of all thank you to my supervisor Dr. Deepali Kumar for taking me on as a student, providing me with two really interesting projects and supporting me throughout this endeavour. I would like to thank all of my Master's committee for all of the help and support I have received throughout this degree: Drs. Jutta Preiksaitis, Atul Humar, Patricia Campbell, Luis Hidalgo and Lilly Pang. Thank you to Drs. Luis Hidalgo and Shaun Wasilenko for their invaluable technical insight and support. Thank you to Kimberly Martin and Matthew McEwan for their help and support in this long project. Finally, I would like to thank Ronald Butler and my family for their support, love and belief in me.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A/Aichi – Influenza Virus Strain A/Aichi/2/68

A/PR – Influenza Virus Strain A/PR/8/34

APC – Antigen Presenting Cell

B-cells – B Lymphocyte

B/Lee – Influenza Virus Strain B/Lee/40

BOS - Bronchiolitis Obliterans Syndrome

$CD4^{+}/8^{+}$ T-cells – T Lymphocyte with CD4/8 present

CDC – Centers for Disease Control and Prevention

CMI – Cell Mediated Immunity

CNI – Calcineurin Inhibitor

cRNA – copy RNA

DSA – Donor Specific Antigen

ELISA – Enzyme-Linked Immunosobent Assay

ELISpot - Enzyme-Linked Immunosobent Spot

FasL – Fas Ligand

FITC – Fluorescein Isothcyanate

FPRA – Flow Panel Reactive Antibody

HA – Hemagglutinin

HAU – Hemagglutinin Unit

HEF – Hemagglutinin-Esterase-Fusion Glycoprotein

HD – High Definition

HLA – Human Leukocyte Antigen

HV – Healthy Volunteer

i.d. – Intradermal injection

Ig – Immunoglobulin

IL-2/10 – Interleukin two/ten

i.m. – Intramuscular Injection

IMPDH – Inosine Monophosphate dehydrogenase

IFN-α/β/γ – Interferon alpha/beta/gamma

ISCOM – Immune Stimulating Complex

KTx – Kidney Transplant

LTx – Lung Transplant

M2 – Influenza Ion Channel

MHC – Major Histocompatibility Complex

MMF – Mycophenolate Mofetil

MOI – Multiplicity of Infection

mRNA – messenger RNA

NA – Neuraminidase

NACI – National Advisory Committee on Immunization

OB – Bronchiolitis Obliterans/Obliterative Bronchiolitis

OD – Optical Density

PBMC – Peripheral Blood Mononuclear Cell

PHA – Phytohemagglutinin A

RBC – Red Blood Cell

T-cells – T Lymphocytes

T_C cell – Cytotoxic T-cell

T_{CM} cell – Central Memory T-cell

T_{EM} cell – Effector Memory T-cell

T_H cell – Helper T-cell

T_H1 cells – T_H cells specific to the cytotoxic response

T_H2 cells – T_H cells specific to the humoral response

TRC – T-cell Receptor

Vp – Virus Particle

WHO – World Health Organisation

Overview Introduction

Influenza

The family *Orthomyxoviridae* consists five genera of RNA viruses of which influenza viruses comprise three of the five genera [1]. Each of the three genera of influenza viruses contains only a single species of influenza; genus Influenzavirus A contains the species influenza A virus, Influenzavirus B contains the species influenza B virus and Influenzavirus C contains the species influenza C virus. While these viruses do share a common ancestry as determined by genomic sequencing, the divergent evolution of each virus has left them unable to share genomic RNA segments between species through genetic reassortment [1]. Influenza virus particles are compositionally similar between the three viruses and are characterised by the segmentation of the viral negative-sense, single-stranded RNA genomes into seven or eight pieces [1, 2]. In general the viruses consist of the segmented genome surrounded by a viral protein matrix which is enveloped in a lipid bilayer obtained from the infected cell [1]. The lipid layer is studded with glycoproteins that are used by the virus in attachment and invasion of target cells [1]. The different glycoproteins and matrix proteins and the structure of the virus particles on cell surfaces are how the different species are distinguished microscopically [1, 2]. The influenza A viruses have three proteins embedded in the lipid bilayer. There are two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are found in a four to one

ratio, and one matrix protein that functions as an ion channel (M2) [1, 2]. The M2 protein is found in an approximate ratio with HA of one to 10^1 to 10^2[1]. The lipid envelope sits on top of a matrix made of another matrix protein called M1 which encases the virion core [1]. The virion core consists of the nuclear export protein and the eight-part segmented RNA genome, each segment coated in nucleoprotein and bound to a heterotrimeric RNA-dependent polymerase [1]. Influenza B has a similar makeup but there is a different set of proteins embedded in the lipid bilayer; both NA and HA are present but instead of M2, influenza B viruses have NB and BM2 proteins [1]. Influenza C viruses on the other hand, have only two proteins embedded in the lipid bilayer. There is the hemagglutinin-esterase-fusion (HEF) glycoprotein, that is the functional equivalent of HA and NA, and the CM2 protein [1, 2]. The genome of influenza C viruses is also segmented into seven not eight pieces like influenza A and B [1, 2]. Another difference of the three viruses is the range of hosts infected [2]. Influenza B and C viruses have been found to be almost exclusive human pathogens (these viruses have also been recovered from seals, pigs, dogs) [2]. In contrast, influenza A viruses have been found to infect a wide variety of warm blooded animals including mammals other than humans [2]. Research and genetic sequencing indicate that aquatic birds, ducks in particular, are the reservoir for all influenza A viruses [2, 3].

Influenza is transmitted between humans by the inhalation of airborne droplets containing infectious virus particles or possibly by indirect contact of

infected surfaces leading to self-infection of the respiratory tract or conjunctival mucosa [4]. Once inside a host the influenza virus goes through its replication cycle which consists of six steps: attachment, entry, synthesis of positive-sense viral RNA, synthesis of new viral proteins and genomes, packaging and assembly of new viral particles and budding and release of new viral particles. The first step is the attachment of the virus to the host cell surface; this is mediated by the HA spikes (or HEF in influenza C) on the virus [1]. Hemagglutinin binds to the *N*-acetylneuraminic (sialic) acid moiety that is found at the terminus of many glycosylated proteins and carbohydrates [1]. The binding of HA to sialic acid has some specificity as some HA recognise the α-2,3-linkage while others the α-2,6-linkage [1, 4]. Both linkages can be found in the lungs of humans but with site specificity; the α-2,6-linkage is the predominate linkage in tracheal and respiratory epithelium; in small amounts the α-2,3-linkage can also be found on the surface of respiratory epithelial cells in the bronchioles and alveoli of the lower respiratory tract [1, 4]. Human influenza viruses specifically recognise the α-2,6-linkage and avian viruses the α-2,3-linkage [1]. After attachment, the virus subverts the endocytosis pathway to gain entry to the target cell [1]. Once endocytosed, the endosome is acidified as part of the cell's normal process and which leads to uncoating of the virus and release of the RNA segments into the cytoplasm [1]. Once inside the cytoplasm, the viral RNA traffic to the nucleus and undergo transcription by the virus' RNA-dependent polymerase to produce positive-sense strands used in the production of viral proteins (mRNA) and new

copies of the negative-sense viral genome (cRNA) [1]. The mRNA leaves the nucleus and is transcribed using the infected cell's own protein production line and precursors to produce the viral proteins, which upon completion are shuttled to the cell membrane to wait for all viral components to be assembled and virion production [1]. The cRNA remains in the nucleus and is transcribed into new viral genomes that are then sent to the cell membrane to meet with the rest of the virion components [1]. Once all components are present at the cell membrane in sufficient numbers, assembly of virion cores and the packaging of RNA segments into virion cores begins [1]. As whole viral particles are constructed they begin to bud off from the infected cell taking part of the lipid bilayer with it [1]. As the new virus particles bud off they are attached to the infected cell via their HA spikes but NA cleaves these bonds thereby releasing the new viral particle to infect new cells [1].

The pathogenicity of each species of influenza virus in the human host is also a method of distinguishing them; influenza C viruses are considered endemic to the area they are found and while rare, can occasionally cause a mild respiratory infection [2]. Influenza B viruses are the next most pathogenic species as they are capable of causing significant disease and large epidemics but are not capable of sustaining pandemics [2]. Influenza A viruses are the most pathogenic of the three species; they are the most significant cause of influenza infections and cause the most severe disease [2]. Influenza A viruses are also the only species that have caused pandemics [2]. One of the reasons that influenza A

viruses are so pathogenic is that their genome undergoes two processes known as antigenic drift and antigenic shift [1, 2]. Antigenic drift is the result of the accumulation of multiple point mutations created in the viral genome during replication [2]. Mutations occur because the RNA polymerase has no proofreading ability; this combined with high speed replication leads to mutation rates of $1x10^{-3}$ to $8x10^{-3}$ substitutions per site per year [2]. Antigenic drift becomes advantageous for the virus and a problem for the host when the mutations accumulate in antigenic viral proteins, like HA and NA, that allow the virus to evade the host's pre-existing immunity [2]. While antigenic drift occurs on a small scale, antigenic shift is a much larger event that occurs when segments of viral genome are traded between different viral subtypes when more than one subtype has infected a host cell [1, 2]. Like antigenic shift, this becomes a major issue for hosts and advantage for the virus when it gains entirely new genes, one of which must be the HA gene, thereby creating an entirely new virus to which the host is immunologically naive [1, 2]. It is these new viruses created through antigenic shift that are thought to be the cause of all human influenza pandemics of the last 200 years when an avian and human virus has reassorted bringing previously unknown avian genes into the human virus [1, 2]. This is not thought to be the case with the 1918 pandemic, the most deadly pandemic recorded, as the reconstructed genome appears to be wholly avian and is thought to have arisen through global adaptation of the genome for infection in humans [2]. Interestingly, the influenza strain responsible for the

current H1N1 pandemic was found to have originated in swine; however, the genes were derived from various avian influenza virus species over a period of several years [5, 6]. Most of the genes directly reassorted to swine viruses from avian viruses but in some cases the avian genes reassorted into human viruses before then reassorting into swine viruses [5, 6]. The crucial novel HA gene is of a H1 variety that up until this point had only been found to be circulating in the pig population [5, 6]. Since there are human influenza strains of the H1 subtype currently circulating it is possible that previous exposure to these strains, either through infection or vaccination, will provide cross-protection to infection from the pandemic strain. While all influenza A, B and C viruses experience antigenic drift and shift it is the multitude of HA and NA variants that influenza A viruses possess that make these processes especially problematic for hosts. In influenza A viruses, sixteen different HA and nine different NA genetically distinct subtypes have been found in avian virus species [1, 2]. However, of these only three HA and two NA have been found in human virus species that are capable of sustained widespread person-to-person transmission [1]. However, it is possible that any of the remaining HA and NA subtypes could reassorting into the human influenza virus population leading to the creation of a new and novel influenza virus.

Influenza is for the most part considered a seasonal disease in that while the virus may be isolated a various times during the year, and indeed in regions along the equator throughout the year, there is a point, or several, in the year in

which infection rates increase and an outbreak occurs [7]. This seasonal influenza outbreak is commonly called the 'flu season' and in temperate zones strikes during the winter months and in countries along the equator during the rainy seasons [7]. A typical seasonal outbreak will move through a community in an average of twelve weeks [7]. If during a seasonal outbreak the morbidity and mortality rates surpass what is considered typical, in the United States this is about 200,000 people hospitalised and 36,000 deaths due to infection, not including those infected but do not seek medical treatment [2, 7], the outbreak is then labelled an epidemic. While mortality does increase during epidemics death is more often due to secondary infections like pneumonia or complications arising from underlying and chronic diseases [2, 3]. Though influenza infects people of all ages and health, like most pathogens influenza has a preferred niche within its host and in humans influenza seems to preferentially infect the immunocompromised or suppressed [3, 7]. This group of persons includes both the children school-age and younger who have an underdeveloped immune system, the elderly who have a waning immune system, those with chronic diseases that compromise the immune system such as diabetes mellitus or those affecting the cardiopulmonary system and those who are on drugs that suppress the immune system such as rheumatoid arthritis suffers and those that have received a transplant [2, 3, 7]. While both influenza A and B viruses are the most common cause of disease during[2, 7] outbreak it is possible to define a severity ranking, based on the duration of uncompleted influenza infections, within the

healthy non-niche population; influenza A (H3N2) viruses cause the most severe disease, influenza B viruses are in the middle and influenza A (H1N1) viruses cause the lease severe disease [7]. The different viral serotypes that emerge each outbreak season come from a pool of influenza viruses that are circulating worldwide at low endemic levels and each year an influenza A (H1N1) and (H3N2) strain and an influenza B strain become more prominent and become the cause of that season's outbreak [4]. Interestingly, the serotypes causing the seasonal outbreaks in the Northern and Southern hemispheres are generally different and of course the outbreaks are offset from one another as the winter months occur at different times in the year.

Clinically influenza is acute respiratory illness with a sudden onset of symptoms [2]. Symptoms most commonly involved are high fever, chills, sore throat, cough, nasal congestion, headache, myalgia, and malaise lasting 7-10 days [2, 4]. Accompanying these are symptoms of weakness and fatigue that can last for many weeks following the resolution of initial symptoms and illness [2]. As influenza infects the respiratory tract infection also leads to the inflammation of the upper, respiratory tree and trachea in immunocompetent hosts [2, 4].

Vaccination

Influenza vaccination is recommended annually for the prevention of influenza infection globally by the World Health Organization (WHO), in Canada by the National Advisory Committee on Immunization (NACI) [8] and in the

United States by the Centers for Disease Control and Prevention (CDC). According to the WHO, any healthy person over the age of six months without contraindications can and should be immunized if they so choose [9]. The WHO also recommends that all at risk persons be vaccinated with priority over those not at risk [9]. Those that are at risk as defined by the WHO, and in decreasing priority, are: 1. disabled and elderly residents of long-term care facilities; 2. elderly persons not in long-term care facilities but who have chronic conditions including pulmonary and cardiovascular disease, metabolic disease including diabetes mellitus or are immunosuppressed either from disease including HIV/AIDS or from treatment including transplant recipients; 3. any person over six months who has any of the above conditions; 4. any persons, irrespective of other risk factors, who are above their nation's determined age limit: in most countries 65 years and greater; 5. any other groups of persons at risk as defined by a nation's own data; 6. health care workers that have frequent and sustained contact with persons identified as at risk; 7. persons who live with persons identified as at risk [9]. The contraindications for receiving the annual influenza vaccine according the WHO are having a serious egg allergy, previous influenza vaccination associated with an episode of Guillain-Barré syndrome and current acute febrile illness until symptoms have abated [9].

There are three types of globally available vaccine preparations as identified by the WHO: (i) whole inactivated virus, (ii) split virion preparations that utilise virus particles disrupted through treatment with detergents and (iii)

subunit preparations that consist of primarily only the viral HA and NA proteins with all other viral components removed [9]. Only available in the United States is a live-attenuated vaccine that is administered intranasally as a mist and is safe for use in healthy persons from two to forty-nine years of age. There are three different vaccines currently available on the Canadian market; two are split virion preparations while the third is a subunit preparation [8]. The vaccine, which is injected into the deltoid muscle, consists of 15µg of each of the component virus strains: influenza A (H1N1), influenza A (H3N2) and influenza B, [3, 8, 9]. The annual strains comprising the vaccine are determined by the WHO Global Influenza Program six to eight months prior to the onset of flu season so that manufactures have time to prepare the vaccine [3, 8, 9]. The virus strains are chosen from the population of currently circulating endemic pool as the strains most likely to cause significant numbers of infection and are most virulent [3, 8, 9]. Side effects are few when receiving the standard injected vaccine and can include swelling, redness and tenderness at the site of injection, a low-grade fever and general aches [8]. When the vaccine is antigenically well matched to the actual seasonal strains it has been proven to prevent illness in 70-90% of healthy people who are vaccinated [9].

Human Immune System

The human immune system is comprised of two main components: the innate and adaptive systems [10]. These systems function independently with

their own specific cells and mechanisms but there is cross-talk between systems, indeed the innate system is able to help activate the adaptive system, and the systems are integrated to work together to fight infections. The innate immune system refers to the first line of defences that a microbe will encounter upon entering the body. There are four types of defences that comprise the innate system: anatomic, physiologic, phagocytic and inflammatory, but all are a method of barrier preventing the establishment of microbes [10]. Anatomic barriers act as physical barrier that serves to prevent the entry of microbes and includes the skin and mucosal membranes throughout the body. Physiologic barriers act to inhibit or kill microbes and include raised body temperature (fever), low pH of the skin and stomach, which is outside the optimal range of many microbes, and chemical mediators that serve to lyse microbes, activate an antiviral state in uninfected cells limiting spread and alert innate immune cells. The phagocytic barrier is comprised of immune cells that internalize and kill foreign microbes. Inflammatory barriers are activated through tissue damage during infection resulting in vascular fluid leaking into the infection site, bringing with it proteins that have antibacterial activity and more phagocytic cells. In addition to these barriers, individual cells have an antiviral response that is activated by the binding of interferon alpha (IFN-α) and interferon beta (IFN-β) [10]. The production of IFN-α and IFN-β is triggered in infected cells by the presence of double stranded RNA that can be produced during the virus replication cycle [10]. The binding of IFN-α and IFN-β to the IFN-α/β receptor on

infected and surrounding uninfected cells, turns on antiviral state, which occurs through activation of the JAK-STAT pathway, leading to the transcription of genes responsible for the degradation of viral RNA and inactivating protein synthesis; thus, preventing viral replication [10]. The IFN-α and IFN-β produced by infected cells also serves to activate the lytic ability of NK cells allowing them to kill virally infected cells [10]. This activation of the antiviral response in uninfected cells and the lytic ability of NK cells slows the spread of the virus within the body [10]. These defences are always in place whether the body is experiencing an active infection. Since they are not directed against specific viruses, bacteria or parasites but any foreign microbe the innate system is considered non-specific compared to the adaptive system. The innate system is activated immediately upon infection with a microbe and does not require time to produce the necessary mediators.

The adaptive system itself is comprised of two arms: the humoral and cell-mediated immunities [10]. These two arms have different effects during an infection but do not function independently in that activation of both is required for effective neutralization and clearance of pathogens. Unlike the innate system, the adaptive system is antigen-specific and is able to distinguish between different viruses, bacteria and parasites and respond to only that particular antigen. Because of this specificity the adaptive system is highly diverse and is able to distinguish between pathogen and self. The disadvantage of this specificity is that it takes days and energy to activate and produce the

required components for fighting infections [10]. However, the activation and production mechanisms produce immunologic memory making it easier and quicker for the adaptive system to respond the next time it encounters the same antigen.

Cell-mediated immunity is comprised of two different T lymphocytes (T-cells), those that have CD8 molecules on their surface ($CD8^+$ T-cells) and those that have CD4 molecules on their surface ($CD4^+$ T-cells) [10]. $CD8^+$ T-cells are also known as cytotoxic T-cells (T_C cells) because they target and kill infected cells; while, $CD4^+$ T-cells are also known as helper T-cells (T_H cells) as they help activate certain cells of the adaptive system. Progenitor T-cells are produced in the bone marrow and then are exported to the thymus were they are educated to remove self-reactive cells. The naive T-cells circulate through the lymphatic and circulatory systems patrolling for antigen to be activated [10]. Activation of the T_C and T_H cells differs slightly because of the restrictions within the T-cell receptor (TCR) of each type. The TCR of T_C cells is restricted to the recognition of major histocompatibility complex (MHC) class I molecules; while, the TCR of the T_H cell only recognises MHC class II molecules. MHC molecules are part of the human leukocyte antigen (HLA) complex, which contains three MHC molecules designated class I, class II and class III [10]. Here we are only concerned with classes I and II. MHC class I molecules are found on all cells of the body and display the proteins that are being assembled inside the cell, whether they are from self or from an infecting pathogen. MHC class II molecules are only present

on cells of the immune system that specifically take up and digest pathogens and then present these pathogenic proteins on the MHC molecules. These cells are known as antigen presenting cells (APCs) and include dendritic cells, macrophages and B lymphocytes (B-cells) [10].

The activation of naive T_H cells occurs in secondary lymphoid tissues and requires two signals provided by the APC [10]. The first signal provided is from the interaction of the TCR with the antigen-loaded MHC class II molecule on the APC. The T_H cell is specific for a single antigen that must be present on the MHC molecule or activation will not occur. Once the TCR is engaged, the CD28 molecule on the T_H cell interacts with the B7 molecule on the APC providing the second activation signal [10]. Once activated the T_H produces interleukin-2 (IL-2) which binds to self IL-2 receptors and promotes proliferation. The cytokine environment in which the T_H cell proliferates significantly impacts what subset of T_H cell is created. If the cytokine environment is rich in IL-12, IL-18 and interferon-gamma (IFN-γ) the T_H cell will become a T_H1 cell and produce cytokines and activate cells that are important in the creation of a cytotoxic T-cell response. If the T_H cell proliferates in an IL-4 rich environment it will become a T_H2 cell and produce cytokines and activate cells important in the creation of an antibody response [10]. During proliferation of either T_H cell subset, both memory and effector T-cells are created. The memory cells are long-lived serving as the immunological memory for this antigen and reactivate when antigen is

encountered again. The effector cells are responsible for the production of cytokines and activation of cells required for the current immune response [10].

The activation of the naive T_C cells is very similar to that of the T_H cells but there is no production of differing subsets [10]. The first signal required by the T_C cell is the engagement of its TCR with a MHC class II molecule that is loaded with a pathogen protein on the surface of an APC. The T_C cell is specific for a single antigen that must be present on the MHC molecule or activation will not occur. They also require the interaction between CD28 and B7 molecules as a second signal. Unlike T_H where IL-2 served as a self-made promoter of proliferation, in T_C cells IL-2 is the third activation signal and is produced by T_H1 cells [10]. Once fully activated the T_C cells have cytolytic activity and return to the periphery searching for infected cells displaying the specific antigen on MHC class I molecules. When an activated T_C cell encounters an infected cell displaying the correct peptide, through engagement of the TCR and MHC molecule, the T_C and target cells create a conjugate with a large area of close contact. The target cell is killed in one two fashions: either through the release of apoptotic mediators or through the interaction of cell surface receptors [10]. In the former method, the Golgi and granules reorient towards this site of close contact with the target cell and the granules fuse with the T_C cell membrane releasing the apoptotic mediators. These mediators include perforin and serine proteases called granzymes. Perforin is a pore-forming molecule that creates holes in the target cell`s membrane giving the granzymes, most notably Granzyme B, access to the

cytoplasm and initiating a cascade resulting in apoptosis of the target cell. Once the mediators have been released the T_C disengages from the target cell and still primed continues to search for other infected cells [10]. The other method of cell killing T_C cells can use is to couple the Fas ligand (FasL) protein on its surface with the Fas protein on the target cell surface. The interaction of Fas with FasL results in the activation of an internal cascade tied to Fas leading to apoptosis of the target cell. Again, after this cascade has been set in motion the T_C disengages from the target cell and still primed continues to search for other infected cells.

Humoral immunity is comprised of B-cells and the antibodies they produce [10]. Naive B-cells, those that have not encountered antigen, are produced and educated to remove self-reactive cells in the bone marrow and then home to peripheral lymphoid organs or nodes. It is in the periphery that activation, proliferation and differentiation occur if the naive B-cell encounters antigen; if no antigen is encountered within a few weeks the cell will die by apoptosis [10]. Each naive B-cell is specific for a single antigen and will only respond to activation by that antigen. Activation of naive B-cells begins when the membrane bound immunoglobulin (Ig) on their cell surface is cross-linked by antigen (signal 1). These antibody-antigen complexes are then internalized and the antigen is processed and loaded onto MHC class II molecules which are then expressed on the B-cell surface. At this point, T_H2 cells are required for further activation of the B-cell. The loaded MHC molecule is recognised through the TCR of the T_H2 cell which then engages the B-cell through binding of the B-cell B7

molecule with its CD28 molecule [10]. The next step in the activation of the B-cell is the interaction of the B-cell CD40 molecule with the T_H2 cell's CD40L; this is considered signal 2. The final step in the activation of the naive B-cell is binding of activation cytokines produced by the T_H2 cell. Once activated the B-cell undergoes class switching to produce a wider variety of antibodies and affinity maturation to increase the binding affinity of the produced antibodies. After these steps, the activated B-cells begin to proliferate and differentiate into two different phenotypes: the memory B-cell and the effector B-cell called plasma cells [10]. The memory B-cells are long-lived, serve as the immunological memory for this antigen, and are activated during antigen re-encounter. The plasma cells secrete neutralizing antibodies to the HA and NA glycoproteins which both serve to prevent infection but in different manners. Antibodies against HA prevent influenza from infecting cells by preventing HA from binding sialic acid on cell surfaces. Antibodies to NA on the other hand, prevent newly made virus particles from infecting new cells by preventing the activity of the NA protein thereby preventing the detachment of new virus particles from the infected cell [10].

When influenza viruses infect humans the immune system uses all facets to combat it. Innate systems are always in place to prevent and slow the infection. Activation of both the humoral and cell-mediated immunity components of the adaptive system is required to efficiently eliminate the virus.

Transplantation

For immunological purposes transplantation is the transfer of cells, tissues or organs from one source to another location [10]. Clinically this refers to transfer of cells, tissues or organs into one individual from another source for the purpose of replacing a non-functional, damaged or nonexistent component. Since it is currently not feasible to grow the required component from the individual's own cells the transplant must come for another source; therefore, is not immunologically identical. Immunological identity is largely controlled by the expression of various HLA alleles on the surface of cells [10]. Again, the MHC classes of most concern are class I and II. Both MHC classes I and II are encoded by three different genes each of which has various allelic forms[10]. In humans, the genes for MHC class I are called HLA-A, HLA-B and HLA-C while those for class II are DP, DQ and DR [10]. Each person has a different complement of MHC class I and II alleles expressed on their cell surface, half are of maternal and half are of paternal origin [10]. The adaptive branch of the immune system is able to distinguish each allele from each other; thus, when transplantation occurs the recipient's immune system distinguishes the new MHC alleles present on the transplant as non-self [10]. MHC does not have an exclusive role in immunological identity. Red blood cells (RBCs), epithelial cells and endothelial cells also express antigens on their cell surface that can be distinguished and targeted by the immune system; these antigens are the basis of the ABO blood-group system. If RBCs of one ABO group are introduced into a person of a

different ABO group, they will be recognised as non-self and targeted. Because of these reactions, donors and recipients are ABO and MHC typed to determine the best match, which is having the same ABO group and fewest differences in MHC alleles between donor and recipient, prior to transplantation. Recipients are screened for pre-existing antibodies against ABO or MHC alleles that would contraindicate transplantation of certain alleles. The recognition of donor ABO or MHC alleles as foreign and subsequent activation of the adaptive system against the graft leads to injury and loss of the graft in a process termed rejection. To prevent this process of rejection transplant recipients undergo lifelong immunosuppression regimen [10]. Immunosuppression regimens often include a corticosteroid, calcineurin inhibitor and anti-proliferative agent [10, 11].

The most widely used corticosteroids are prednisone, metabolised to prednisolone by the liver, and prednisolone (here both referred to as prednisone) though both have the same clinical effects [10, 11]. The effects of prednisone are a global suppression of T-cells by many means. It suppresses the release of inflammatory mediators, like histamine and prostaglandins, that increase permeability of capillaries reducing movement of T-cells from the circulation into grafts and increasing the stability of the lysosome membrane resulting in decreased release of lysosomal enzymes from T-cells into the graft [10, 11]. Prednisone also enters the cytoplasm of T-cells, binds to glucocorticoid receptor that then transit to the nucleus, and up-regulates the transcription of the NF-κB inhibitor. This in turn leads to the reduction in the production of the

inflammatory cytokines controlled by NF-κB- IL-1, IL-2, IL-6, IFN-γ and TNF-α [10, 11]. Prednisone also negatively affects macrophage/monocyte activity and decreases the number of circulating $CD4^+$ T-cells by reducing chemotaxis [10, 11]. The global immunosuppressive effect of prednisone and the reduced numbers of circulating $CD4^+$ cells could theoretically reduce response to vaccination though probably not to a significant degree, as while there are less T_H cells present they are still able to be activated and activate B-cells and T_C cells to some extent.

There are two different calcineurin inhibitors (CNI) in use and while both inhibit the effects of calcineurin, they bind to different proteins within the T-cell [10, 11]. Cyclosporine binds to the protein cyclophilin, while tacrolimus binds to a protein called FK506-binding protein [11]. Binding of the drug to its target protein inhibits the phosphatase activity of calcineurin that is required for the formation and translocation of NFATc to the nucleus. NFATc is a signal molecule activated when the TCR engages the protein loaded MHC molecule, which then leads to the up-regulation of transcription and production of pro-inflammatory cytokines responsible for T-cell proliferation and activation [10, 11]. T_H cells seem particularly sensitive to the effects of these drugs and the inhibition of their normal function prevents the normal function of other immune cells important to rejection including T_C cells, NK cells and B-cells [10]. The mechanism of action of CNIs suggests that persons on CNIs would have a decrease immune response to influenza vaccination. This decrease would be both antibody and T_C cell

related as CNIs prevent the activation of T_H cells that are required for activation of humoral and cell-mediated immunity.

Currently there are two anti-proliferative drugs used as part of immunosuppressive therapy: azathioprine and mycophenolate mofetil (MMF) [11]. These two drugs do not have the same mechanism of action [11]. Azathioprine is converted to the active forms 6-methyl-MP and 6-thioguanine which then insert into replicating DNA of cells and inhibit the replication process [11]. Inostinic acid is a precursor required for purine synthesis and through the formation of thio-inostinic acid, 6-methyl-MP and 6-thioguanine prevent the *de novo* synthesis of purine again leading to the inhibition of DNA replication [10, 11]. The inhibition of DNA replication inhibits the proliferation of activated T- and B-cells [10, 11] This effect on purine synthesis is limited to only lymphocytes as other cells have alternative mechanisms to utilize when this pathway is blocked [11]. MMF is converted to its active form mycophenolic acid which then binds to inosine monophosphate dehydrogenase (IMPDH) inside cells [11]. Once bound to IMPDH, it prevents the *de novo* synthesis of guanosine nucleotides which are required in DNA replication and without DNA replication activated B- and T-cells cannot proliferate [11]. This effect on guanosine nucleotide synthesis is limited to only lymphocytes as other cells have alternative mechanisms to utilize when this pathway is blocked [11]. In addition, there are two isoforms of IMPDH one of which is only present in activated lymphocytes and is the target of MMF leading to selective inhibition of only lymphocyte proliferation [11]. Since

the result of anti-proliferative agents is only against proliferation and not activation, it is possible that an initial immune response to influenza vaccination would be generated. However, without the ability to proliferate there should be limited generation of effector or memory cells, thereby leading to no observable long-term benefit of vaccination.

Overview Introduction Bibliography:

1. Bouvier, N.M. and P. Palese, *The biology of influenza viruses.* Vaccine, 2008. **26 Suppl 4**: p. D49-53.
2. Taubenberger, J.K. and D.M. Morens, *The pathology of influenza virus infections.* Annu Rev Pathol, 2008. **3**: p. 499-522.
3. Gross-Schulman, S., D.E. Dassey, and L. Mascola, *Influenza.* West J Med, 1998. **169**(6): p. 377-8.
4. Kuiken, T. and J.K. Taubenberger, *Pathology of human influenza revisited.* Vaccine, 2008. **26 Suppl 4**: p. D59-66.
5. Garten, R.J., et al., *Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1) Influenza Viruses Circulating in Humans.* Science, 2009.
6. Smith, G.J., et al., *Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic.* Nature, 2009.
7. Monto, A.S., *Epidemiology of influenza.* Vaccine, 2008. **26 Suppl 4**: p. D45-8.
8. National Advisory Committee on Immunization, *Canadian Immunization Guide*. 2006, Public Health Agency of Canada.
9. World Health Organization, *Weekly Epidemiological Report/Relevé épidémiologique hebdomadaire.* 2002. **77**(28): p. 229-240.
10. Richiard A. Goldsby, T.J.K., Barbara A. Osborne, Janis Kuby, *Immunology*. 5th ed. 2003: W.H. Freeman and Company.
11. Taylor, A.L., C.J. Watson, and J.A. Bradley, *Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy.* Crit Rev Oncol Hematol, 2005. **56**(1): p. 23-46.

Study #1 – Response method & potentiality of renal transplant recipient T-cells to influenza virus after seasonal influenza vaccination

Introduction

The first successful human transplant took place in Boston 1954 and was a kidney transplant between identical twins [1]. Today in Canada and the United States, kidney transplants make up about 60% of all solid organ transplants performed [2, 3]. In 2008 in Canada of the total of 2,048 solid organ transplants performed, 1,204 (59%) were kidneys [2]. In 2007 in the United States of the total 27,578 solid organ transplants performed, 16,119 (58%) were kidneys [3]. Given the large number of kidney transplants performed each year it is important to research not only the protocols of transplantation and maintenance but also other factors that have significant impact on the patient's quality of life after transplantation. As immunosuppression leaves patients more susceptible to infections with greater chances of complications and increased severity of disease, vaccination becomes an important factor in the quality of life and survival after transplantation as it may prevent infection, reduce disease severity and decrease the probability of long-term complications.

Previous investigations into transplantation and influenza vaccination

It is not unreasonable to expect that due to lifelong immunosuppressive therapy given post-transplant, there is poor response to influenza vaccination. If

this holds true, is the vaccine even effective in transplant patients? The data from published literature on the efficacy of influenza vaccination in kidney transplant (KTx) recipients is conflicting. Some studies have found that indeed KTx recipients do have a suboptimal response to the influenza vaccine [4-8]. Studies in lung [6, 9, 10], heart [6, 11, 12] and liver [6, 13, 14] transplant recipients have also shown that these patients have a decreased response to influenza vaccination. However, other studies in KTx recipients have found that there is no difference in the ability of the transplant patients' immune system to respond to influenza vaccination compared to healthy persons not on therapy [15-18]. Indeed, studies in lung [19, 20] and heart [21] transplant recipients have also found that the immune response to influenza vaccination does not decrease in these patients. In theory, due to the differing mechanisms of action of immunosuppressives, certain drugs or classes of drugs may have a greater or lesser effect on a patient's immune response when challenged with the influenza vaccine. In fact a study by Mazzone *et al.* in lung transplant recipients found that while all participants had a lower antibody response than the healthy controls, patients that were receiving cyclosporine as a calcineurin-inhibitor had poorer responses than those receiving tacrolimus [9]. However, a study in of KTx recipients by Lindemann *et al.* found that tacrolimus suppressed the immune response more than cyclosporine [22]. Other studies involving KTx patients and influenza vaccination have found that that MMF suppresses the patients' ability to produce antibody when challenged with the influenza vaccine [8, 17], though

Keshtkar-Jahromi, M. *et al* found no difference in antibody response in KTx patients compared to healthy controls when MMF was used [16]. In part this inconsistency in the literature could also be due to what appears to be a difference in immunogenicity of the different influenza vaccine strains in different transplant recipients: kidney [4-6], heart [6, 11], lung [9, 10] and liver [6], children [23] and the elderly [24]. It could also be due to a mismatch between the vaccine and actual circulating strains: as of the 2007-2008 influenza [25] season the vaccine strain has only matched the circulating strain in sixteen years of the past twenty.

Measuring the immune response

Most studies investigating the efficacy of the influenza vaccine in any population including the transplant and specifically the KTx population have assessed the change in serum antibody titres following vaccination. The test for determining the serum antibody titre is called the hemagglutination inhibition assay (HAI) and is able to determine the titre of anti-influenza antibodies present in the serum. The titres before and after immunization are compared to determine if the person experienced a response (seroresponse) to vaccination and was protected (seroprotection) from infection by the strains within the vaccine. A positive seroresponse is defined as at least 4-fold increase in the antibody titre from prevaccination levels and seroprotection is defined as having an absolute antibody titre ≥1:40 [20]. As they do not directly measure the

activity of the plasma cells, serum antibody titres are a surrogate marker of activation [26]. A more indicative way to assess the efficacy of the influenza vaccine would be to study the cell-mediated immune (CMI) response looking at the activation level of $CD8^+$ and $CD4^+$ T-cells. Another reason to directly assess the activity of cells in the CMI response is that effective clearance of the influenza virus requires the activity of $CD8^+$ T-cells. While antibody levels may indicate, a person is protected, if the CMI response is lacking they will not be able to respond to and clear an infection as quickly or efficiently as when the CMI response is activated.

There are three methods by which the CMI response can be evaluated: each gives unique information and specificity: enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunosorbent spot (ELISpot) and flow cytometry. ELISA can detect the presence of cytokines in the supernatant of cell culture media. For this assay, lymphocytes are stimulated for a period of time allowing for the release of the cytokines into the supernatant media as they are activated. The supernatant is removed and transferred to a well that has been coated with capture antibodies that are specific for the cytokine in question followed by detection and enzyme-linked antibodies are added. Finally, a substrate of the enzyme is added to the wells and a colour change occurs. The darker the colour in a well, the more cytokine is present in that supernatant and therefore the more activated the stimulated cells. A drawback of this method is that a few over activated cells producing large quantities of cytokine cannot be

distinguished from numerous normal-level activated cells; therefore, results may be misleading.

The second method of assessing CMI activation is the ELISpot assay, which is a modification of the ELISA and has a similar procedure. The major advantage of ELISpot is that cells are stimulated in a well already coated with capture antibody specific for the cytokine in question. This difference allows for cytokines to be captured immediately and locally upon release from the cell which in turn allows for the determination of the number of cells responding (the number of spots) and to what degree (the size of spot; larger spot means more cytokine produced). This also means that the ELISpot assay is more sensitive than the ELISA is and removes the confounder of a small population of over activated cell producing large quantities of cytokine. This method can also be used to directly assess the antibody production of stimulated B-cells and was originally created for this purpose. The disadvantage to ELISpot is it cannot distinguish $CD4^+$/$CD8^+$ T-cells.

The third method of assessing CMI is flow cytometry. This method is increasingly used in vaccination studies. Flow cytometry involves the detection and differentiation of cells based on the binding of various fluorescently tagged antibodies both on the surface of cells and intracellularly. The cells are stimulated in the same fashion as ELISA but for part of the time, a chemical that inhibits intracellular trafficking is added if detection of intracellular cytokines is

of interest. After the stimulation, the cells are removed from the media, fixed to prevent the loss of intracellular components, permeabilized to allow for the entry of antibodies into the cell, and stained with fluorescently tagged antibodies. In the flow cytometer the cells are passed by detection lasers in a stream of single cells that collect information concerning the size of the cell, cell granularity and what antibodies are bound to it. Every cell that is read by the machine is placed on a dot-plot based on what was detected by the machine indicating the properties of the cell and the population of cells as a whole. The amount of information obtainable by flow cytometry is restricted by the number of lasers present in the machine; the more lasers means that more antibodies can be used allowing for the detection of more specific cells and populations. In vaccine efficacy analysis flow cytometry can be used to detect which specific populations of cells are being activated and what cytokines are produced prior to and following vaccination. One major advantage flow cytometry has over ELISA and ELISpot is that hundreds of thousands of cells can be rapidly counted. Another major advantage of this method is that distinct subset cell populations can be distinguished and analysed in flow cytometry such as memory T_H2 cells, which can indistinguishable from naive T_H2 cells in ELISpot. It is also possible to look at the production of all cytokines produced by a specific population of cells simultaneously within a single cell instead of separate wells for the detection of individual cytokines, e.g. the pro-humoral immune response cytokines produced by T_H2 cells.

The influenza vaccine is an annual vaccine with a very defined vaccination and outbreak window. This coupled with the strong recommendation that transplant recipients receive the vaccine annually make it ideal for study. That kidney transplantation is the most numerous transplant preformed and has good survival rates makes this group an ideal study group. Flow cytometry, along with serum antibody titres, was chosen to assess the CMI response because of its high throughput and ability to look at individual populations of cells.

Methods

Participant Enrolment

In the fall of 2008, fifteen adult kidney transplant (KTx) recipients were recruited from the University of Alberta Hospital's transplant outpatient clinics and fifteen healthy volunteers (HV) were recruited from laboratory and hospital staff. All attempts were made to match KTx and HV for age and gender. The University of Alberta Research Ethics Board approved the study and written informed consent was obtained from all participants. Persons with allergies to eggs, a previous life-threatening reaction to influenza vaccination, on anticoagulants, had febrile illness in the past two weeks, KTx patients currently receiving therapy for rejection, were less than six months post transplantation, HV on immunosuppressives, or with underlying immunological disorders, were excluded. Participants were followed for six months after vaccination to determine if participants became infected with influenza or experienced

influenza infection-like symptoms. Adverse events including rejection and loss of graft function were also noted during follow-up in KTx patients. Demographic data including age, previous vaccination history for previous five years and time since transplantation collected from all participants.

Schedule of Blood Draws

Baseline venous blood samples were taken prior to vaccination in sodium heparin vacutainer tubes for the purpose of peripheral blood mononuclear cell (PBMC) isolation and serology vacutainer tubes for antibody titre testing. Participants received the 2008-2009 influenza vaccine as the standard 0.5 ml dose intramuscularly in the deltoid muscle of the non-dominant arm. Vaccine was administered at influenza clinics held at the University of Alberta Hospital or at the kidney transplant clinic during the 2008 fall season. At four weeks post vaccination, venous blood samples were again collected from patients.

Peripheral Blood Mononuclear Cell Isolation (Personal Communication with Dr. Wasilenko) (Figure 1.)

Blood samples were processed on the same day they were drawn. Serology tubes were centrifuged at 3,000 rpm for 10 minutes. Resulting layer of serum was removed and stored in liquid nitrogen for influenza antibody titre testing at the Health Protection Agency in London, United Kingdom. Blood collected for PBMC isolation was transferred from the vacutainer tubes to a 50mL conical tube and the volume of blood collected was recorded. Following the transfer of blood

each vacutainer tube was rinsed with 10mL of RPMI 1640 media supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin-antimycotic solution, 1% nonessential amino acids and 1mM sodium pyruvate (media) which was then added to the collected blood. Ficoll-Paque™ PREMIUM (Ficoll) (GE Healthcare Life Sciences cat#17-5442-03) was used as the cell density gradient and was layered under the blood. To layer under the blood, 13.5 mL of Ficoll was drawn into a 10mL plastic pipette then the tip of the filled pipette was gently placed at the bottom of the blood containing conical tube. The pipetteman, motorised pipette filler, was removed allowing gravity to pull the Ficoll out of the pipette and under the blood resulting in a clean blood-Ficoll interface. To pellet the RBCs and polymorphonuclear leukocytes and suspend the PBMCs blood-Ficoll tubes were centrifuged at room temperature for 20 minutes at 1200xg with the centrifuge rotor's acceleration set to maximum and the deceleration set to zero. After centrifugation, the PBMCs are located in the layer located between the plasma and Ficoll layers. This layer and the lower Ficoll layer were slowly and delicately removed by sweeping a 5mL pipette with minimal pipetteman suction over the plasma-Ficoll interface until a minute amount of Ficoll was remaining over the pelleted red blood cells and polymorphonuclear leukocytes as a buffer. The volume of removed Ficoll-PBMCs was recorded and then media was added to a final volume of 40mL and the cells resuspended. To count the number of cells recovered, 50µL of the cell suspension was mixed with 50µL of trypan blue and layered on a standard hemocytometer. The large outer

four corner squares, made of 16 smaller squares, were counted and averaged to obtain a count number. Only cells that were completely round, with clear not blue cytoplasm and not of extremely small size were counted. The count number was then inputted into the following calculation to determine the number of recovered PBMCs: count number ***x*** $1x10^4$ ***x*** 2 ***x*** 40 **=** recovered number. From the recovered number of cells the volume of cells required for the stimulation experiment was calculated and removed to a separate conical tube. Both cells for stimulation and remaining cells were centrifuged at room temperature for 10 minutes at 600xg with the centrifuge rotor's acceleration set to maximum and the deceleration set to zero in order to pellet the cells. After centrifugation the supernatant was removed from all tubes, the required volume of RPMI 1640 media supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin-antimycotic solution, 1% nonessential amino acids and 1mM sodium pyruvate was added to the tube of cells to be used in the stimulation and the cells resuspended. The remaining cells were frozen and stored in liquid nitrogen; to do so the cells were resuspended in heat inactivated fetal bovine serum at a final concentration of $2x10^7$ cells/mL. Then while gently shaking/agitating the cells an equal volume of freezing media (20% dimethyl sulfoxide in heat inactivated fetal bovine serum) to generate a final concentration of $1x10^7$ cells/mL. Cells were aliquoted at 1mL /tube, immediately placed in a Mr. Freezy™ in a -80°C freezer overnight, and then transferred to liquid nitrogen for long-term storage.

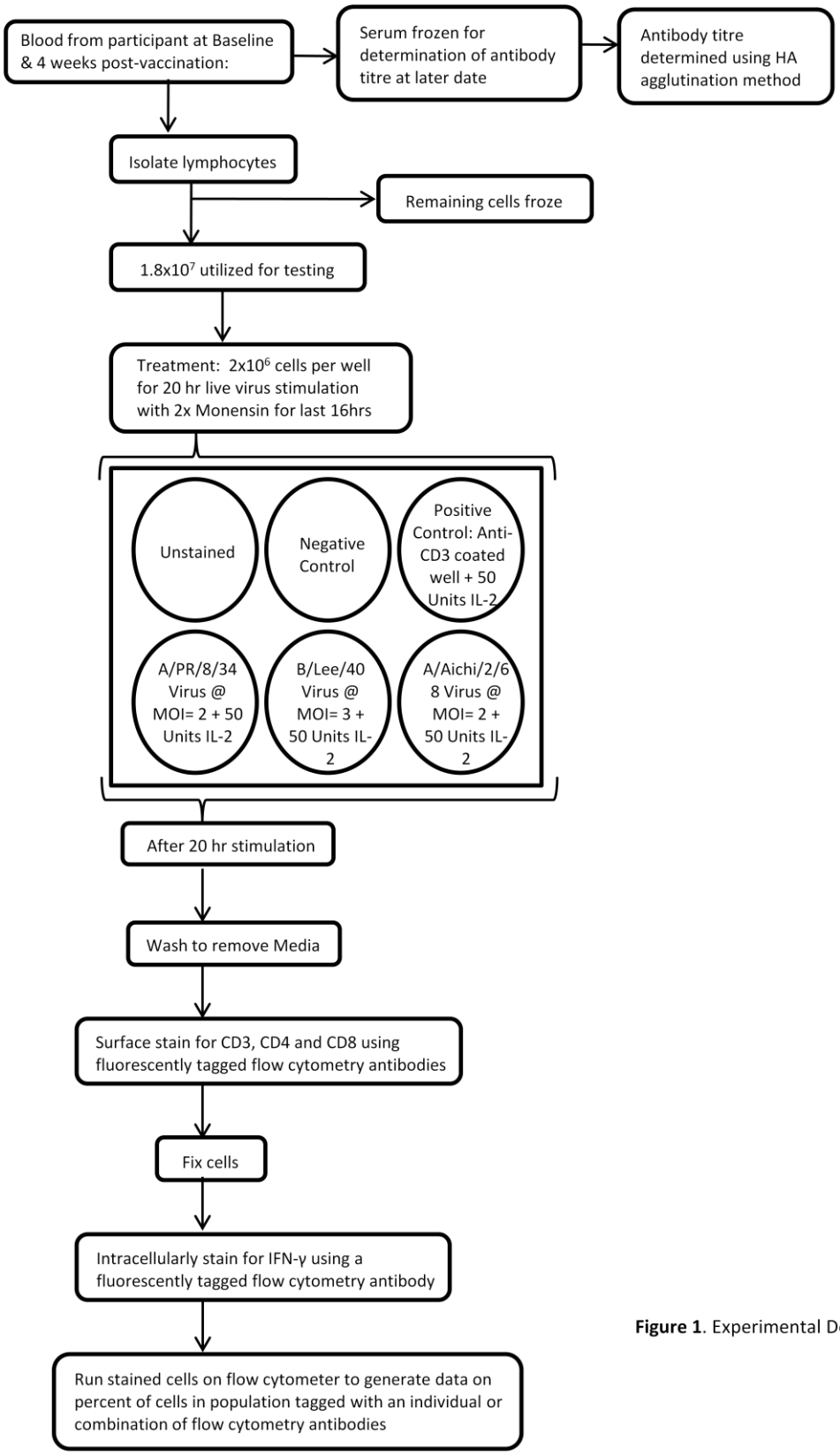

Figure 1. Experimental Design

Peripheral Blood Mononuclear Cell Stimulation (Figure 1.)

For stimulation, experiments Corning 48 well plates were used and cells were aliquoted at $2x10^6$ cells/well. A total of six different wells were involved in testing: unstained, negative control, anti-CD3 (positive control), A/PR/8/34 (A/PR) (Charles River Laboratories cat#490710), B/Lee/40 (B/Lee) (Charles River Laboratories cat#490735) and A/Aichi/2/68 (A/Aichi) (Charles River Laboratories cat#490715 and American Type Culture Collection cat#VR-547). These viruses were chosen for their extensive use in the literature and their subtype to match the ones in the vaccine: A/PR is an H1N1 subtype, A/Aichi is an H3N2 subtype and B/Lee is a B subtype. A/PR/8/34 and B/Lee/40 virus stimulations were completed in duplicate (Figure 2). The unstained and negative control wells only contained the cells and media. The unstained cells were not stained with flow cytometry antibodies, but were treated identically to stained cells, to assess the cells' natural immunofluorescence. The negative cells were stained to assess the level of spontaneous activation due to the isolation and stimulation procedures. For the positive control, wells were coated with 150µL of anti-CD3 antibody at a concentration of 10µg/mL (1:100 dilution of 10mg/mL stock in sterile phosphate buffered saline) for either 2 hours at 37°C or overnight at 4°C to coat the bottom of the well. Anti-CD3 antibody was chosen as the positive control as cross-linking of the CD3 molecule is required for activation of T-cells and has been shown to significantly increase the proliferation and IFN-γ production in response to heat-inactivated influenza virus particles [27]. After coating, the wells were blocked

with 1mL of RPMI 1640 media supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin-antimycotic solution, 1% nonessential amino acids and 1mM sodium pyruvate for 45 minutes at 37°C. The virus multiplicity of infection (MOI), that is the ratio of virus particles to target cells, was determined experimentally by panel testing several MOIs leading to A/PR/8/34 and A/Aichi/2/68 viruses used at an MOI of 2 while B/Lee/40 was used at a MOI of 3. Please see appendix A for information concerning the investigation of the optimal MOI for each virus and sample MOI calculations. Fifty units of interleukin-2 were included in the anti-CD3 and virus containing wells as a supplementary proliferation cytokine. No additional supplements were added to the negative or unstained wells. Cells were plated in 200µL of RPMI 1640 media supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin-antimycotic solution, 1% nonessential amino acids and 1mM sodium pyruvate and incubated for two hours at 37°C to bring the cells and virus particles in closer proximity to enhance infection rates. After the initial two-hour infection period, 300µL of RPMI 1640 media supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin-antimycotic solution, 1% nonessential amino acids and 1mM sodium pyruvate was added. At four hours into the stimulation, monensin (eBioscience cat#00-4505) was added at a concentration of 2X. After the addition of monensin, the cells were left to incubate for 16 hours at 37°C.

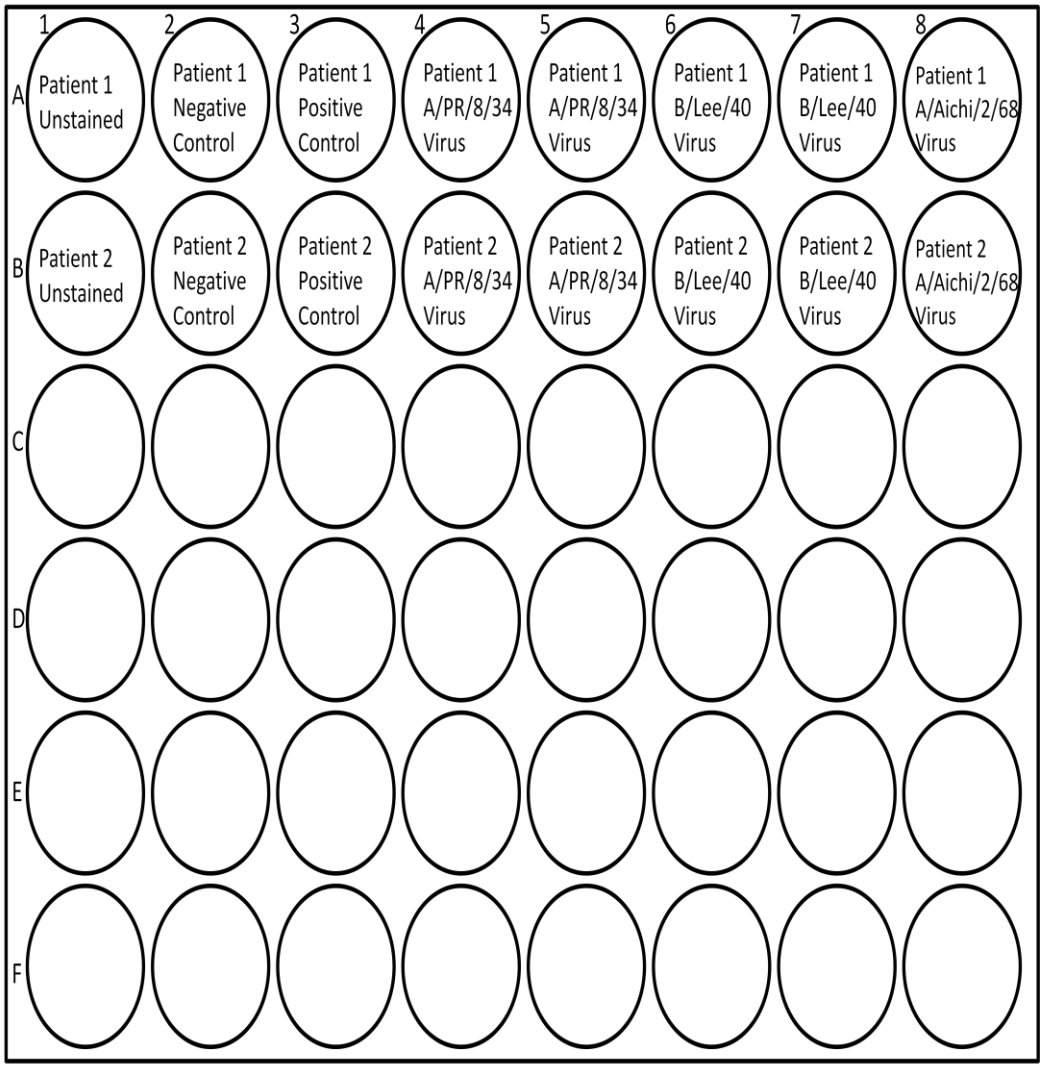

Figure 2. Stimulation Plate Layout

Staining and Flow Cytometry (Personal communication with Dr. Luis Hidalgo) (Figure 1.)

After the 20 hour stimulation 500µL of ICS wash (sterile phosphate buffered saline plus 1X monensin (eBioscience cat#00-4505), 0.5% fetal bovine serum, 2mM ethylenediaminetetraacetic acid and 0.05% sodium azide) was added to each well to help lift cells that had adhered to the bottom of the well. A pipette tip was then drawn gently over the bottom of the well surface to mechanically detach cells. Cells were transferred to 12x75 mm tubes containing an additional 500µL of the ICS wash and then centrifuged at 1200xg for 5 minutes. After centrifugation, the supernatant was poured off and 40µL of each of the surface markers, volume used based on manufacture specifications, was diluted in the remaining ICS wash, about 100µL, in each tube. Surface markers used were phycoerythrin-Cy7 tagged anti-human CD3 (eBioscience cat#25-0038), allophycoerythrin-alexa fluor 750 anti-human CD4 (eBioscience cat#27-0049) and allophycoerythrin anti-human CD8 (eBioscience cat#17-0086). Tubes were gently vortexed and incubated for 10 minutes in the dark at room temperature. After incubation, 2mL of ICS wash was added to each tube and the tubes centrifuged at 1200xg for 5 minutes. After centrifugation the supernatant was poured off and cell pellet resuspended in 500µL of fixation buffer (eBioscience cat#00-8222), vortexed gently and incubated for 20 minutes in the dark at room temperature. Following incubation, 1mL of 1X permeabilization buffer (eBioscience cat#00-8333) was added to each tube and then centrifuged at

1200xg for 5 min. The supernatant was poured off and 1mL of 1X permeabilization buffer (eBioscience cat#00-8333) was added to each tube, gently vortexed and centrifuged at 1200xg for 5 min. The supernatant was poured off and the cell pellet was resuspended in the remaining buffer, about 100 µL, and 2.5µL, volume based on manufacturer's specifications, of phycoerythrin tagged anti-human IFN-γ (eBioscience cat#12-7319) was added and tubes gently vortexed. Tubes were incubated for 20 minutes in the dark at room temperature. After incubation, 1mL of 1X permeabilization buffer (eBioscience cat#00-8333) was added to each tube and then centrifuged at 1200xg for 5 min. The supernatant was poured off and 500µL of FACS wash (0.5% fetal bovine serum, 2mM ethylenediaminetetraacetic acid and 0.05% sodium azide) was added, tubes gently vortexed and 250µL of each cell suspension was transferred to a 96-well plate for reading on BD FACSArray flow cytometer.

The FACSArray is a digital, not analogue, flow cytometer and was optimised for the detection of lymphocytes with the following laser voltage settings: forward scatter – 180V; side scatter – 305V; far red – 100V; yellow – 540V; near-infrared – 200V and red – 630V.. During the testing a no less than 100,000 and up to 200,000 cells were counted for a single sample. The FACSArray flow cytometer is a four-colour, six-channel machine using two lasers: a green at 532 nm for the yellow and far-red parameters and red at 635 nm for the red and near-infrared parameters. Weekly calibration was completed by Dr.

Hidalgo on weeks in which the machine had been used an bi-weekly when the machine was not in use.

Generated flow cytometry data was analyzed with the program FCS Express (De Novo Software). Pre-vaccination negative control data from each subject was used to set gates and quadrants for that individual. The first gate set was to isolate the lymphocyte population on the side scatter vs. forward scatter plot that displays all PBMCs counted by the flow cytometer. These cells were then displayed on a dot plot of side scatter vs. $CD3^+$ in order to create a gate around only $CD3^+$ lymphocytes thereby excluding other IFN-γ producing lymphocytes like natural killer cells. Further dot plots were then created using either the gated lymphocyte population or the $CD3^+$ population to display $CD3^+$ vs. IFN-γ, $CD8^+$ vs. IFN-γ, $CD4^+$ vs. IFN-γ and $CD8^+$ vs. $CD4^+$. Quadrants were set on each of these graphs to define where the population of negative cells was located, negative quadrant. Any cells located in the quadrant right of the negative quadrant were considered positive for the marker in question (Figure 3). In the case of the negative control, this displayed the percent of cells normally over-expressing the marker; in anti-CD3 and virus treatments, this quadrant displayed the percent of cells activated by the treatment. Using these gates and quadrants, a layout was created for the patient, which was used for all treatments pre- and post-vaccination. The subject's layout was used to analyse the anti-CD3 and virus stimulated cells for evidence of activation based on change in the percentage of cells ($CD3^+$, $CD4^+$, and $CD8^+$) expressing intracellular IFN-γ both pre- and post-

vaccination. Representations of generated FACS data plots for negative, positive and virally stimulated cells are shown in figures 4-6.

Statistical Analysis

For the purpose of statistical analysis, a ratio of the median post-vaccination percent IFN-γ positive T-cells to the median baseline percent IFN-γ positive T-cells with a value greater than one was considered a positive response. A participant was considered a responder if they had a positive response of at least one T-cell type to at least one of the three viruses. Response ratios were compared within KTx/HV groups and within responder/non-responders groups using the Mann-Whitney U test with a p-value <0.05 considered statistically significant. Response ratios were compared to median baseline percent IFN-γ positive T-cells using the Mann-Whitney U test with a p-value <0.05 considered statistically significant. Correlations between median baseline percent IFN-γ positive T-cells and response ratios and between having reactive $CD8^+$ and $CD4^+$ T-cells were considered statistically significant at p-values <0.05. All analyses were completed using SPSS version 11 (SPSS Inc.).

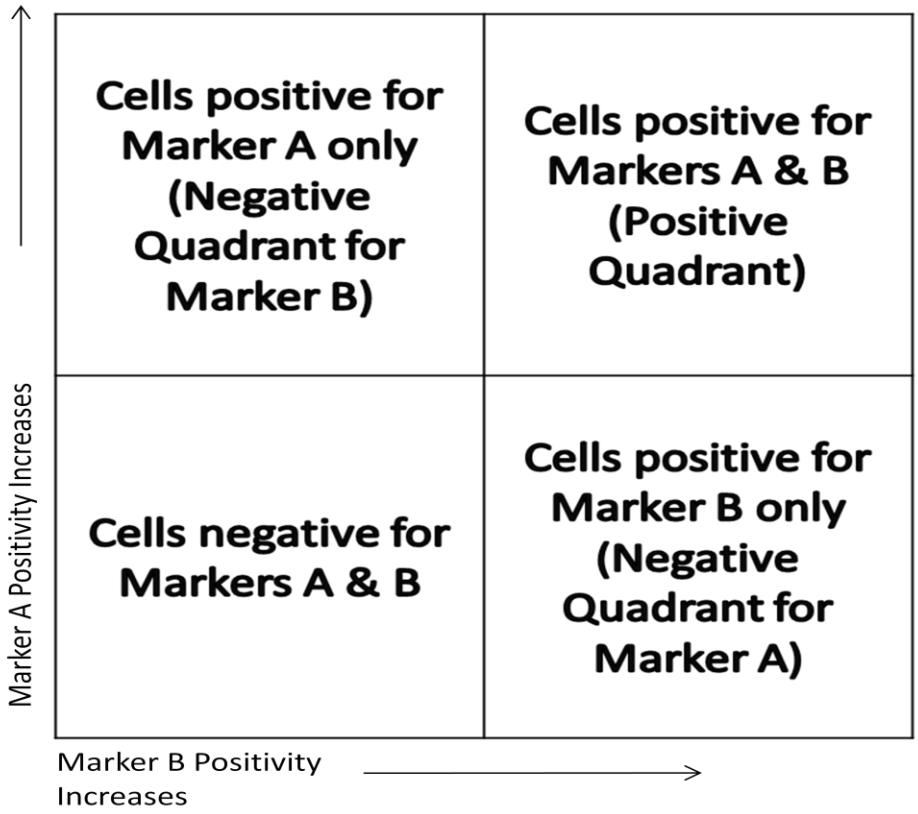

Figure 3. Basic flow cytometry dot plot quadrant organisation

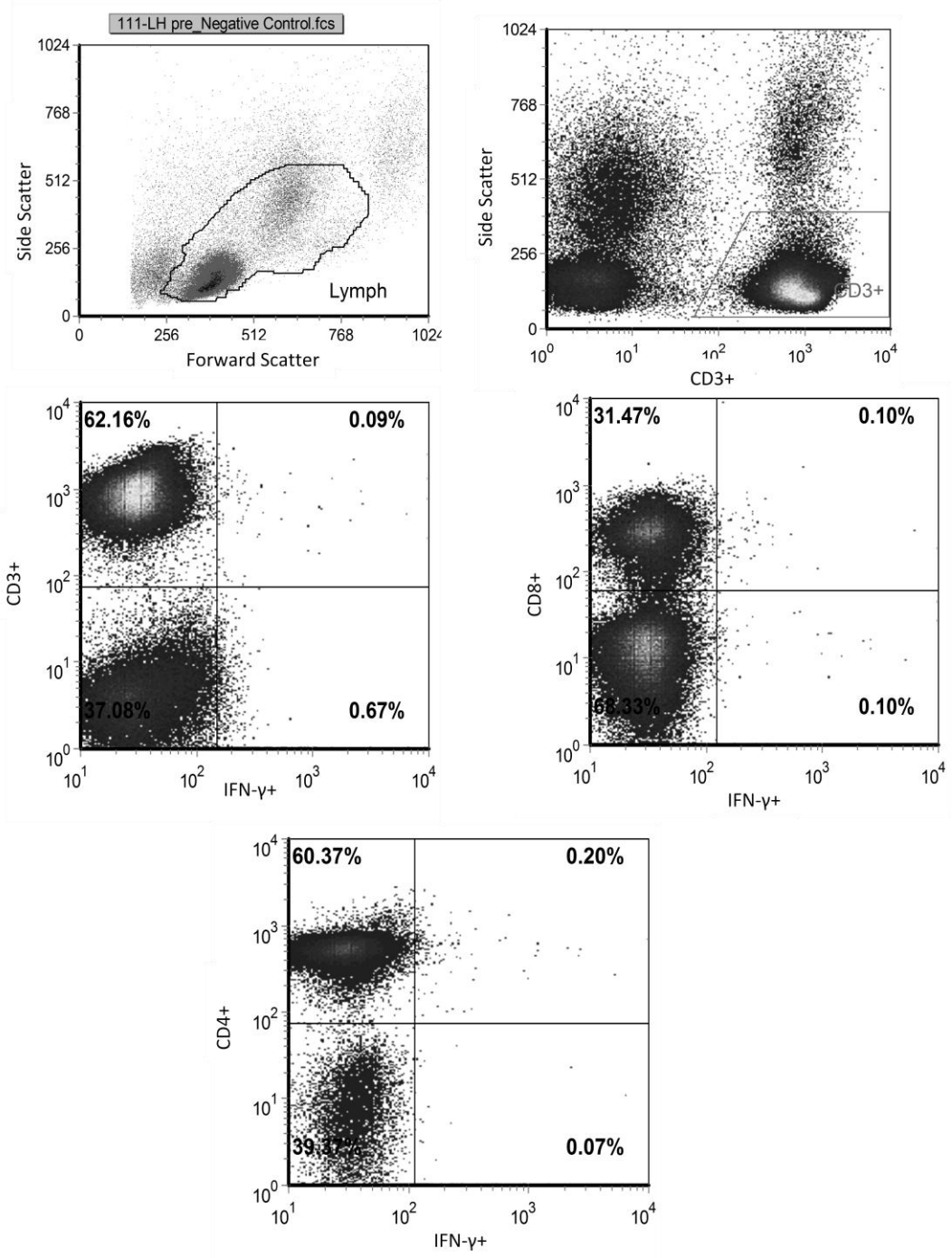

Figure 4. Representative Negative FACS Data

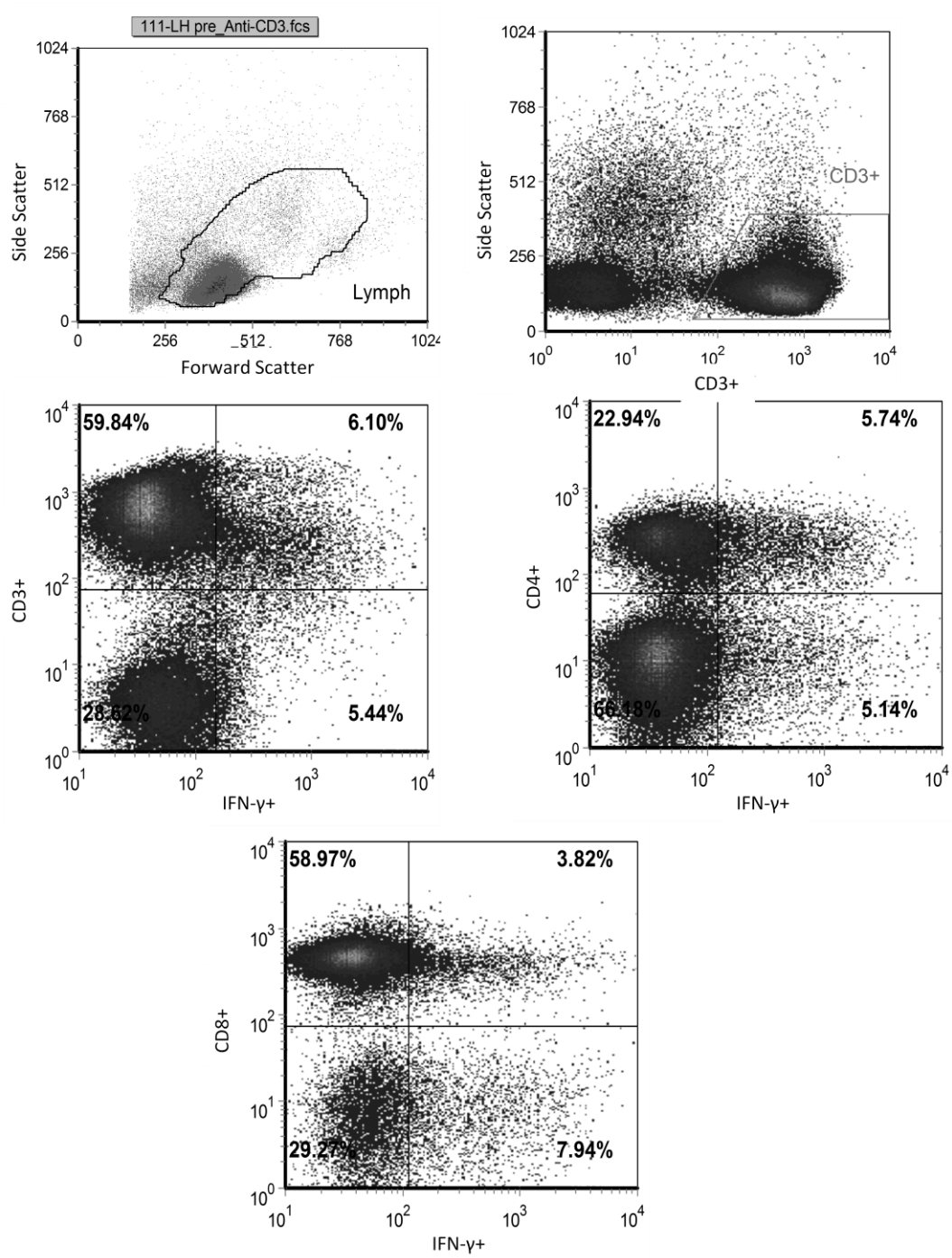

Figure 5. Representative Positive FACS Data

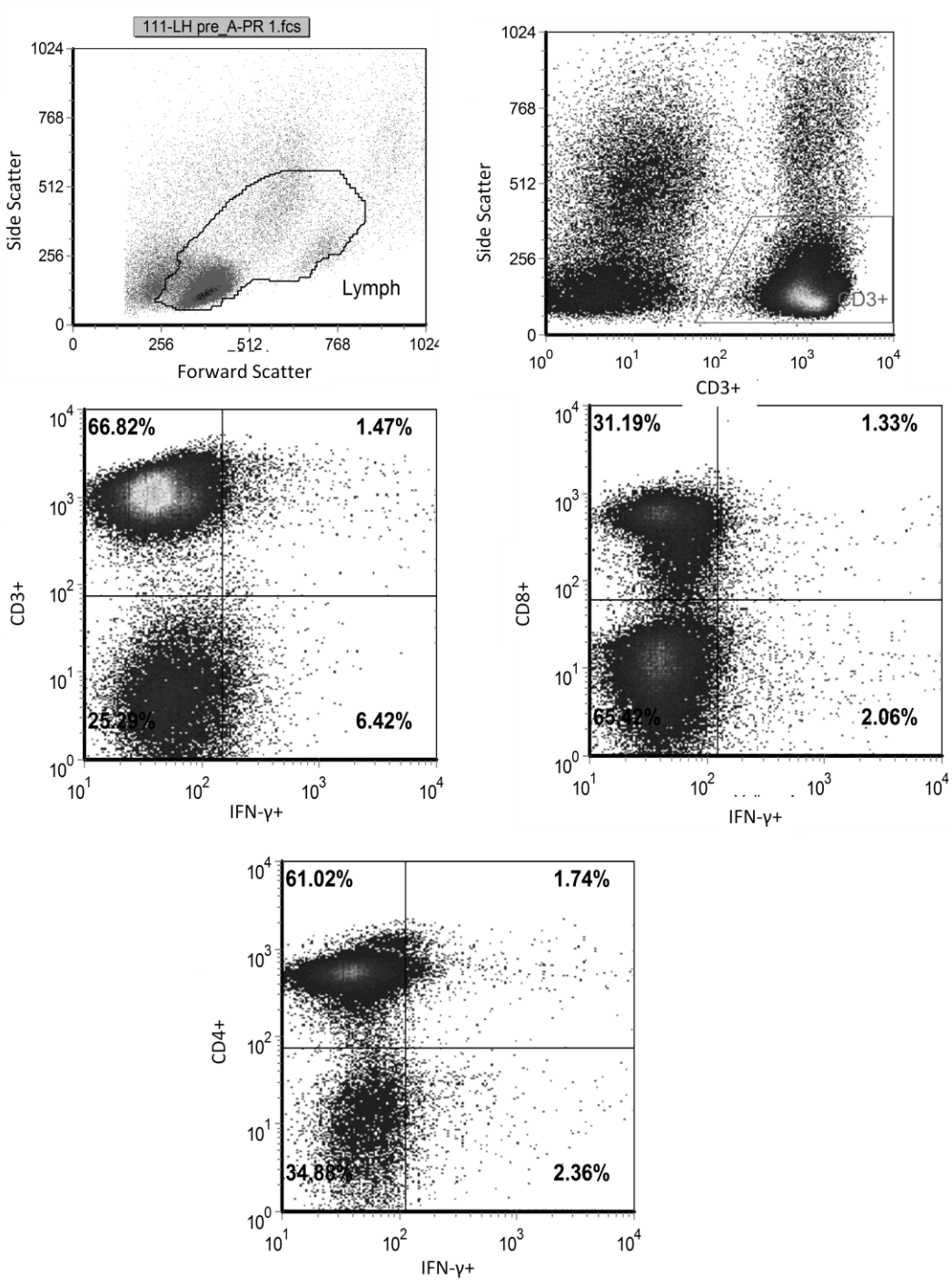

Figure 6. Representative Virus Stimulation FACS Data

Results

Study population characteristics

Thirty participants, fifteen KTx and fifteen HV were enrolled in the study and received the standard i.m. 2008-2009 trivalent-inactivated influenza vaccine. All fifteen HV subjects and fourteen KTx patients returned for follow-up blood work. Blood work from one KTx patient had to be later excluded due to inaccurate phlebotomy technique that collected an inadequate volume of blood in the vacutainer tubes to produce the correct ratio of blood to sodium heparin. This exclusion resulted in thirteen KTx patients for final analysis. The clinical characteristics of the participants are listed in Table 1. The HV group had a median age of 36 years (range 22-50) and comprised seven males and eight females. The KTx group had a median age of 45 years (range 27-72) and consisted of nine males and four females. The mean time from transplant was 6.9 years (range 0.6-12.9). The thirteen KTx patients were on nine different immunosuppression regimens with the majority on the standard immunosuppression regimen consisting of a combination of a calcineurin inhibitor (cyclosporine or tacrolimus), an antimetabolite agent (azathioprine or MMF) and prednisone. Thirteen (86.7%) of the HV and twelve of the KTx (92.3%) had been previously vaccinated with the trivalent-inactivated influenza vaccine during the 2007-2008 influenza season. There were no episodes of acute

rejection occurring in any KTx patients during the study or the six-month follow-up period.

Table 1. Participants' Clinical Characteristics

	KTx (n=13)	HV (n=15)
Age (years; median, range)	45 (27-72)	36 (22-50)
Gender (M/F)	9/4	7/8
History of influenza vaccination	12 (92.3%)	13 (86.7%)
Time of vaccination from Transplantation (years; mean, range)	6.9 (0.6-12.9)	-
Maintenance Immunosuppression		
Prednisone	10 (76.9%)	-
Calcineurin Inhibitors	10 (76.9%)	-
MMF	3 (23.1%)	-
Azathioprine	3 (23.1%)	-

Table 2. Breakdown of Responders in KTx & HV Groups According to Virus

	KTx (n=13)	HV (n=15)
A/PR $CD8^+$	4 (30.8%)	4 (26.7%)
A/PR $CD4^+$	5 (38.5%)	3 (20.0%)
B/Lee $CD8^+$	3 (23.1%)	5 (33.3%)
B/Lee $CD4^+$	4 (30.8%)	4 (26.7%)
A/Aichi $CD8^+$	8 (61.5%)	6 (40.0%)
A/Aichi $CD4^+$	6 (46.2%)	7 (46.7%)
Response to any Virus	12 (92.3%)	10 (66.7%)

Immunogenicity after standard influenza vaccination

Overall responders in the KTx and HV groups were twelve of thirteen (92.3%) and ten of fifteen (66.7%) enrolled, respectively (Table 2). The stimulating virus and responding cell type was used to subdivide overall responders within each group. A/Aichi virus was the most effective at stimulating PBMCs when compared to B/Lee or A/PR viruses (Table 2). The difference in the number of overall responders, those that responded to at least one virus, between the KTx and HV groups was found to not be statistically significant ($p = 0.254$). Strong and statistically significant correlation was found between having virus reactive $CD8^+$ T-cells and virus reactive $CD4^+$ T-cells to all three viruses (A/PR: Spearman's $\rho=0.825$, $p\leq0.001$; B/Lee: Spearman's $\rho=0.650$, $p\leq0.001$; A/Aichi: Spearman's $\rho=0.716$, $p\leq0.001$).

Interferon-gamma response ratios

The IFN-γ positive response ratios of the KTx and HV groups were compared for each virus according to cell type. No significant difference between groups was found (A/PR: $CD8^+$ $p = 0.964$, $CD4^+$ $p = 0.683$; B/Lee: $CD8^+$ $p = 0.683$, $CD4^+$ $p = 0.751$; A/Aichi: $CD8^+$ $p = 0.539$, $CD4^+$ $p = 0.872$). Therefore, we chose to analyze IFN-γ response ratios of the total cohort for the remainder of the analyses. With this analysis, IFN-g response ratios were found to be significantly different between responders and non-responders as shown in Table 3. For example, median response ratio of A/PR specific $CD8^+$ T-cells was

2.87 (n=8) vs. 0.35 (n=20) for non-responders ($p < 0.001$. Table 3, Figure 7). Similarly, results for B/Lee specific CD8+ and CD4+ T-cell response ratios is shown in Table 3 and Figure 8. A/Aichi stimulation showed similar results (Table 3, Figure 9).

Table 3. Number and median of positive IFN-γ response ratios of Responders & Non-responders according to virus and T-cell type in the overall cohort (A/PR & B/Lee n=28; A/Aichi n=22)

	Responders	Median Positive IFN-γ Response Ratio for Responders	Non-responders	Median Positive IFN-γ Response Ratio for Non-responders	p-value[a]
A/PR $CD8^+$	8	2.868 (1.16-4.04)	20	0.351 (0.004-0.71)	<0.0001
A/PR $CD4^+$	8	1.984 (1.220-51.000)	20	0.517 (0.004-0.905)	<0.0001
B/Lee $CD8^+$	8	1.756 (1.052-37.500)	20	0.519 (0.004-0.905)	<0.0001
B/Lee $CD4^+$	8	2.056 (1.123-36.000)	20	0.638 (0.004-0.980)	<0.0001
A/Aichi$CD8^+$	14	1.946 (1.160-18.486)	8	0.424 (0.004-0.616)	<0.0001
A/Aichi$CD4^+$	13	2.500 (1.030-138.000)	9	0.450 (0.003-0.900)	<0.0001

a=p-value corresponds to the comparison of median positive response ratio between responders & non-responders (Mann-Whitney U test)

Table 4. Number and median of baseline percent IFN-γ^+ cells of KTx & HV according to virus and T-cell type in the overall cohort (A/PR & B/Lee: n=28; A/Aichi: n=22)

	KTx	Median Baseline Percent IFN-γ^+ Cells for Responders	HV	Median Baseline Percent IFN-γ^+ Cells for Non-responders	p-value[a]
A/PR $CD8^+$	13	1.13 (0.12-4.00)	15	1.01 (0.60-3.84)	0.363
A/PR $CD4^+$	13	0.51 (0.01-4.76)	15	0.90 (0.33-1.62)	0.387
B/Lee $CD8^+$	13	1.52 (0.01-3.53)	15	1.61 (0.44-7.07)	0.856
B/Lee $CD4^+$	13	0.93 (0.01-3.88)	15	1.25 (0.36-2.51)	0.856
A/Aichi $CD8^+$	13	1.80 (0.22-3.78)	15	1.51 (0.36-5.10)	1.000
A/Aichi $CD4^+$	13	2.01 (0.01-5.55)	15	1.19 (0.20-3.94)	0.418

a=p-value corresponds to the comparison of median baseline IFN-γ values between responders & non-responders (Mann-Whitney U test)

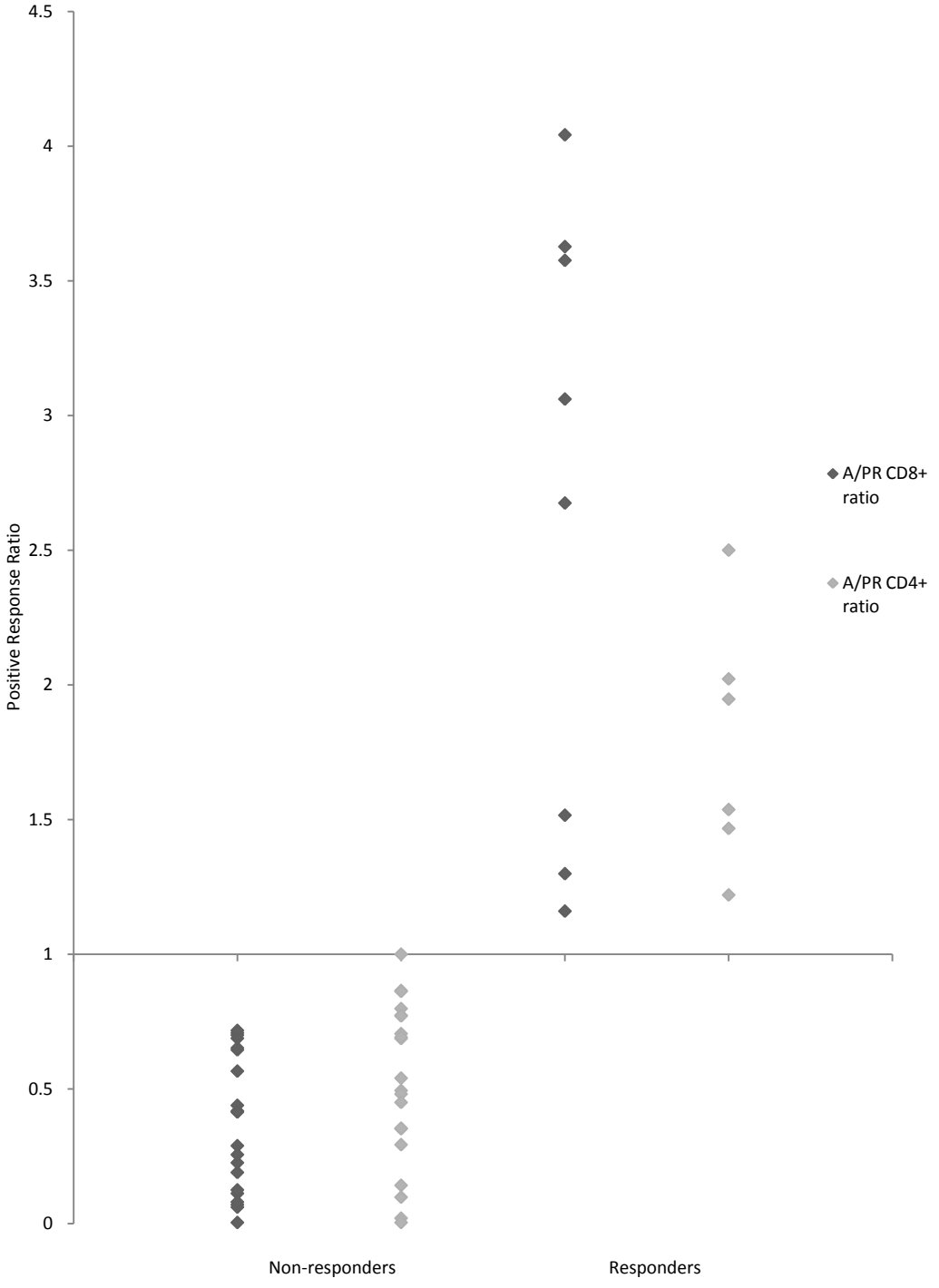

Figure 7. A/PR virus positive IFN- γ response ratios of responders and non-responders by T-cell type (n=28)*

*=Outlier $CD4^+$ postive response ratios of 50.0 and 51 .0 omitted for graph clarity

Baseline percent of interferon-gamma positive T-cells

We also found that participants who were classified as non-responders in fact had higher median baseline percent of IFN-γ positive T-cells. When the median baseline percent of IFN-γ positive T-cells of non-responders were compared between KTx and HV groups by virus and cell type, no significant difference was found (Table 4). Therefore, following analyses of the baseline percent of IFN-γ positive T-cells were completed by pooling the KTx and HV groups to compare responders to non-responders. Significance was seen in some of the virus specific T-cells but not all (Table 5; Figures 10-12). There was a significant difference in the median baseline percent of IFN-γ positive A/PR specific $CD8^+$ T-cells; the eight participants classified as responders had 0.63% IFN-γ positive $CD8^+$ T-cells; while the twenty non-responders had 1.49% IFN-γ positive $CD8^+$ T-cells ($p = 0.018$; Table 5, Figure 10). There was no significant difference in the median baseline percent of IFN-γ positive B/Lee specific $CD8^+$ T-cells; the eight participants classified as responders had 1.12% IFN-γ positive $CD8^+$ T-cells; while the twenty non-responders had 1.63% IFN-γ positive $CD8^+$ T-cells ($p = 0.199$; Table 5, Figure 11). Strong statistically signifficant inverse correlations were found between having a high median baseline percent of IFN-γ positive $CD8^+$ and $CD4^+$ T-cells and a positive response to the vaccine for stimulation with A/PR virus ($CD8^+$: Spearman`s $\rho=-0.450$, $p=0.016$; $CD4^+$: Spearman`s $\rho=-0.597$, $p=0.001$) (Figures 13 and 14). Weak inverse correlation, without statistical significance, was found between having a high median

baseline percent of IFN-γ positive $CD8^+$ T-cells and a positive response to the vaccine for stimulation with B/Lee virus (Spearman's ρ=-0.255, p=0.191). However, the correlation with $CD4^+$ T-cells was found to be a strong inverse correlation with statistical significance (Spearman's ρ=-0.607, p=0.001) (Figures 15 and 16). Modest inverse correlations were found between having a high median baseline percent of IFN-γ positive $CD8^+$ and $CD4^+$ T-cells and positive response to the vaccine for stimulation with A/Aichi virus; however, the correlation was only statistically significant for $CD8^+$ T-cells ($CD8^+$: Spearman's ρ=-0.477, p=0.025; $CD4^+$: Spearman's ρ=-0.372, p=0.089) (Figures 17 and 18).

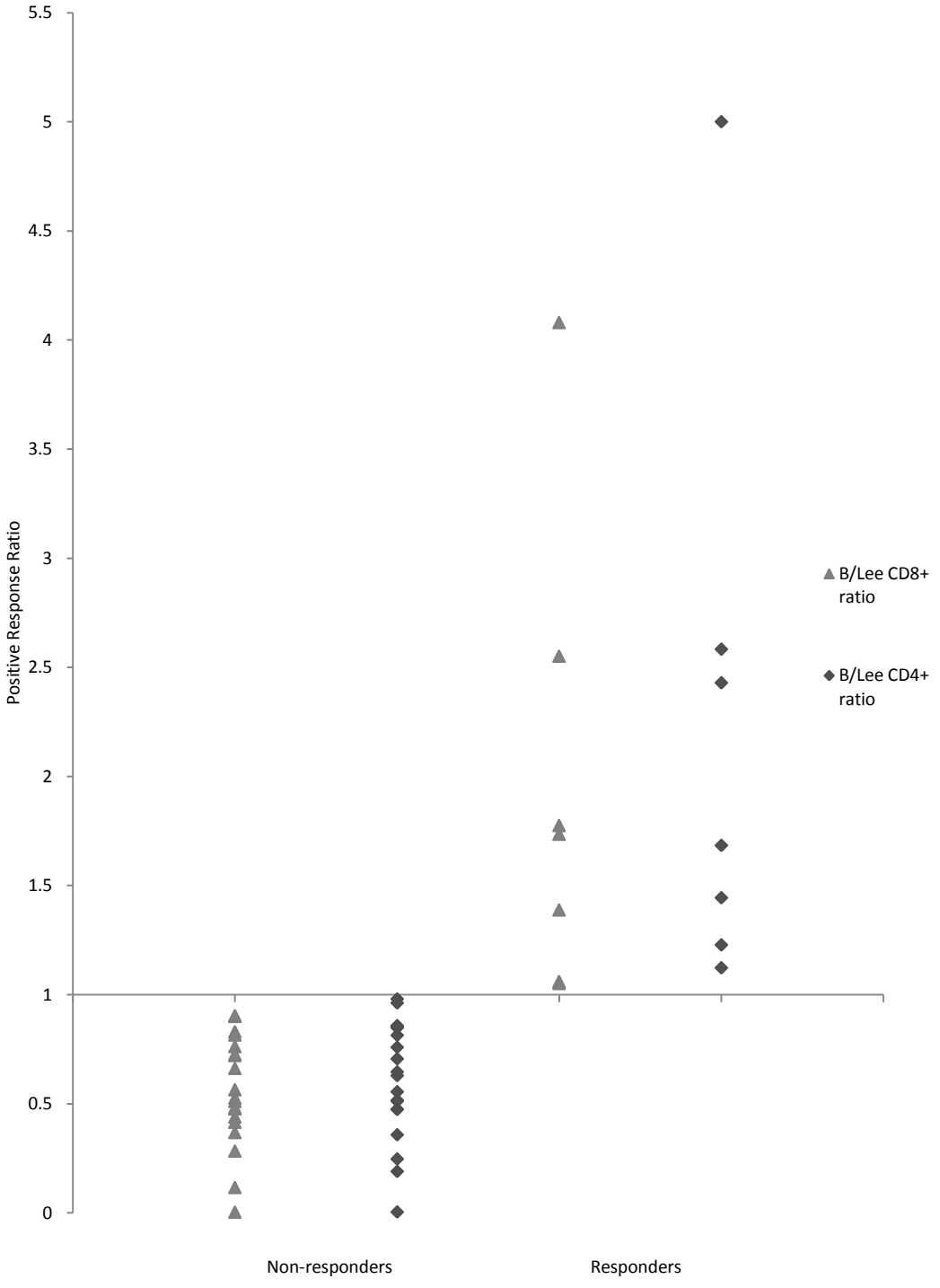

Figure 8. B/Lee virus positive INF- γ response ratios of responders and non-responders by T-cell type (n=28)*

*=Outlier $CD8^+$ positive response ratio of 37.5 and $CD4^+$ positive response ratio of 36.0 omitted for graph clarity

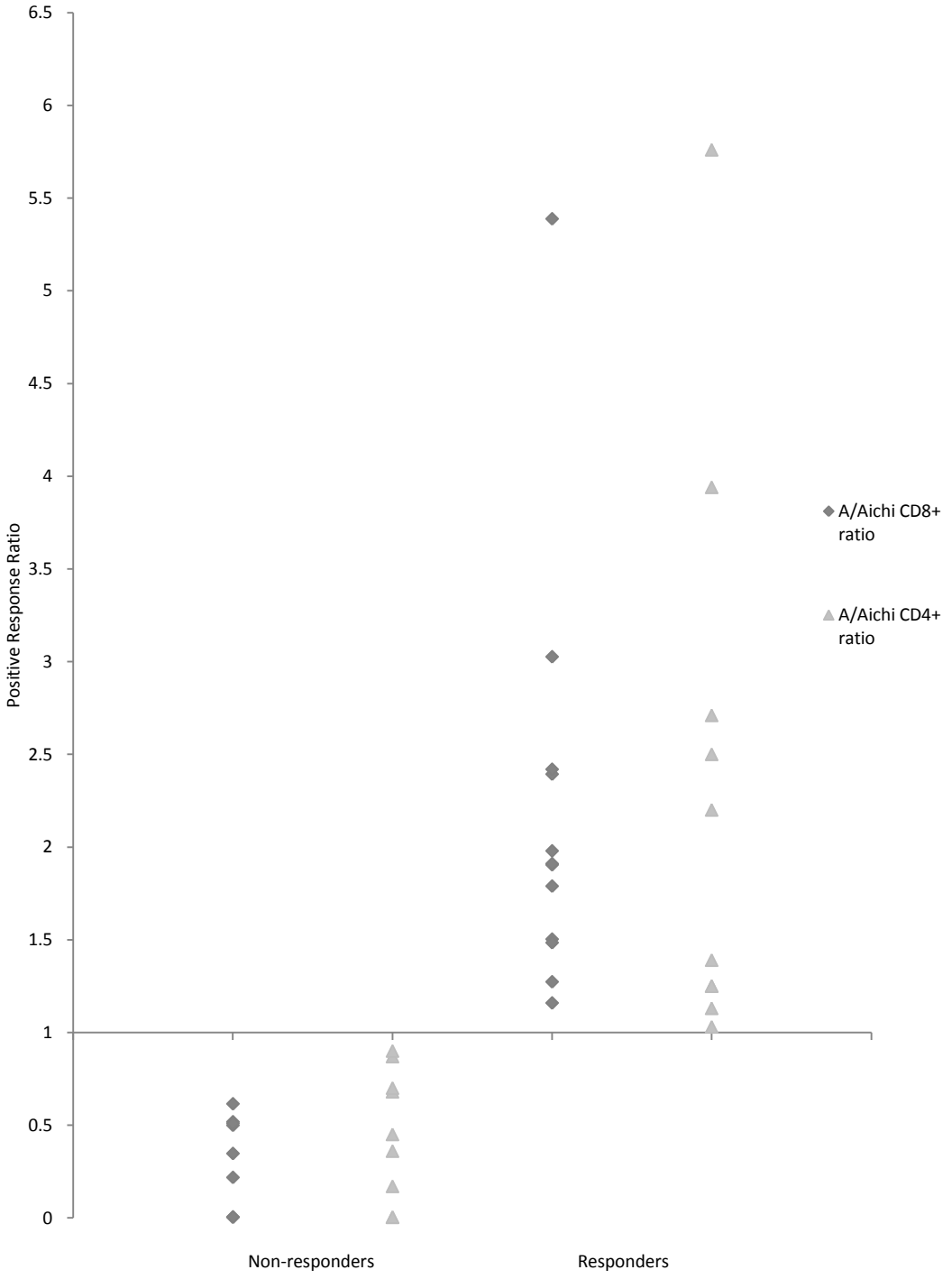

Figure 9. A/Aichi virus positive INF- γ response ratios of responders and non-responders by T-cell type (n=22)*

*=Outlier $CD8^+$ positive response ratios of 12.4 and 18.5 and $CD4^+$ positive response ratios of 138.0, 22.0 and 8.5 omitted for graph clairity

Table 5. Number and median of baseline percent IFN-γ^{+} cells of responders & non-responders according to virus and T-cell type in the overall cohort (A/PR & B/Lee: n=28; A/Aichi: n=22)

	Responders	Median Baseline Percent IFN-γ^{+} Cells for Responders	Non-responders	Median Baseline Percent IFN-γ^{+} Cells for Non-responders	p-value[a]
A/PR CD8^{+}	8	0.63 (0.12-1.16)	20	1.49 (0.15-4.00)	0.018
A/PR CD4^{+}	8	0.32 (0.01-0.84)	20	1.04 (0.01-4.76)	0.001
B/Lee CD8^{+}	8	1.12 (0.01-2.59)	20	1.63 (0.44-7.07)	0.199
B/Lee CD4^{+}	8	0.41 (0.01-1.02)	20	1.56 (0.37-3.88)	0.001
A/Aichi CD8^{+}	14	1.22 (0.22-3.78)	8	2.53 (0.57-5.10)	0.029
A/Aichi CD4^{+}	13	1.04 (0.01-4.47)	9	2.47 (0.41-5.55)	0.096

a=p-value corresponds to the comparison of median baseline IFN-γ values between responders & non-responders (Mann-Whitney U test)

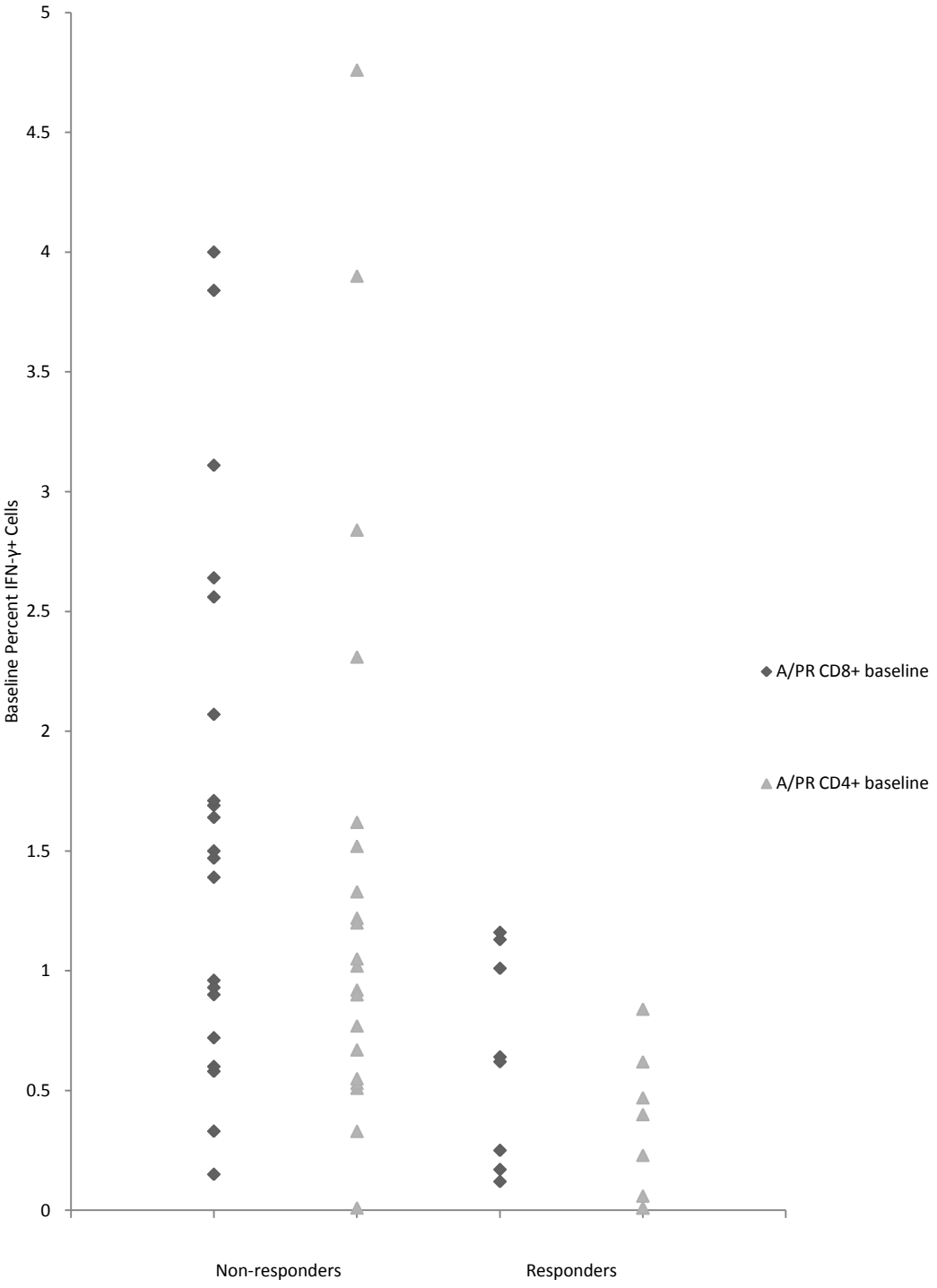

Figure 10. A/PR baseline percent IFN-γ+ cells of responders and non-responders by T-cell type (n=28)

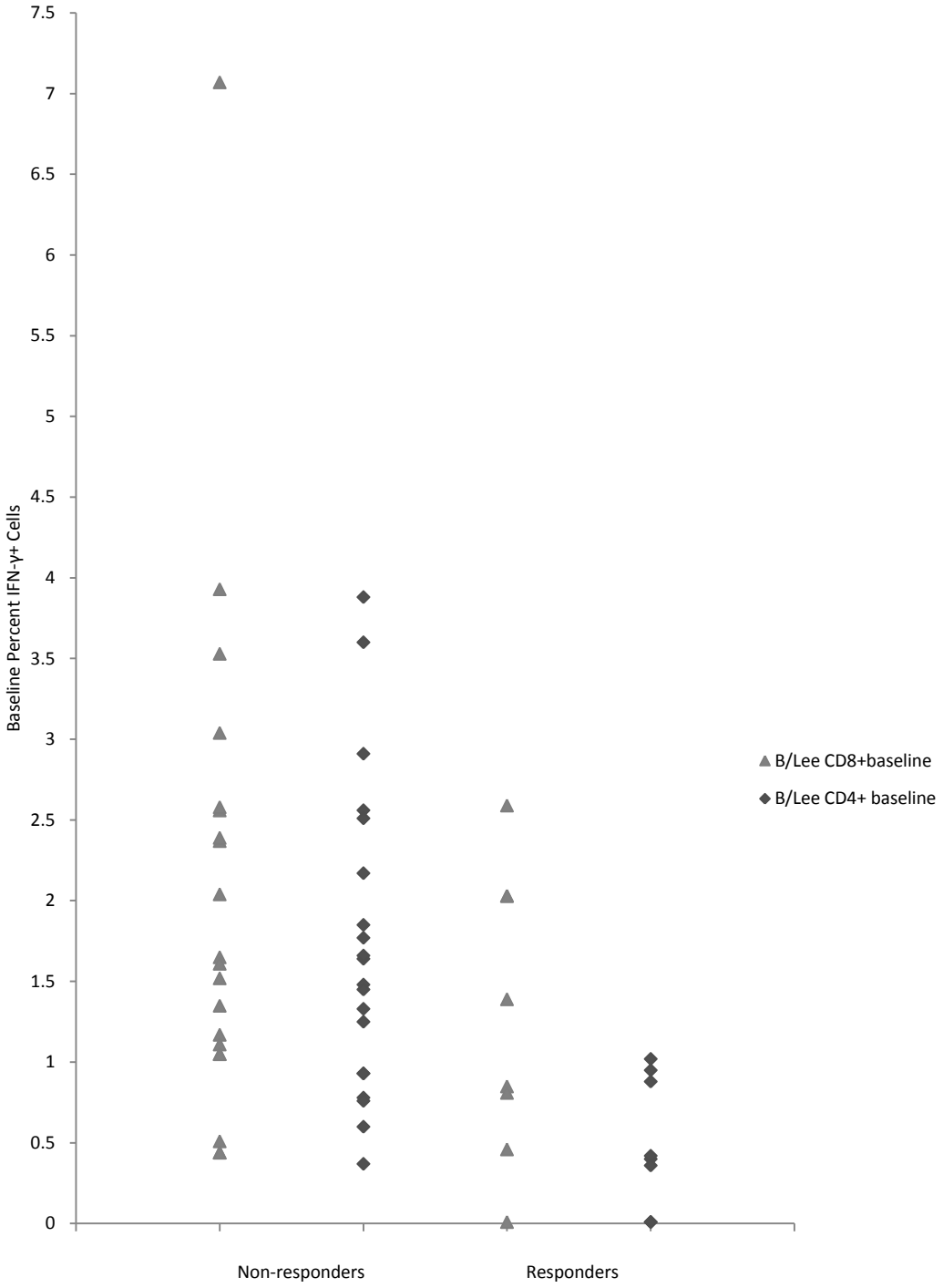

Figure 11. B/Lee baseline percent IFN-γ+ cells of responders and non-responders by T-cell type (n=28)

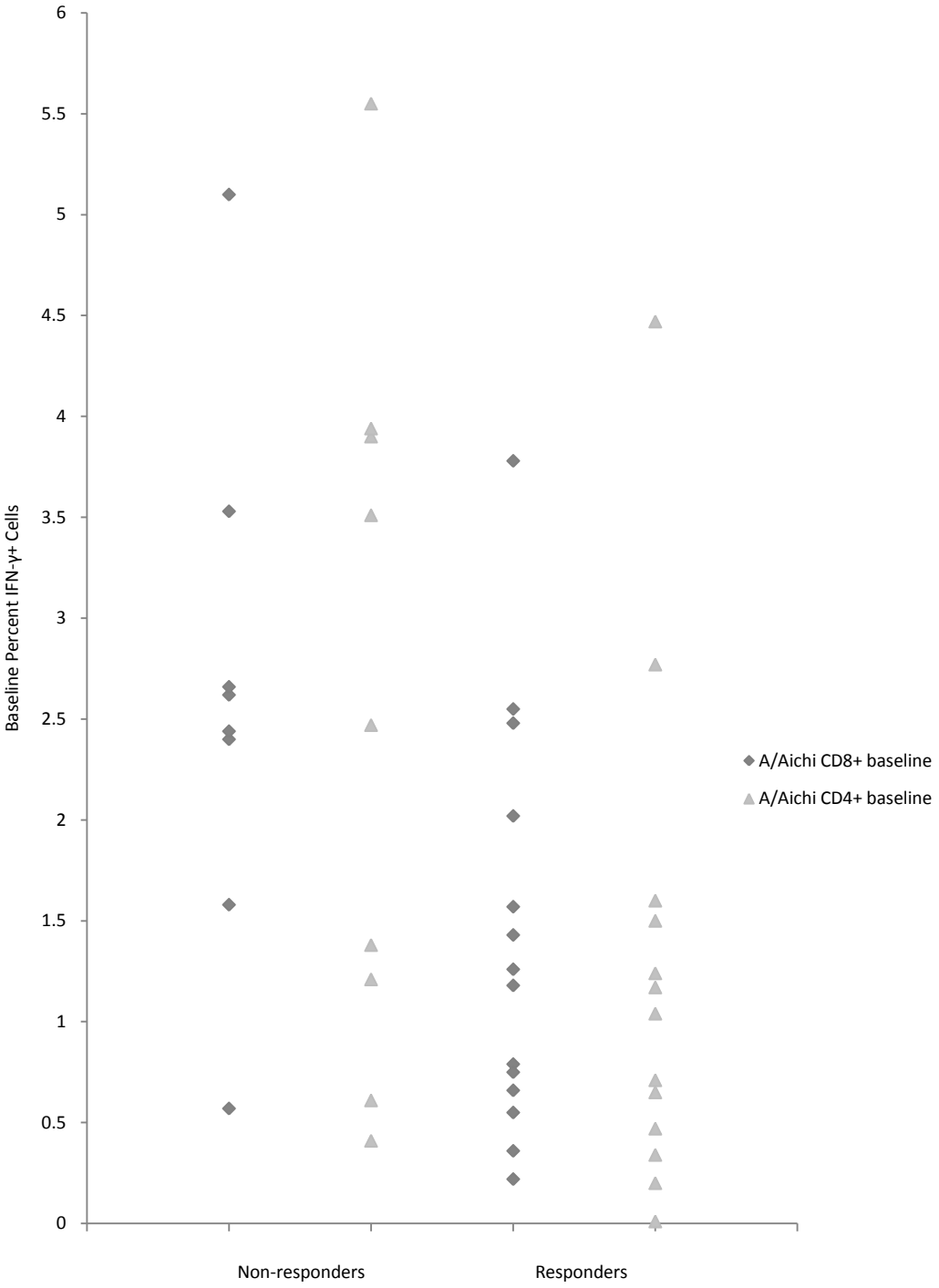

Figure 12. A/Aichi baseline percent IFN-γ+ cells of responders and non-responders by T-cell type (n=22)

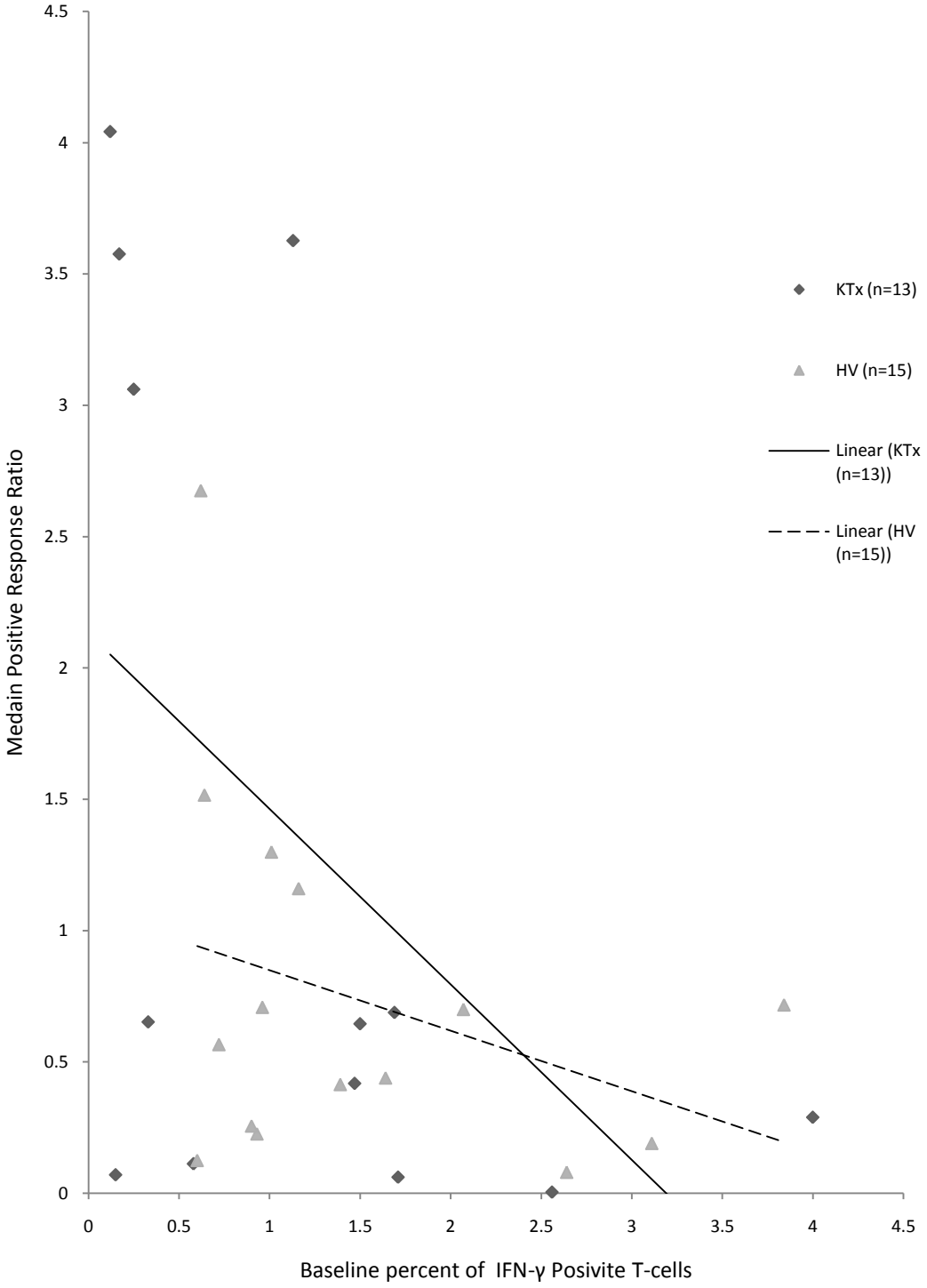

Figure 13. A/PR CD8+ T-cell positive response ratios versus baseline percent of INF-γ positive CD8+ T-cells

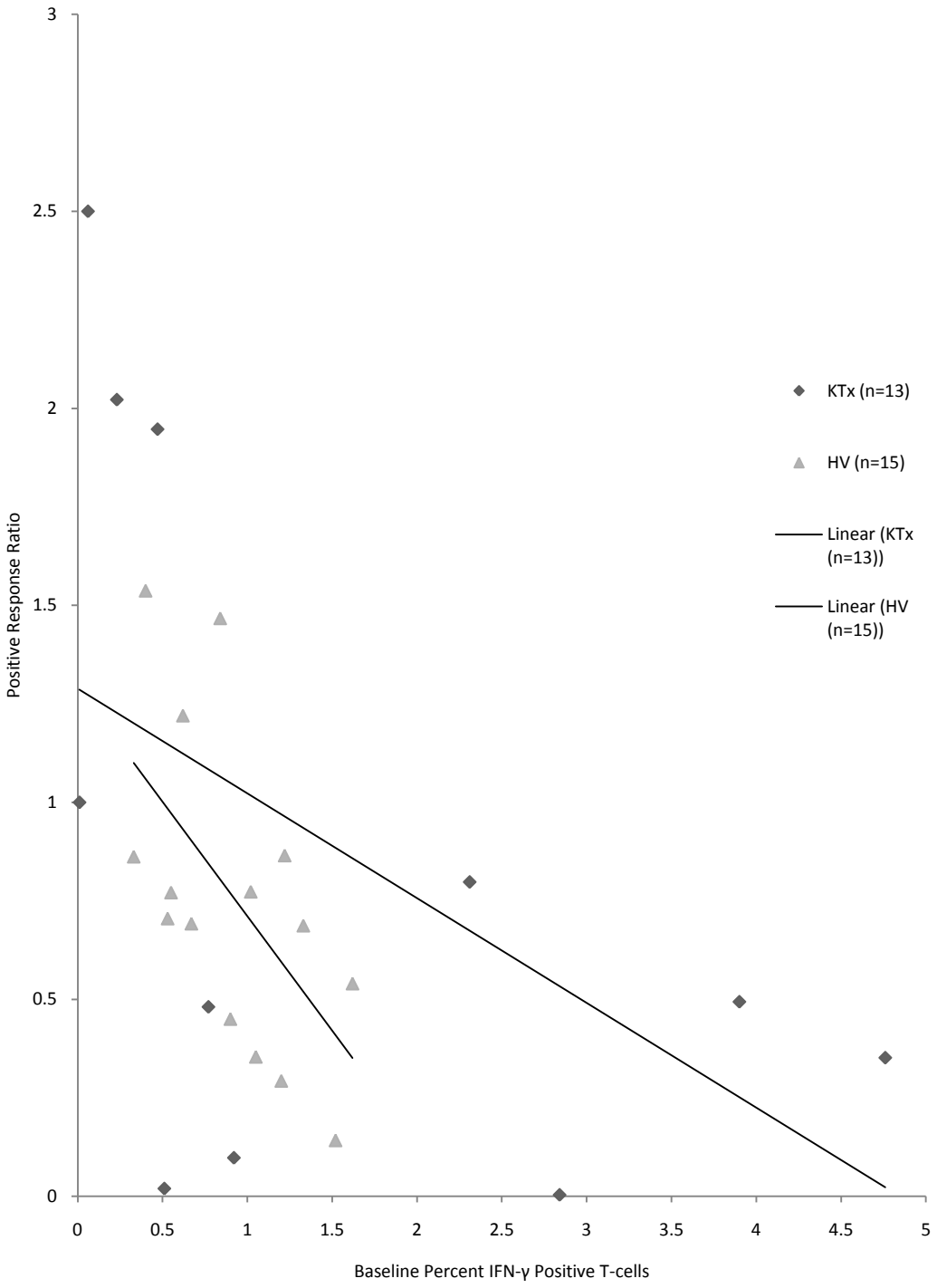

Figure 14. A/PR CD4+ T-cell positive response ratios versus baseline percent of INF-γ positive CD4+ T-cells*

*=Outlier postive response ratios of 50.0 and 51 .0 omitted for graph clarity

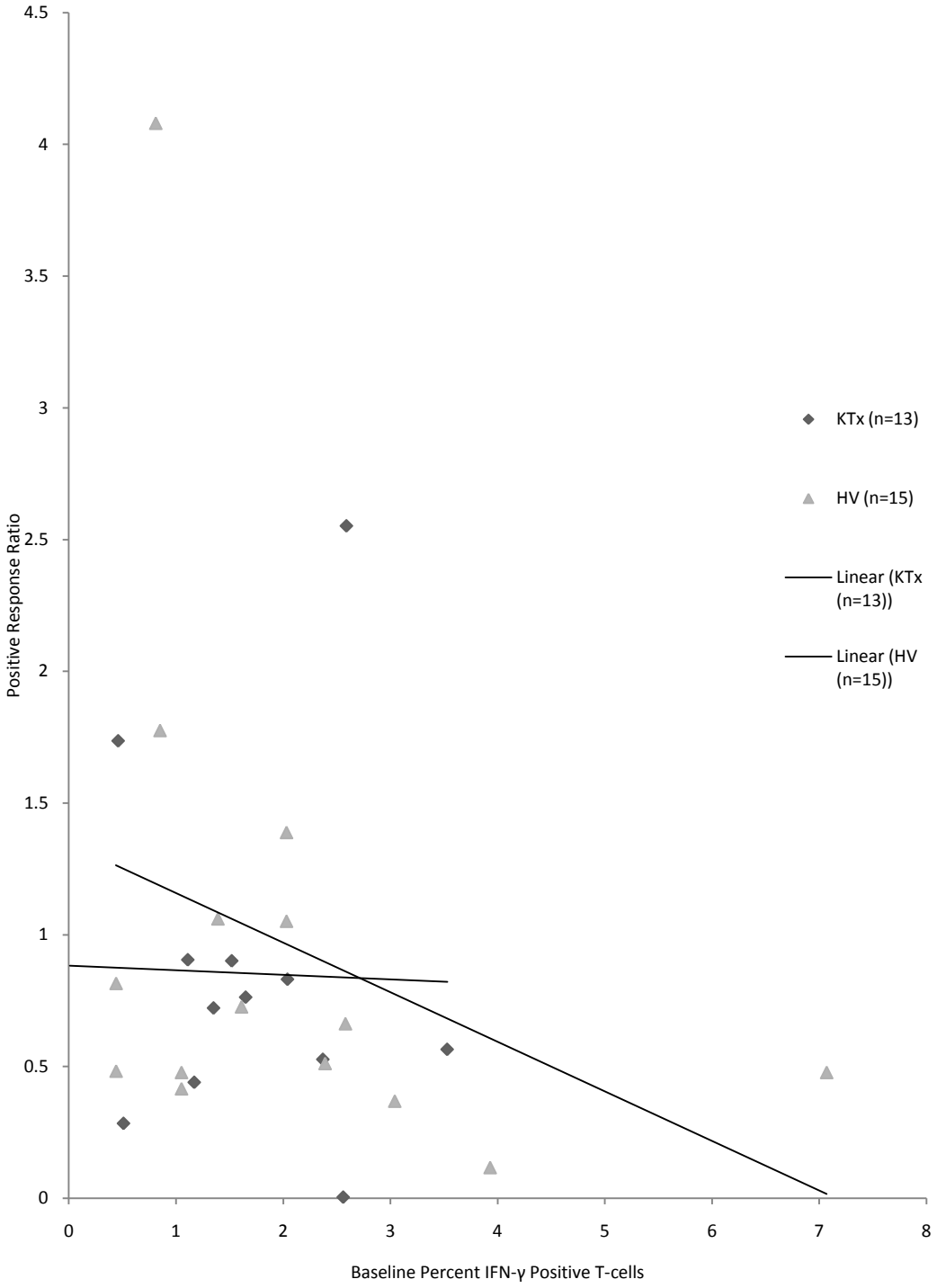

Figure 15. B/Lee CD8+ T-cell positive response ratios versus baseline percent of INF-γ positive CD8+ T-cells*

*=Outlier positive response ratio of 37.5 omitted for graph clarity

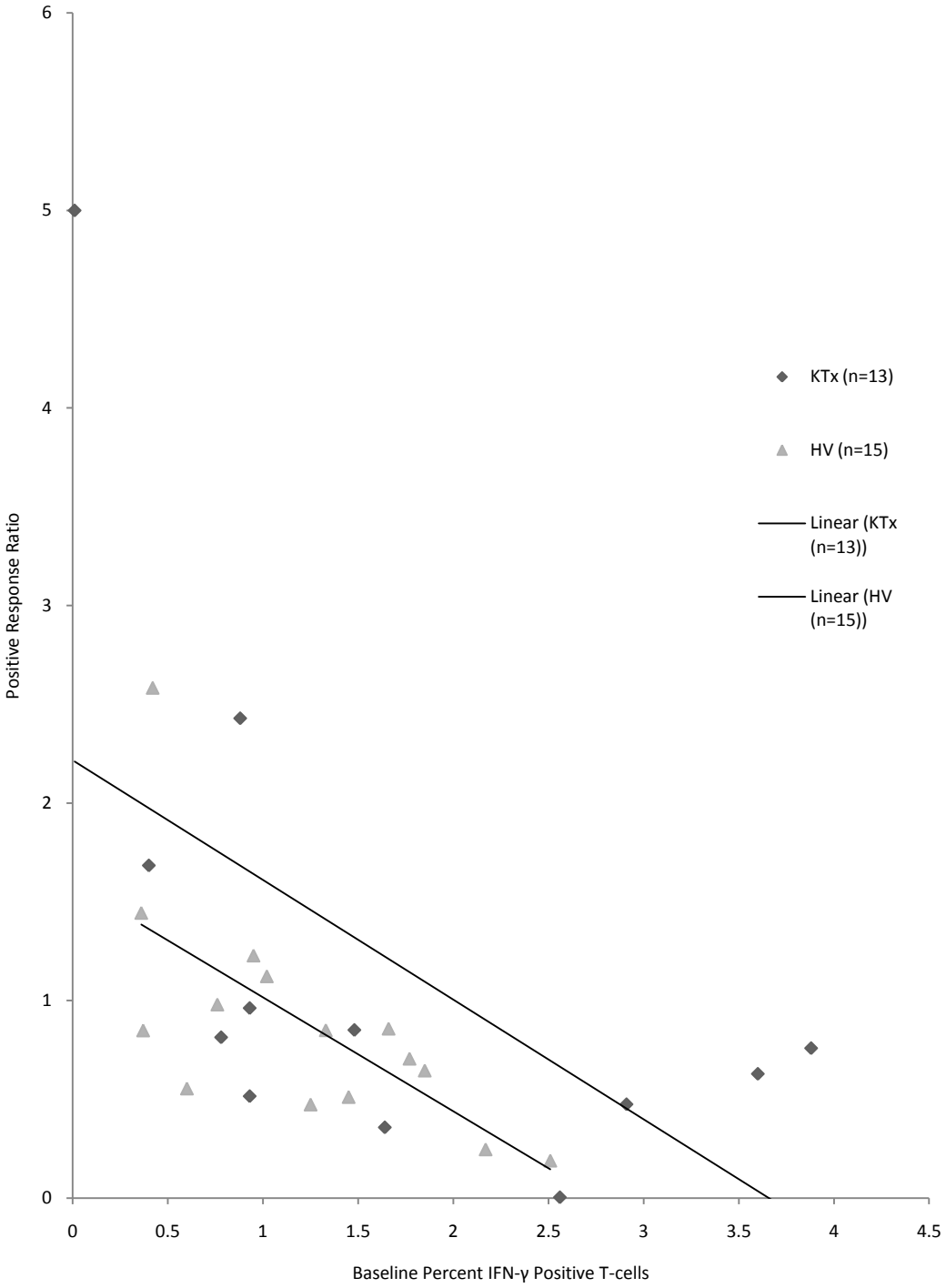

Figure 16. B/Lee CD4+ T-cell positive response ratios versus baseline percent of INF-γ positive CD4+ T-cells*

*=Outlier positive response ratio of 36.0 omitted for graph clarity

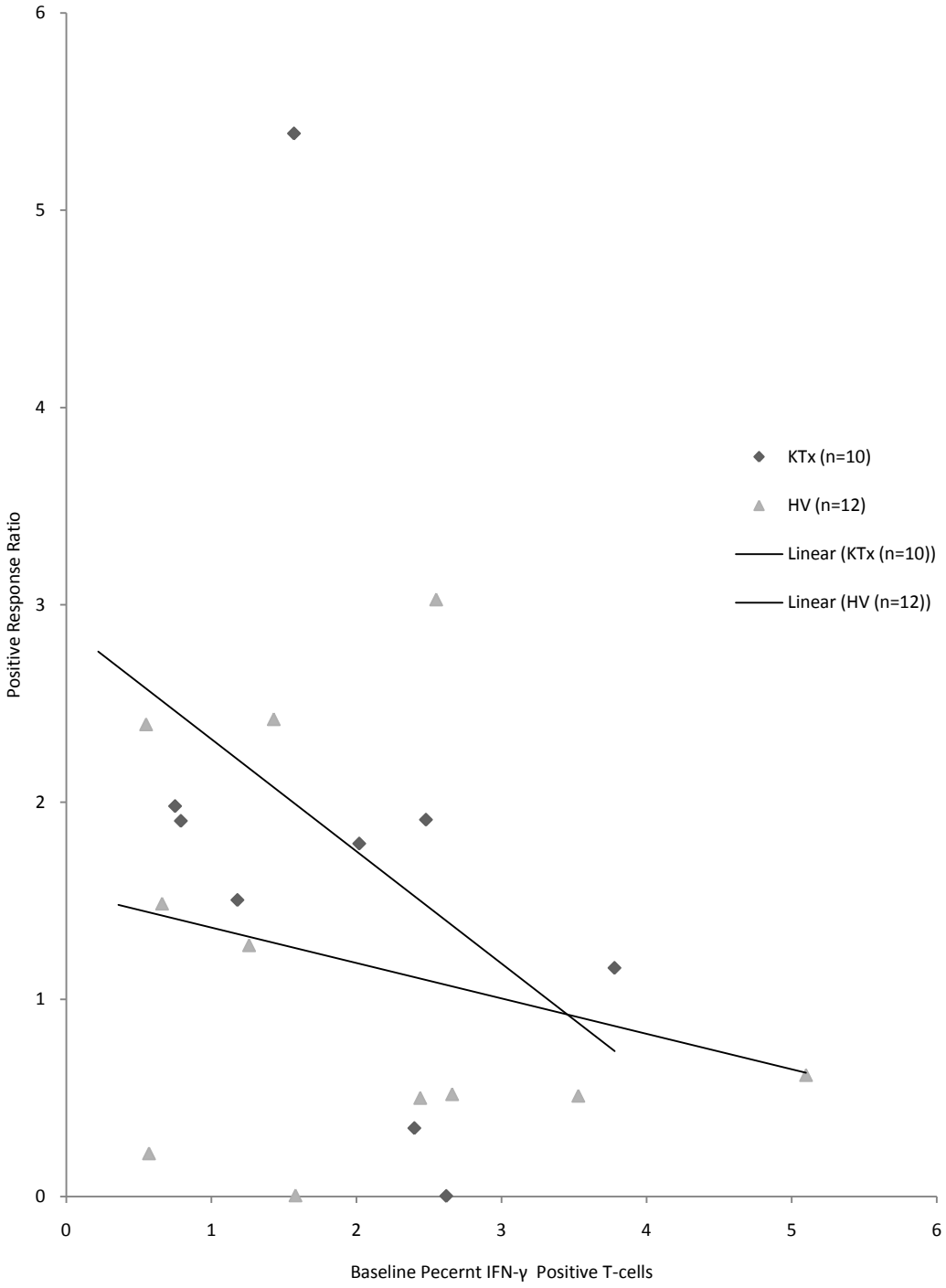

Figure 17. A/Aichi CD8+ T-cell positive response ratios versus baseline percent of IFN-γ positive CD8+ T-cells*

*=Outlier positive response ratio of 37.5 omitted for graph clarity

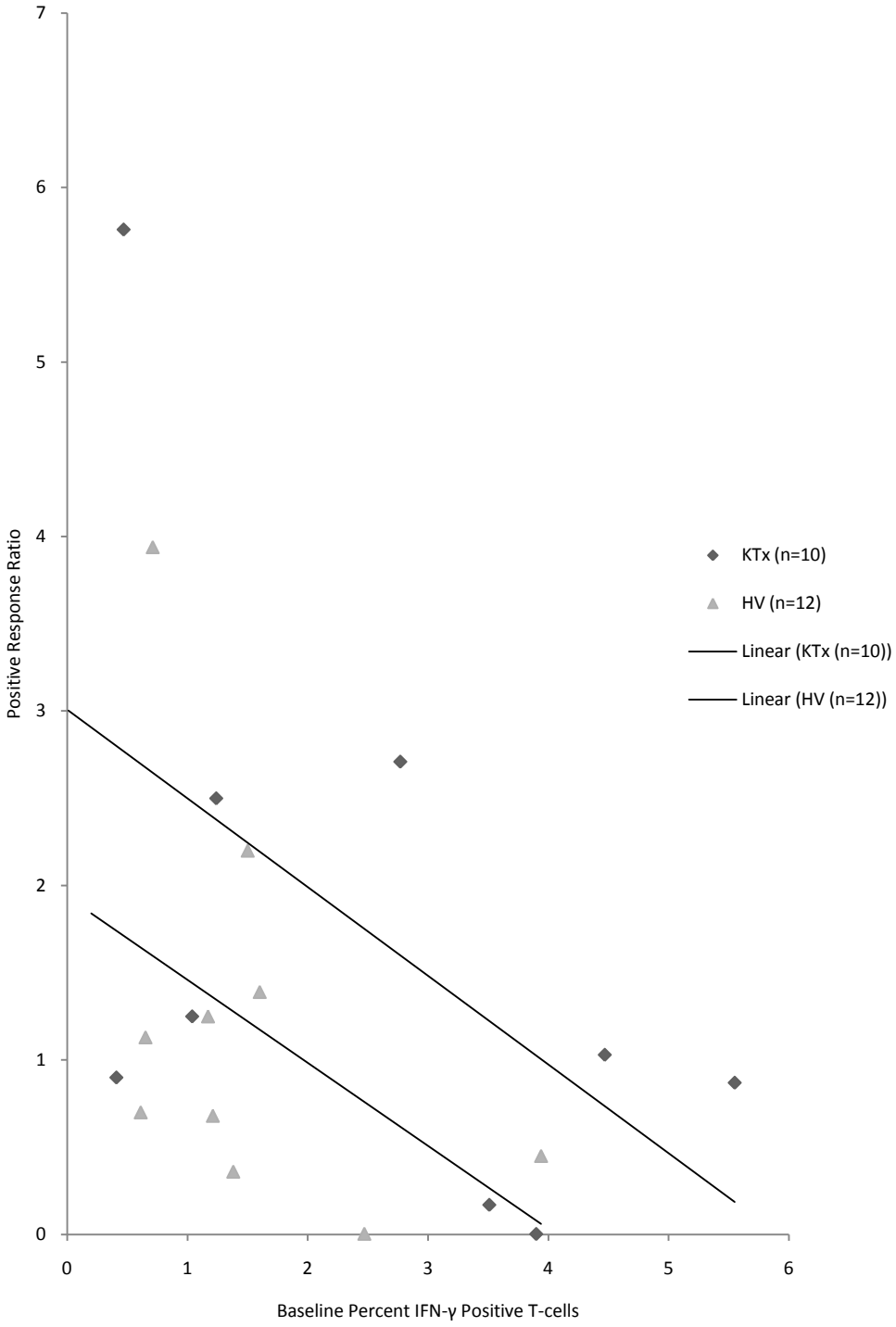

Figure 18. A/Aichi CD4+ T-cell positive response ratios versus baseline percent of IFN-γ positive CD4+ T-cells*

*=Outlier positive response ratio of 36.0 omitted for graph clarity

Serology Results

The serum of all thirteen KTx recipients and fifteen HV were tested in duplicate for antibodies against the influenza strains contained in the 2008-2009 vaccine at the Health Protection Agency in London, U.K.. A 4-fold increase in antibody levels following vaccination was considered seroconversion for a virus. In a few instances, in one replicate a participant met the 4-fold increase to be considered seroconverted but in the other replicate had not; in these instances the result was called as indeterminate. Overall more HV participants seroconverted than KTx recipients did; this was significantly different only for A/Brisbane/10/07 (Table 6). There were six instances of indeterminate results, one KTx recipient and five HV participants (Table 6). The overall responders as determined by T-cell activation and those as determined by seroconversion were compared and a weak inverse correlation without statistical significance was found (Spearman's ρ=-0.084, p=0.670). The geometric means of the titres according to virus are listed in table 7. Overall, the mean titres were higher post-vaccination than prior. Seroprotection was defined as having a titre greater that 1:40 and the number of participants with seroprotection pre- and post-vaccination is listed in table 8. There was no change in the number of KTx patients seroprotected following vaccination (Table 8). While number of seroprotected HV increased following vaccination for B/Florida/4/06 and A/Brisbane/59/07 but not A/Brisbane/10/07 (Table 8). All of the HV were seroprotected against A/Brisbane/10/07 prior to vaccination. Weak inverse

correlations without statistical significance were found between those determined to be responders by T-cell activation and being seroprotected prior to vaccination (Spearman's ρ=-0.213, p=0.276) or following vaccination (Spearman's ρ=-0.213, p=0.276).

Other results

Within the KTx group, immunosuppressive drugs individually were not factors in response to vaccination (prednisone: Pearson Chi-Square value=0.325, p=0.769; CNIs: Pearson Chi-Square value=0.325, p=0.769; MMF: Pearson Chi-Square value=0.481, p=0.692; azathioprine: Pearson Chi-Square value=0.325, p=0.769). Using linear regression significant association was found between response to vaccination and age ($b=1.655x10^{-2}$, p=0.009). However, linear regression failed to find significant association between response to vaccination and time from transplantation within the KTx recipients ($b=4.272x10^{-3}$, p=0.877).

Table 6. Seroconversion Results of KTx and HV According to Virus (KTx: n=13; HV: n=15)

	KTx			HV			
	Seroconversion	No Seroconversion	Indeterminate	Seroconversion	No Seroconversion	Indeterminate	p-value*
B/Florida/4/06	2 (15.4%)	11 (73.3%)	0 (0.0%)	7 (46.7%)	8 (53.3%)	0 (0.0%)	0.086
A/Brisbane/59/07	2 (15.4%)	11 (73.3%)	0 (0.0%)	6 (40.0%)	6 (40.0%)	3 (20.0%)	0.077
A/Brisbane/10/07	0	12 (92.3%)	1 (7.7%)	5 (33.3%)	8 (53.3%)	2 (13.3%)	0.024

*Chi-Square Test; Indeterminates removed

Table 7. Geometric Means Pre- & Post-Vaccination Titres according to Virus (n=28)

	Pre-vaccination	Post-vaccination
B/Florida/4/06	26.1	55.1
A/Brisbane/59/07	18.6	45.9
A/Brisbane/10/07	91.9	159.6

Table 8. Seroprotection Results Pre- & Post-Vaccination of KTx and HV according to Virus (KTx: n=13; HV: n=15)

	Pre-vaccination					Post-vaccination				
	KTx		HV			KTx		HV		
	Sero-protection	Without sero-protection	Sero-protection	Without sero-protection	p-value *	Sero-protection	Without sero-protection	Sero-protection	Without sero-protection	p-value *
B/Florida/4/06	5 (38.5%)	8 (61.5%)	5 (33.3%)	10 (66.7%)	0.544	5 (38.5%)	8 (61.5%)	11 (73.3%)	4 (26.7%)	0.069
A/Brisbane/59/07	2 (15.4%)	11 (84.6%)	3 (20.0%)	12 (80.0%)	0.572	2 (15.4%)	11 (84.6%)	12 (80.0%)	3 (20.0%)	0.001
A/Brisbane/10/07	7 (53.8%)	6 (46.2%)	15 (100.0%)	0 (0.0%)	0.005	8 (61.5%)	5 (38.5%)	15 (100.0%)	0 (0.0%)	0.013

*Chi-Square Test

Discussion

In this study, the CMI response of KTx recipients to the influenza vaccine was evaluated and compared to that of HV. In total 28 participants were evaluated, thirteen KTx recipients and fifteen HV. Immediately following vaccination and in the six-month follow-up period, no adverse reaction or influenza-like illness was reported; no KTx recipient was diagnosed or treated for acute rejection. This is in agreement with other studies that found the influenza vaccine safe for use in transplant recipients [4, 7-10, 12, 15-17, 20, 21].

Response rates of kidney transplant recipients and healthy volunteers

We used the definition that a positive response was a baseline to post-vaccination value of percent IFN-γ positive T-cells ratio greater than one. The definition of response is not standardized in the literature and we deemed this a conservative estimate of response. Using this criteria, 92.3% (12/13) KTx recipients and 66.7% (10/15) HV had a response to at least one virus (Table 2). No difference in response rates was seen for any comparison between HV and KTx. Therefore, by cell-mediated immunity, the immunogenicity of influenza vaccine in KTx was similar to HV. This finding that transplant patients can mount an immune response to the influenza vaccine comparable to that of healthy non-transplanted individuals is comparable to studies in KTx recipients [15-17] and in a study by Hayney *et al.* involving lung transplant recipients [19]. The three KTx studies compared the level of antibody production following vaccination and found that transplant recipients produced protective antibody titres and had

seroconversion rates similar to their non-transplant counterparts [15-17]. Briggs *et al.* and Hayney *et al.* investigated the ability to generate T-cell responses to vaccination in their transplant populations and found that here also transplant recipients generated T-cell responses similar to those produced by the HV [15, 19]. However, other studies in transplant recipients have found that either they respond poorly to the influenza vaccine [10, 12] or they respond less than healthy persons [4-9, 13, 14].

Non-responders versus responders

When comparing the number of responders to non-responders in the KTx and HV groups there are two methods to do so: as responses to individual viruses according to T-cell type or as overall an overall response any virus regardless of T-cell type. When looking at the overall response to any virus the number of responders is greater than the number of non-responders (Table 2). However, when considering the response to individual viruses by T-cell type the number of non-responders was greater than the number of responders (Table 2). This occurred because those classified as non-responders had higher baseline values of percent IFN-γ positive T-cells. Baseline values of non-responders were similar to post-vaccination values in responders. This high baseline value made it appear that the individuals were not responding to vaccination when in fact they already possessed adequate levels of immunity so no further response to activation was detected. The lack of response to vaccination as a result of

already high baseline titres has been found in other studies involving both transplant recipients [15-17] and healthy persons alone [23, 28-30]. The studies involving transplant recipients were all conducted in KTx recipients and evaluated the humoral immune response. Briggs *et al.* and Scharpe *et al.* both found that seroresponse rates were inversely related to the baseline antibody titre for all virus strains [15, 17]. Keshtkar-Jahromi *et al.* interestingly only found this inverse relationship for the influenza A strain viruses in their patient cohort [16]. He *et al.*, Sasaki *et al.* and Zeman *et al.* all invested the effect of previous immunization/baseline values had on the responsiveness of healthy persons to the influenza vaccine [23, 28, 29]. The study by He *et al.* evaluated both CMI and humoral responses to vaccination based on baseline T-cell levels and found that lower baseline percentages of IFN-γ positive $CD4^+$ T-cells predicted a larger antibody response to vaccination [28]. Zeman *et al.* also studied both the humoral and CMI responses related to the number of previous vaccinations finding that both $CD4^+$ and $CD8^+$ T-cells responses to vaccination were inversely related to the number of previous vaccinations [23]. Research by Sasaki *et al*. looked at the effect of prior vaccination on B-cell response and antibody production finding that previous vaccination lead to higher baselines of B-cell response and antibody titres [29]. These in turn were inversely related to the magnitude of response following vaccination [29]. It is unknown if the low response rate seen in many studies involving transplant recipients could be an

effect of high baseline levels because individual response rates are not reported and such an association has not been investigated by others.

Immunological memory and influenza vaccination

The large proportion of individuals in this study with already protective baseline percentages of IFN-γ positive $CD8^+$ and $CD4^+$ T-cells coupled with an overall vaccination rate (prior to the current study) of 89.3% indicates that not only does the vaccine induce a CMI response but KTx recipients, with a vaccination rate of 92.3% (12/13), are capable of producing and sustaining a CMI response following influenza vaccination. The research of Keshtkar *et al.*, Briggs *et al.* and Scharpe *et al.* also supports this conclusion, as their KTx recipients had also been previously vaccinated and many recipients had protective baseline values indicating that memory from previous vaccinations had been created and was maintained [15-17]. However, a study conducted by Kosor Krnic *et al.* in healthy subjects found opposite results [31]. In this study the subjects did not already have protective baseline levels of antibody or percentages IFN-γ positive $CD8^+$ T-cells but they did mount successful CMI and humoral responses following vaccination. However, one year later the levels of antibody and percentages IFN-γ positive $CD8^+$ T-cells had returned to the original baseline, pre-vaccination levels indicating that here the memory response was not maintained [31].

The results from this study and others [15-17] indicate that KTx recipients are capable of responding to and maintaining a memory response against

influenza following vaccination. This data seems irreconcilable unless one remembers that memory cells are not created for or able to prevent infection from occurring. Their purpose is to decrease the duration and severity of infection when it recurs, to levels unnoticed by the infected individual. However, the duration and symptoms experienced by the patients during influenza infection seem to mimic infection of naive individuals. If the patients are protected according to baseline levels, how is this occurring? The most obvious reason is that patients are being infected with non-vaccine strains. This most commonly occurs when the vaccine strains do not match the outbreak causing strains. However, a study by Jelley-Gibbs *et al.* on the effect of repeated stimulation on the protective function of $CD4^+$ T-cells in mice found that the repeated stimulation caused the $CD4^+$ t-cells to be less effective at providing help. This resulted an overall lack of responsiveness by both the type 1 (CMI) and type 2 (humoral) response systems [32]. It was also found that the repeatedly stimulated cells were less able to protect mice from lethal infection by influenza virus [32]. While the repeat stimulations happened on a much shorter time scale than what is encountered with typical vaccination strategies, it is possible that receiving the annual vaccine each year combined with being infected with influenza viruses could essentially lead to a state of 'over-stimulation'. This over-stimulation would mean that when an infection later occurred while the patient had protective baseline levels, the $CD4^+$ T-cells were

no longer capable of providing the help necessary to efficiently combat the infection before symptoms developed and leading to their prolonged duration.

Another study in mice by Legge *et al.* found that high titres of influenza virus in the lungs during infection inhibited the efficient activation of $CD8^+$ T-cells and even led to the increased apoptosis of activated $CD8^+$ T-cells [33]. This could be an important factor in influenza infection in transplant recipients. Because transplant patients are immunosuppressed, it is possible that during the time it takes to mount an effective immune response, the virus could replicate to high titres. These high titres could then trigger the inhibition described by Legge *et al.* [33] leading to more severe symptoms and longer durations even though the patient is protected as indicated by baseline levels.

Increasing vaccine efficacy: Adjuvants

Given that there are conflicting studies on the immunogenicity of influenza, vaccination coupled with there being a possible suboptimal state of immune protection, other methods to increase immunogenicity need to be sought. One method to increase the immunogenicity of a vaccine is to include an adjuvant; this is any substance that non-specifically enhances the immune response to a particular vaccine. Several different studies have been conducted in mice and humans to assess the effect of various adjuvants on the immunogenicity of the influenza vaccine. Guebre-Xabier *et al.* used mice to evaluate the use of an immunostimulatory patch that could be placed on or near

the site of injection [34]. The idea of using a patch is not unusual and is under investigation as an alternative to injected cold-storage vaccines that makes vaccinating large numbers of people in developing countries and remote areas difficult. The patch used by Guebre-Xabier *et al.* contained heat-labile enterotoxin from *Escherichia coli* as an adjuvant that was applied to the skin from one hour up to overnight following vaccination to determine the impact on vaccine immunogenicity [34]. The patch increased the production of both serum and mucosal antibodies as well as the CMI response [34]. The cause of this increased immunogenicity was due to the activation of dermal dendritic cells that went on to directly activate the immune cells or indirectly activated by the production of pro-inflammatory cytokines [34]. Importantly the antibodies produced and the T-cells activated were specific for influenza and not for the enterotoxin, suggesting that the toxin caused a specific increase in immunogenicity of the vaccine not just an overall nonspecific increase in the activation of the immune system [34].

Soo Hoo *et al.* also used mice to evaluate the ability of antibodies against T-cell immunoglobulin mucin-1 (TIM-1) to act as an adjuvant for the influenza vaccine [35]. Mice were vaccinated with vaccine preparation containing TIM-1 antibodies or placebo. The production of influenza-specific antibodies and proliferation and activation of influenza specific T-cells was evaluated [35]. Soo Hoo *et al.* found that the addition of the TIM-1 antibodies made the vaccine more immunogenic then the vaccine alone and the antibodies and T-cells

generated were specific for influenza [35]. Interestingly the TIM-1 antibodies specifically activated the T_H1 response by activating and directing the $CD4^+$ cells [35].

In humans, Brignone *et al.* tested a natural high affinity ligand for MHC class II known as IMP321, which induces the maturation and migration of dendritic cells to lymph nodes allowing for the activation of T-cells [36]. Healthy volunteers were vaccinated with mixture of the standard split virion vaccine preparation and IMP321 or saline and the production of antibodies and T-cell activation were measured [36]. Brignone *et al.* found that the addition of IMP321 resulted in activation of T_H1 responsive $CD4^+$ T-cells specific for influenza but no increase in antibody production [36].

A second study in humans evaluating the immunogenicity of vaccines based on immune stimulating complexes (ISCOM™) was conducted by Rimmelzwaan *et al.* [30]. There were two different ISCOM™ vaccines: one in which the ISCOM™ lattice formed in the presence of the influenza antigen and a second in which the ISCOM™ lattice formed without the influenza antigen and then was added to the vaccine preparation afterwards [30]. Healthy volunteers were either vaccinated with an ISCOM™ vaccine or the standard split virion vaccine, and resulting antibody production and T-cell activation were evaluated [30]. The study found that there was no difference in the ability of the different ISCOM™ vaccine preparations to increase the immunogenicity of the influenza

vaccine [30]. Both ISCOM™ vaccines resulted in more rapid production of influenza-specific antibody titres, enhanced the proliferation of influenza-specific T-cells and increased the number of vaccinated people achieving a CMI response compared to the conventional vaccine [30].

Despite the promising developments in the research detailed above in which an influenza vaccine containing the MF59 adjuvant, an oil-in-water emulsion, was compared to the standard split virion vaccine, in transplant patients the results were anything but stellar [21]. Magnani *et al.* compared the two vaccine preparations in heart transplant patients and found no difference in immunogenicity between the two when antibody response and clinical symptoms of infection were compared [21].

Since adjuvants work by non-specifically activating the immune response likely through the innate immune system, there is concern that this could lead to allograft rejection. The nature of non-specificity of the innate immune system does make it possible that activated dendritic cells will cause the activation of T- and B-cells that will recognise the graft as foreign leading to rejection. However, the method by which dendritic cells process antigen appears to be antigen-specific. Since vaccination occurs at a site distant from the allograft, this makes it less likely that a misdirected response of the immune system towards the graft would occur. Even though the study by Magnani *et al.* did not find any difference in immunogenicity of the MF59 vaccine compared to the standard vaccine there

were no cases of rejection or other graft-related complications occurring as a result of the use of adjuvant in these heart transplant recipients [21]. The area of adjuvants in influenza vaccination needs to be investigated more to find new safe adjuvants and determine their safety and immunogenicity in transplant recipients.

Increasing vaccine efficacy: Antigen preparation

The most commonly used influenza vaccine preparation is a split virion that is made up of components of the inactivated influenza virus. This means that the virus has been denatured in some form, most often chemically, to create the components and this can cause the denaturation of the virus proteins as well. This denaturation of the virus proteins can create epitopes that are not actually present in the whole virus state creating antibodies and T-cells that have no real use during an infection. Using inactivated whole virus vaccine preparations prevents this issue and allows for more epitopes that are natural to be recognised and activated against. The drawback of using a whole virion vaccine preparation is that they generally have higher reactogenicity, the capability of causing an immunological reaction, then other types can leading to adverse effects following vaccination [37, 38]. In addition, because it is inactivated whole virus depending on what method was used to inactivate the virus reversion could occur after vaccination leading to acute atypical infection. Inactivated whole influenza virus vaccine preparations are licensed for and are

used but with less frequency than the split virion preparation. Szyszko *et al.* used mice to evaluate if there was a difference in the immunogenicity of inactivated whole virus vaccine preparations and the split virion preparations [37]. Mice were vaccinated with either one of the preparations and the resulting cytokine production was evaluated for a difference in immunogenicity and direction of response- type 1 or type 2 [37]. Szyszko *et al.* found that the whole vaccine preparation was more effective at overall stimulation of the immune response to influenza but particularly effective in directing the immune system towards a type 1-CMI response [37].

Increasing vaccine efficacy: Booster vaccination

Booster vaccination works on the presumption that during the response to the vaccine if a second dose is given it will further the activation of the immune system because of an influx of more antigens whiles the system is partially primed. This system is employed in vaccination strategies for other diseases like Hepatitis B but often employs multiple booster injections. When the ability of a booster to increase the immunogenicity of the influenza vaccine was investigated in kidney transplant recipients by Smith *et al*. and Scharpe *et al.* it was found that the booster had no effect on the production of influenza specific antibodies [8, 17]. This was also found to be the case in lung [39] and heart transplant recipients [6]. In pediatric liver transplant recipients, boosters had no effect on the production of antibodies against influenza [13]. Conversely, when

boosters were evaluated by Soesman *et al.* it was found that the booster did increase the influenza-specific antibody response but did not affect the CMI response [14]. More study in this area needs to be conducted to determine if boosters are a viable option to increase immunogenicity and in what preparation and schedule.

Influenza vaccination and heterosubtypic immunity

In theory, an effective vaccine would require a single dose or series of doses and provide lifetime protection against all the strains of one virus. The influenza vaccine is an annual vaccine primarily due to variation in annual circulating strains. In addition, infection with one strain does not guarantee protection to another strain. It is thought that the HA and NA proteins are too varied between influenza strains for effective heterosubtypic immunity or cross-protection to occur. While the results obtained in this study of KTx patients are not a direct measure of heterosubtypic immunity, the ability of influenza lab strains to effectively activate the participants' T-cells indicates that at some level there is cross-recognition of strains. This phenomenon was also described in mice by two different researchers evaluating the ability of the influenza vaccine to cause heterosubtypic immunity [35, 40]. In the study by Soo Hoo *et al.,* the addition of TIM-1 antibodies to the vaccine preparation resulted in activation of serotype cross-protection that was type 1-CMI in nature between H1N1 strains and H3N2 strains when the mouse was immunised with a H1N1 strain and

challenged with H3N2 strains [35]. In the study by Droebner *et al.,* mice were immunised with a low pathogenic H5N2 influenza virus strain then lethally challenged with a highly pathogenic H5N1 strain [40]. The mice survived the H5N1 challenge because of the production of cross-reactive antibodies and $CD4^+$ T-cells from the H5N2 immunization [40]. Researching methods of increasing the heterosubtypic immunity of the influenza vaccine is important for seasons in which the circulating and vaccine strains do not match and in times of pandemics.

This study of the CMI response in KTx recipients found that it was equal to that of HV and that the vaccine is safe for use in this population and should continue to be received by all KTx recipients annually. It was also discovered that having a protective CMI response prior to vaccination results in lower response rates to the new vaccine. In addition, many KTx recipients and HV had these protective levels prior to vaccination and if one was to assume that these groups were not unique within the KTx and HV populations then many people already may have protective levels. In the case of healthy individuals with no other indication to receive the vaccine, i.e. the general healthy population, vaccination every other year may be sufficient as long as circulating strains are not highly varied. However, this must be extensively researched before such a recommendation could be made clinically. As further study to evaluate the interaction between the vaccine and the transplant recipient immune system, it

would also be interesting to evaluate the production of anti-HLA antibodies in this cohort following vaccination.

Study limitations

Our study had some limitations. One limitation of the study was the small sample size with only thirteen KTx and fifteen HV evaluated. This sample size, however, allowed us to develop our assay and gather enough results to be hypothesis generating for future studies. Studies still need to be completed in larger groups before concrete recommendations can be created from the results.

Another limitation is that the memory T-cell population is made up of two different cells located in two different areas of the body. The central-memory T-cells (T_{CM}) are located within the lymphoid organs and are responsible for replacing effector-memory T-cell (T_{EM}) during re-infection [41]. On the other hand, T_{EM} cells are located in the periphery and are responsible for the immediate protection during re-infection and are incapable of proliferation [41]. Therefore, it is possible that the majority of T-cells recovered from the participants were the T_{EM} cells resulting in lower numbers of generated activated cells after a 20-hour stimulation because the lack of proliferation and could be the reason that in non-responders an increase to vaccination was not seen.

The blood contains both naive and memory T-cells but when flow cytometry was done no distinction was created; thus, the resulting response is a mixture of naive and memory cells responding to the stimulation by viruses

either directly or through heterosubtypic immunity. To alleviate this T-cells could have been stained for the presence of one or more memory-specific markers such as CD69 [23, 31, 33], CD80 [33] or CD45RO [41] of the lack of CD27 [41]. However, the FACSArray flow cytometer is only capable of detecting four different fluorescence colours and these were all required for the identification and distinction of $CD8^+$ and $CD4^+$ cells and the intracellular expression of IFN-γ.

A further limitation was that the T_H1 and T_H2 responses were not separated from each other in the $CD4^+$ T-cell response to determine whether there was a difference in activation of the components of the immune system in response to vaccination. However, this again would have required a flow cytometer that could recognise more than four colours at a time and while such a machine was available, it was not feasible for use this study.

The viruses used for stimulation provided another limitation as they were laboratory strains of the same HA and NA subtypes but not identical to the strains in the vaccine or that circulated during the 2008-2009 influenza season. This means that the response observed of the patient T-cells to the stimulatory virus may be less specific than if using actual vaccine strains. Vaccine strains in the quantity required for the study were not feasible to grow. Future studies could use the vaccine strain although appropriate MOIs for cell stimulation would again need to be determined.

A final limitation may be the use of IL-2 and anti-CD3 antibodies alone to increase activation and proliferation. Interleukin-2 is a nonspecific proliferation signal for activated T-cells and anti-CD3 antibody results in cross-linking of the TCR, signal one, but neither of these provides co-stimulatory signals required. It is possible to use anti-CD28 antibodies alone [32] or together with anti-CD49d antibodies [23, 31, 36]. CD28 is a co-stimulatory molecule that interacts with B7 to provide the second activation signal in T-cells, while CD49d can also act as a co-stimulatory molecule on T-cells [1]. However, as memory T-cells are partially primed and do not require the same amount of stimulation for activation, the use of anti-CD28 antibodies is not necessary and in fact stimulation through CD28 is not required for memory T-cell activation [1].

Bibliography

1. Richiard A. Goldsby, T.J.K., Barbara A. Osborne, Janis Kuby, *Immunology*. 5th ed. 2003: W.H. Freeman and Company.
2. CIHI, *Cumulative Report - 2008*, in *e-Statistics on Organ Transplants*, *Waiting Lists and Donors*. 2009: Ottawa.
3. UNOS, *2008 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 1998-2007*. . 2009.
4. Birdwell, K.A., et al., *Decreased Antibody Response to Influenza Vaccination in Kidney Transplant Recipients: A Prospective Cohort Study.* Am J Kidney Dis, 2009.
5. Ballet, C., et al., *Humoral and cellular responses to influenza vaccination in human recipients naturally tolerant to a kidney allograft.* Am J Transplant, 2006. **6**(11): p. 2796-801.
6. Blumberg, E.A., et al., *The immunogenicity of influenza virus vaccine in solid organ transplant recipients.* Clin Infect Dis, 1996. **22**(2): p. 295-302.
7. Sanchez-Fructuoso, A.I., et al., *Influenza virus immunization effectivity in kidney transplant patients subjected to two different triple-drug therapy immunosuppression protocols: mycophenolate versus azathioprine.* Transplantation, 2000. **69**(3): p. 436-9.

8. Smith, K.G., et al., *Suppression of the humoral immune response by mycophenolate mofetil.* Nephrol Dial Transplant, 1998. **13**(1): p. 160-4.
9. Mazzone, P.J., et al., *The humoral immune response to influenza vaccination in lung transplant patients.* Eur Respir J, 2001. **18**(6): p. 971-6.
10. Mazzone, P.J., et al., *Cell-mediated immune response to influenza vaccination in lung transplant recipients.* J Heart Lung Transplant, 2004. **23**(10): p. 1175-81.
11. Blumberg, E.A., et al., *Safety of influenza vaccine in heart transplant recipients.* J Heart Lung Transplant, 1998. **17**(11): p. 1075-80.
12. Kimball, P., S. Verbeke, and D. Tolman, *Influenza vaccination among heart transplant recipients.* Transplant Proc, 2001. **33**(1-2): p. 1785-6.
13. Madan, R.P., et al., *A prospective, comparative study of the immune response to inactivated influenza vaccine in pediatric liver transplant recipients and their healthy siblings.* Clin Infect Dis, 2008. **46**(5): p. 712-8.
14. Soesman, N.M., et al., *Efficacy of influenza vaccination in adult liver transplant recipients.* J Med Virol, 2000. **61**(1): p. 85-93.
15. Briggs, W.A., et al., *Influenza vaccination in kidney transplant recipients: cellular and humoral immune responses.* Ann Intern Med, 1980. **92**(4): p. 471-7.
16. Keshtkar-Jahromi, M., et al., *Antibody response to influenza immunization in kidney transplant recipients receiving either azathioprine or mycophenolate: a controlled trial.* Am J Nephrol, 2008. **28**(4): p. 654-60.
17. Scharpe, J., et al., *Influenza vaccination is efficacious and safe in renal transplant recipients.* Am J Transplant, 2008. **8**(2): p. 332-7.
18. Willcocks, L.C., et al., *The effect of sirolimus therapy on vaccine responses in transplant recipients.* Am J Transplant, 2007. **7**(8): p. 2006-11.
19. Hayney, M.S., et al., *Lung transplant patients' T cell responses to influenza vaccine viruses between seasons.* Vaccine, 2008. **26**(21): p. 2596-600.
20. Manuel, O., et al., *Immunogenicity and safety of an intradermal boosting strategy for vaccination against influenza in lung transplant recipients.* Am J Transplant, 2007. **7**(11): p. 2567-72.
21. Magnani, G., et al., *Safety and efficacy of two types of influenza vaccination in heart transplant recipients: a prospective randomised controlled study.* J Heart Lung Transplant, 2005. **24**(5): p. 588-92.
22. Lindemann, M., et al., *ELISpot assay as a sensitive tool to detect cellular immunity following influenza vaccination in kidney transplant recipients.* Clin Immunol, 2006. **120**(3): p. 342-8.
23. Zeman, A.M., et al., *Humoral and cellular immune responses in children given annual immunization with trivalent inactivated influenza vaccine.* Pediatr Infect Dis J, 2007. **26**(2): p. 107-15.
24. McElhaney, J.E., et al., *T cell responses are better correlates of vaccine protection in the elderly.* J Immunol, 2006. **176**(10): p. 6333-9.
25. Centers for Disease Control and Prevention. *How often are the vaccine and circulating virus strains well matched?: 2007-2008 Influenza (Flu) Season Questions & Answers*. 2008 July 17, 2008 June 17, 2009]; Available from: http://www.cdc.gov/flu/about/qa/season.htm.
26. Gangappa, S., et al., *Immune responsiveness and protective immunity after transplantation.* Transpl Int, 2008. **21**(4): p. 293-303.

27. Eisenthal, A., et al., *Costimulatory activity of inactivated influenza virus in response of human peripheral blood mononuclear cells to phytohemagglutinin A and anti-CD3 antibody.* Viral Immunol, 1998. **11**(3): p. 137-45.
28. He, X.S., et al., *Baseline levels of influenza-specific CD4 memory T-cells affect T-cell responses to influenza vaccines.* PLoS ONE, 2008. **3**(7): p. e2574.
29. Sasaki, S., et al., *Influence of prior influenza vaccination on antibody and B-cell responses.* PLoS ONE, 2008. **3**(8): p. e2975.
30. Rimmelzwaan, G.F., et al., *A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines.* Vaccine, 2000. **19**(9-10): p. 1180-7.
31. Kosor Krnic, E., et al., *Enumeration of haemagglutinin-specific CD8+ T cells after influenza vaccination using MHC class I peptide tetramers.* Scand J Immunol, 2008. **67**(1): p. 86-94.
32. Jelley-Gibbs, D.M., et al., *Repeated stimulation of CD4 effector T cells can limit their protective function.* J Exp Med, 2005. **201**(7): p. 1101-12.
33. Legge, K.L. and T.J. Braciale, *Lymph node dendritic cells control CD8+ T cell responses through regulated FasL expression.* Immunity, 2005. **23**(6): p. 649-59.
34. Guebre-Xabier, M., et al., *Immunostimulant patch containing heat-labile enterotoxin from Escherichia coli enhances immune responses to injected influenza virus vaccine through activation of skin dendritic cells.* J Virol, 2003. **77**(9): p. 5218-25.
35. Soo Hoo, W., et al., *Vaccination with cell immunoglobulin mucin-1 antibodies and inactivated influenza enhances vaccine-specific lymphocyte proliferation, interferon-gamma production and cross-strain reactivity.* Clin Exp Immunol, 2006. **145**(1): p. 123-9.
36. Brignone, C., et al., *IMP321 (sLAG-3) safety and T cell response potentiation using an influenza vaccine as a model antigen: a single-blind phase I study.* Vaccine, 2007. **25**(24): p. 4641-50.
37. Szyszko, E., et al., *Impact of influenza vaccine formulation with a detailed analysis of the cytokine response.* Scand J Immunol, 2006. **64**(5): p. 467-75.
38. World Health Organization, *Weekly Epidemiological Report/Relevé épidémiologique hebdomadaire.* 2002. **77**(28): p. 229-240.
39. Campos, M. and D.L. Godson, *The effectiveness and limitations of immune memory: understanding protective immune responses.* Int J Parasitol, 2003. **33**(5-6): p. 655-61.
40. Droebner, K., et al., *Antibodies and CD4(+) T-cells mediate cross-protection against H5N1 influenza virus infection in mice after vaccination with a low pathogenic H5N2 strain.* Vaccine, 2008. **26**(52): p. 6965-74.
41. Esser, M.T., et al., *Memory T cells and vaccines.* Vaccine, 2003. **21**(5-6): p. 419-30.

Study #2 – *Does seasonal influenza vaccination cause de novo anti-HLA antibody formation in lung transplant recipients?*

Introduction

Lung transplants account for less than ten percent of all annual transplants [1, 2]. In 2008 in Canada lung transplants accounted for six percent (131) of the total 2,048 performed [1]. In the United States five percent (1,461) of the 27,578 transplants performed were lungs [2]. Even though the overall percent of lung transplants performed is not as large as other organs, the system that they are part of and the function they perform makes them vulnerable to insults that other organs are not, including respiratory infections. One of the more prominent respiratory infections that lung transplant (LTx) recipients face is influenza. This is because it is a seasonal virus in which the infection-causing strain is different every year, and previous infection with one strain provides no guarantee of protection from infection with another. As a preventive measure, LTx patients are strongly recommended to receive the annual influenza vaccination every year. It is important to study the response of vaccination in this group to not only optimise treatment thereby increasing quality of life but also to investigate the effect vaccination may have on the graft since the site of influenza infection is the graft.

Influenza infection, vaccination, and the lung transplant

While the influenza vaccine is strongly recommended for all LTx recipients, some patients and health practitioners are reluctant to administer the vaccine because of the theoretical concern of precipitating acute or chronic rejection. In the studies involving transplant recipients and infection with influenza viruses, the majority of patients presented with the standard hallmark symptoms of influenza infection fever, cough, sore throat, dyspnea, rhinorrhea and malaise but these symptoms were more severe, longer in duration and resulted in patient hospitalization [3-7]. In some of the transplant recipients but not all infected with influenza some studies have found infection of the lower respiratory tract by influenza and progression to viral or co-infection with bacterial pneumonia [3-11]. However, these are not the findings that give most people pause when considering the influenza vaccination; it is the findings that in a small number of infected transplant recipients acute episodes of rejection occurred and were documented [3-8]. These episodes of acute rejection were seen to occur at the same time or shortly after symptoms of infection presented and the majority were resolved following anti-rejection treatment with no negative lasting effects to the graft [3-8]. It is important to note that two of the studies were completed in the early 1970's and involved only kidney transplants; a third looked at a period from 1989-1992 involving all solid organ transplants but the majority were kidney [3, 4, 7]. Since these studies were completed there have been advancements in immunosuppressive therapy and transplantation

protocols that make the mismatch of donor and recipient less common. It is conceivable that some episodes of rejection documented in these studies were already present but either aggravated because of infection or only documented because the patient was in hospital under observation. In the past decade Vilchez *et al*. has performed two new studies into influenza infection and transplant recipients: one in solid organ transplants, dominated by LTx, as a group and one in LTx recipients [5, 6]. Again, a potential link between acute rejection episodes and infection was seen in a small number of patients but it was not proven that the infection was directly responsible for the rejection as no viruses were found in the biopsies [5, 6]. In addition it is possible that the rejection episode were already underway at the time of infection but were only documented because the patient was hospitalized [5] or enrolled in the study [6]. A study in 2006 by Milstone *et al.* involving LTx recipients and one by Lopez-Medrano *et al.* [9] found that while severe infection did occur in patients there were no episodes of acute rejection [10]. Another in 1992 by Ljungman, P. *et al* in all solid organ transplant recipients found that severe influenza infection only occurred in the most immunocompromised patients and no episodes of rejection [11].

Influenza infection and the immune response within the lung

While the number of documented cases of rejection following influenza infection is few in LTx recipients there is a possible immunological basis for it

occurring since influenza is a respiratory infection. Influenza infection activates all components, innate, humoral and cell-mediated, of the immune system within the allograft. This may result in viral clearance but also a deleterious response in transplanted lungs due to improper activation of the immune response. Improper activation of the immune response could start with the recognition of the cells of the graft as foreign occurring as a result of damaged and dead lung cells being phagocytosed by macrophages along with virus particles. All cells of the adaptive immune system would be involved including $CD8^+$ and $CD4^+$ T-cells, B-cells and other white blood cells. The activation of these cells and the subsequent production of cytokines like IL-2 and IFN-γ and immune complexes would lead to chronic damage of the graft, through production and activation of memory T- and B-cells, and eventually the loss of graft function [12]. This precise scenario has been theorized to occur after immunization with the influenza vaccine as it has happened with infection. However, there are two significant differences between the vaccine and infection that preclude this occurring. First, the site of infection and vaccination differ: infection occurs in the lungs while vaccine is given in the arm. Second, infection involves live actively replicating virus particles but the most commonly used vaccine preparations utilize preparations of viral proteins not live virus. There are vaccine preparations that use whole virus but it is killed and not capable of infecting and replicating within cells. In studies that investigated influenza vaccine in heart [13, 14], lung [15-17] and kidney [18-23] transplant recipients no correlation was found

between episodes of rejection and vaccination. However, there is one report by Blumberg *et al.* [24] in heart transplant recipient and one by Wertheim *et al.* [25] in corneal transplant recipients in which a small number of patients experienced acute low-level episodes of rejection following influenza vaccination. All episodes were treated with anti-rejection therapy and resolved [24, 25].

Chronic rejection and influenza infection

Even though the first lung transplant was performed nearly three decades ago the 5-year survival rate is only 43% primarily due to chronic rejection [26]. Chronic rejection in LTx recipients is defined histologically by a process of fibroproliferation in the small airways of the lungs that leads to submucosal fibrosis and obliteration of the lumen [26]. This process is named bronchiolitis obliterans/obliterative bronchiolitis (OB). OB is difficult to diagnose by histopathology and can also be diagnosed based on clinical symptoms of at least 20% reduction in lung function from the initial post-transplantation peak values with no other explanation [26]. This clinical diagnosis is known as bronchiolitis obliterans syndrome (BOS) [26]. With the indication, that infection with influenza and other respiratory viruses can trigger acute rejection episodes in transplant recipients and the potential for large amount of damage to occur in such infection a link between OB/BOS has been investigated. Several studies have been conducted and have found that following an episode of infection with a respiratory virus, including influenza; several LTx recipients developed OB or

BOS or were at more risk to do so [27-31]. Two of these studies even found a relation between infection and increased risk of death due to complications [28, 31].

With an apparent link between infection with influenza and the development of acute and chronic rejection, and the theory that this could be a side effect of influenza vaccination, it is appropriate to investigate the activation of the immune system towards the transplanted lung following vaccination. To investigate this possibility, serum samples from LTx recipients that had received not only the standard influenza vaccine but also a booster four weeks later were examined for the presence of antibodies directed against donor HLA prior to and following both immunization events. The donors and recipients are initially HLA typed through serology and DNA with antibody screens completed with flow cytometry. Since this was another antibody screen, flow cytometry was used as well employing the techniques and protocols of the University of Alberta Hospital's Histocompatibility Laboratory. It is expected that because the vaccine is administered in the arm and does not contain any live virus particles that vaccination will not lead to the production of antibodies targeting the HLA molecules of the donor lung.

Methods

The sera from sixty patients who had been enrolled in a previous study investigating a trial of intradermal influenza boosting, were used to investigate

this question. The Research Ethics Board at the University Health Network, Toronto, approved the previous study and written informed consent was obtained from all patients. In the previous study, baseline serum was collected from patients prior to receiving the standard 0.5 ml i.m. vaccination. At four weeks post i.m. vaccination, serum was again collected following which the patients received a non-standard 0.1 ml intradermal (i.d.) booster. Finally, at eight weeks post i.m. vaccination (or four weeks post i.d. booster) vaccinations a final serum sample was collected. To determine if anti-HLA antibodies were present, sera were tested using flow cytometry in the University of Alberta Hospital Histocompatibility Laboratory following their pre-transplantation histocompatibility protocol (Figure 1). The University of Alberta Research Ethics Board approved the current study. To start, the four and eight week post-vaccination sera were analyzed using the flow panel reactive antibody screen (FPRA). Flow cytometry beads that bound anti-MHC Class I and II antibodies were used to detect presence of these antibodies in patient serum samples (OneLambda cat# FL12-60). A flow cytometry bead mixture was created using the basic formula of 5µL of each MHC Class I and Class II beads and 1µL of control beads for each tube/sample in a run. Ten microlitres of the bead mixture was then added to 20µL of either the control sample or patient serum. Each tube was vortexed to mix and incubated in the dark at room temperature for 30 minutes. A second vortex occurred at 15 minutes into

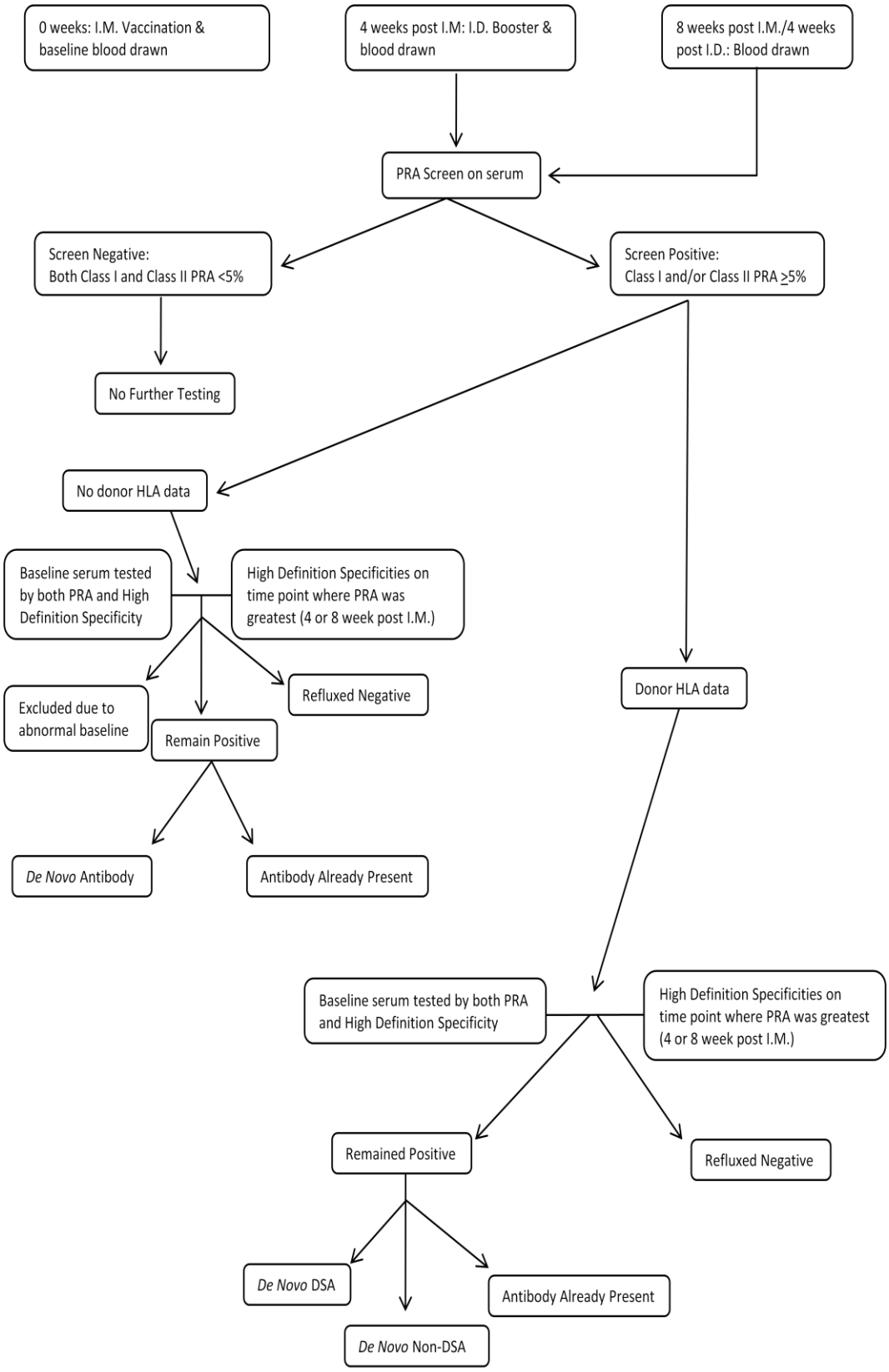

Figure 1. Experimental Design

incubation. After incubation, tubes were washed twice in the following manner: 1mL of wash buffer was added to each tube before vortexing and centrifuging at 9100xg for 2 minutes. After centrifuging supernatant was removed using glass pipettes and discarded. One hundred microlitres of fluorescein isothiocyanate (FITC) conjugate was added to each tube followed by vortexing. Tubes were then incubated in the dark at room temperature for 30 minutes. A second vortex occurred at 15 minutes into incubation. After incubation, tubes were washed twice in the following manner: 1mL of wash buffer was added to each tube before vortexing and centrifuging at 9100xg for 2 minutes. After centrifuging supernatant was removed using glass pipettes and discarded. After washing, 600µL of 0.5% paraformaldehyde fixative was added to each tube and vortexed. Cell suspensions were transferred to 5mL tubes for reading on a BD FACSCalibur flow cytometer.

Analysis of generated flow cytometry data was completed using BD CELLQuest Pro software (BD Biosciences) by members of the University of Alberta Hospital Histocompatibility Laboratory. Using a dot plot displaying all beads picked up by the flow cytometer gates were drawn around the control, MHC Class I and Class II bead populations. Control beads were used to assess level of nonspecific reactivity such as anti-plastic or latex antibodies (OneLambda cat# FL12-60). Negative control samples were used to set negative and positive regions on MHC Class I and II histograms. These gates were then used to analyse patient data. Positive control samples were used to ensure that the staining

procedure had been completed properly. Any samples that had a FPRA greater than or equal to 5% were considered positive.

Samples found to be positive were tested with the high definition (HD) specificity protocol to determine which HLA types the antibodies were specific for and rule out any non-specific binding. To begin 5µL of either MHC Class I or Class II HD beads were aliquoted to a well in a 96 well plate. Twenty microlitres of either control or patient serum were then added to the test wells and the plate vortexed at maximum speed for 10 seconds. The plate was then incubated in the dark at room temperature for 30 minutes. A second vortex occurred at 15 minutes into incubation. After incubation, the plate was vortexed at maximum speed for 10 seconds then 175µL of wash buffer was added to each well before centrifuging at 3000rpm for 10 minutes. After centrifuging, supernatant was removed by flicking the plate straight down, followed by dabbing hard onto paper towel three times. The plate was then washed two more times in the following manner: the plate was vortexed then 200µL of wash buffer was added to each well before centrifuging at 3000rpm for 10 minutes. After centrifuging, supernatant was removed by flicking the plate straight down, followed by dabbing hard onto paper towel three times. After washing, the plate was vortexed and 100µL of FITC conjugate was added to each well followed by vortexing at maximum speed for 10 seconds. The plate was then incubated in the dark at room temperature for 30 minutes. A second vortex occurred at 15 minutes into incubation. After incubation, the plate was vortexed at maximum

speed for 10 seconds then 100µL of wash buffer was added to each well before centrifuging at 3000rpm for 10 minutes. After centrifuging, supernatant was removed by flicking the plate straight down, followed by dabbing hard onto paper towel three times. The plate was then washed two more times in the following manner: the plate was vortexed then 200µL of wash buffer was added to each well before centrifuging at 3000rpm for 10 minutes. After centrifuging, supernatant was removed by flicking the plate straight down, followed by dabbing hard onto paper towel three times. After washing, the plate was vortexed at maximum speed for 10 seconds and 100µL of 0.5% paraformaldehyde fixative was added to each well. The plate was again vortexed at maximum speed, this time until beads came off the bottom of the plate. Bead suspensions were transferred to 5mL tubes containing 400µL of 0.5% paraformaldehyde fixative for reading on a BD FACSCalibur flow cytometer.

Members of the University of Alberta Hospital Histocompatibility Laboratory completed analysis of generated flow cytometry data using CELLQuest Pro software. Control beads were used to assess level of nonspecific reactivity such as anti-plastic or latex antibodies. Negative control samples were used to set background reactivity. Positive control samples were used to ensure that the staining procedure had been completed properly. That allele's bead population shifting to the right of its location on the negative control dot plot determined positive reactivity of an MHC Class I or Class II allele. Shifts were determined by placing a transparency of the negative control dot plot over that

of the patient's and observing any shifts in the populations of MHC allele beads. Based on the degree of shift results were categorised as no specificity (FPRA was borderline positive but no shift occurred with MHC beads and as a result the FPRA changed to zero), weak specificity, moderate specificity or strong specificity.

At this point, the baseline sera corresponding to positive samples were also tested by FPRA and high definition specificity to determine if the antibodies present in the post-vaccination samples were *de novo* or present prior to vaccination. Provided no other factors related to antibody formation were also present, by comparing the specificity of anti-HLA antibodies present in the baseline serum to those present in the post-i.m. vaccination serum it was possible to determine if the anti-HLA antibody was *de novo* (i.e. newly formed after vaccination) and therefore, possibly related to vaccination. Availability of donor typing allowed for the determination of whether the anti-HLA antibodies present were targeting the HLA antigen present on the graft and therefore, considered donor-specific antibody (DSA), or if they were against other HLA antigen not present in the graft or patient. Correlations were made between increased antibody level and episodes of acute rejection and serologic response to vaccination.

Results

Study population characteristics

Sixty serum samples were available from Dr. Manuel's original influenza vaccine booster study [15]. While all 60 patients received both the intramuscular and intradermal vaccinations, post-intradermal vaccination serum was only available for 57 patients; however, all 60 patients were included in this study. Table 1 shows the patients' clinical characteristics. In brief, median patient age was 49.8 years (range 20.7-72.4) with a median time from transplant of 15.7 months (range 2.8-206.7). The majority of patients were on standard maintenance immunosuppression consisting of a calcineurin inhibitor (cyclosporine or tacrolimus), an antimetabolite agent (azathioprine or mycophenolate mofetil (MMF)) and prednisone. Ninety-three percent of the patients had been previously vaccinated with the trivalent-inactivated influenza vaccine during the 2005-2006 influenza season. Anti-lymphocyte globulin therapy was not administered to any patient within the 6 months prior to enrolment in the study.

Results from Dr. Manuel's influenza vaccination booster study [15]

Dr. Manuel used seroconversion as a measure of response to influenza vaccination. Following the criteria that a four-fold or greater increase in hemagglutination inhibition assay was indicative of seroconversion, only 63% of Dr. Manuel's patients responded to the i.m. vaccination. After the i.d. booster

vaccination only a further 13.6% of patients responded. Dr. Manuel did however find that his patients incurred no adverse effects after receiving the i.d. booster vaccination as episodes of rejection did not increase above the published rate for this group or were associated with receiving the booster vaccine. From these results, Dr. Manuel was able to conclude that i.d. booster vaccination does not significantly increase protection against influenza infection but is safe for use in the lung transplant population.

Presence of anti-HLA antibody after initial Flow Panel Reactive Antibody Screen

All 4- and 8-week post-i.m. vaccination serum samples were initially screened using the FPRA screen for the presence of anti-HLA antibody. The screen resulted in two groups: serum free from the presence of anti-HLA antibody and those with anti-HLA antibody present. Sera positive for the presence of anti-HLA antibody were further subgrouped by availability of donor HLA typing data. At this juncture, thirty-nine of the sixty (65%) patients tested negative for the presence of anti-HLA antibody (Table 2). The remaining twenty-one patients (35%) were positive for anti-HLA antibody; of these, twelve had donor HLA typing available and nine did not (Table 2).

Table 1. Patients' Clinical Characteristics (n=60)

Age (years; median, range)	49.8 (20.7-72.4)
Gender (M:F)	30:30
Time of Vaccination from Transplant (months; median, range)	15.7 (2.8-206.7)
History of Influenza Vaccination	56 (93%)
Underlying Disease	
Cystic Fibrosis	22 (37%)
Pulmonary Fibrosis	16 (27%)
Emphysema	9 (15%)
Other	13 (22%)
Previous Induction Therapy	18 (30%)
Maintenance Immunosuppression	
Prednisone	59 (98%)
Calcineurin Inhibitors	58 (97%)
Azathioprine	37 (62%)
MMF	21 (35%)
Sirolimus	4 (7%)

Table 2. Anti-HLA Antibody Presence After FPRA Screening of Post-Vaccination Samples (n=60)

	Presence of Anti-HLA Antibody (n=21)	
Absence of Anti-HLA Antibody	Donor HLA Typing Available	Donor HLA Typing Unavailable
39	12	9

Further testing of anti-HLA antibody positive sera

For the twenty-one patients that tested positive after the initial FPRA, further testing on their sera was completed to determine HLA allele specificity and to correct indeterminate results. The patient's baseline serum and the post-i.m. vaccination serum with the largest FPRA positivity value were used in this determination. Patients were determined to be clinically negative if the high definition specificity screen for both the post-vaccination and baseline sera were negative for specific HLA antigen reactivity and if the baseline FPRA was similar to that of the post-i.m. vaccination FPRA. Of the twelve patients with donor HLA typing available, only one patient was called negative. The remaining 11 had varying degrees of reactivity to various HLA alleles (Table 3). One patient was determined to have donor-specific antibody. Of the nine patients without donor, HLA typing available three had no antibody on high-definition screening. Based on expert advice from D. Campbell, one patient from this group was excluded from further analysis at this point due to a highly abnormal baseline reading; the baseline FPRA indicated the patient was positive for anti-MHC class I alleles while the high definition did not. When testing was completed on the post-vaccination serum, it indicated positivity for anti-MHC class I alleles; however, due to the abnormal baseline it was not possible to determine whether this was a true change in the patient's status. This left five patients without donor HLA typing positive for anti-HLA antibody (Table 3). When compared to pre-vaccination sera, these patients did not have newly formed anti-HLA antibody. In summary, it was

found that one of twenty-one positive patients had *de novo* non-DSA after influenza vaccination (Table 3). This 72-year old patient was more than seventeen years post-transplant and was receiving cyclosporine for immunosuppression. Prior to transplantation this patient had antibodies to antigens in the Class I alleles A:34 and 26 and to the Class II alleles DR:13 and 17 and DQ:4 and 7. After vaccination, all of these antibodies were still present but the antibodies to antigens in DQ7 had greater affinity for their target. In addition antibodies to the antigen in alleles DQ:8 and 9 were now present; however, these alleles or their antigens were not present in the donor tissue. In the other twenty patients, the antibody was not changed in affinity or amount after vaccination. In the twelve positive patients with donor typings available, it was determined that none of the anti-HLA antibodies were specific to antigen in the donor HLA alleles (Table 3). This was also true for the one patient that created new antibody after vaccination. In the nine patients without donor HLA typing available, it was only possible to determine whether the antibodies were *de novo* without relation to DSA (Table 3).

Overall Results

After all testing had been preformed, taking into account the one excluded patient, there were forty-three (72.9%) patients who were anti-HLA antibody negative (Table 4). The remaining sixteen (27.1%) patients had anti-HLA antibody present and could be further subdivided according to what HLA Class

the antibody was against (Table 4). The majority of these patients (12) had antibody only against HLA Class I (75%), two patients had antibody only against HLA Class II (12.5%) and the remaining two patients had antibody against HLA Class I and Class II (12.5%) (Figure 2 and 3). Interestingly, of the sixteen patients who were positive for some form of anti-HLA antibody 62.5% were female, though no significant correlation was found between gender and anti-HLA antibody formation (Spearman's ρ=0.066, p=0.620). No significant correlation between time from transplant at initial immunization and the formation of anti-HLA antibodies was found (Spearman's ρ=0.071, p=0.595). In addition, no significant correlations were found between individual immunosuppressive drugs and the formation of anti-HLA antibodies (prednisone: Spearman's ρ=-0.215, p=0.102; CNIs: Spearman's ρ=-0.096, p=0.468; MMF: Spearman's ρ=0.104, p=0.433; azathioprine: Spearman's ρ=-0.216, p=0.100; sirolimus: Spearman's ρ=0.206, p=0.118). No correlation was found between those patients that experienced acute rejection and those with anti-HLA antibody present.

Table 3. Anti-HLA Antibody Presence After 0 Week FPRA & 0/4 or 8 Week Specificity Testing (n=60)

Donor HLA Typing Available (n=12)				Donor HLA Typing Unavailable (n=9)				
Absence of Anti-HLA Antibody	Presence of Anti-HLA Antibody (n=11)			Absence of Anti-HLA Antibody	Presence of Anti-HLA Antibody (n=5)*			Excluded
	De Novo DSA	*De Novo* Non-DSA	No *De Novo* Antibody		*De Novo* DSA	*De Novo* Non-DSA	No *De Novo* Antibody	
1	0	1	10	3	-	-	5	1

*=*De Novo* DSA vs. *De Novo* Non-DSA undeterminable without donor HLA typing
DSA= Donor Specific Antibody

Table 4. Overall Anti-HLA Antibody Presence (n=59*)

Absence of Anti-HLA Antibody	Only Anti-Class I Antibody	Only Anti-Class II Antibody	Anti-Class I & II Antibody
43 (72.9%)	12 (20.3%)	2 (3.4%)	2 (3.4%)

*=One patient excluded due to abnormal baseline PRA; thus, n=59

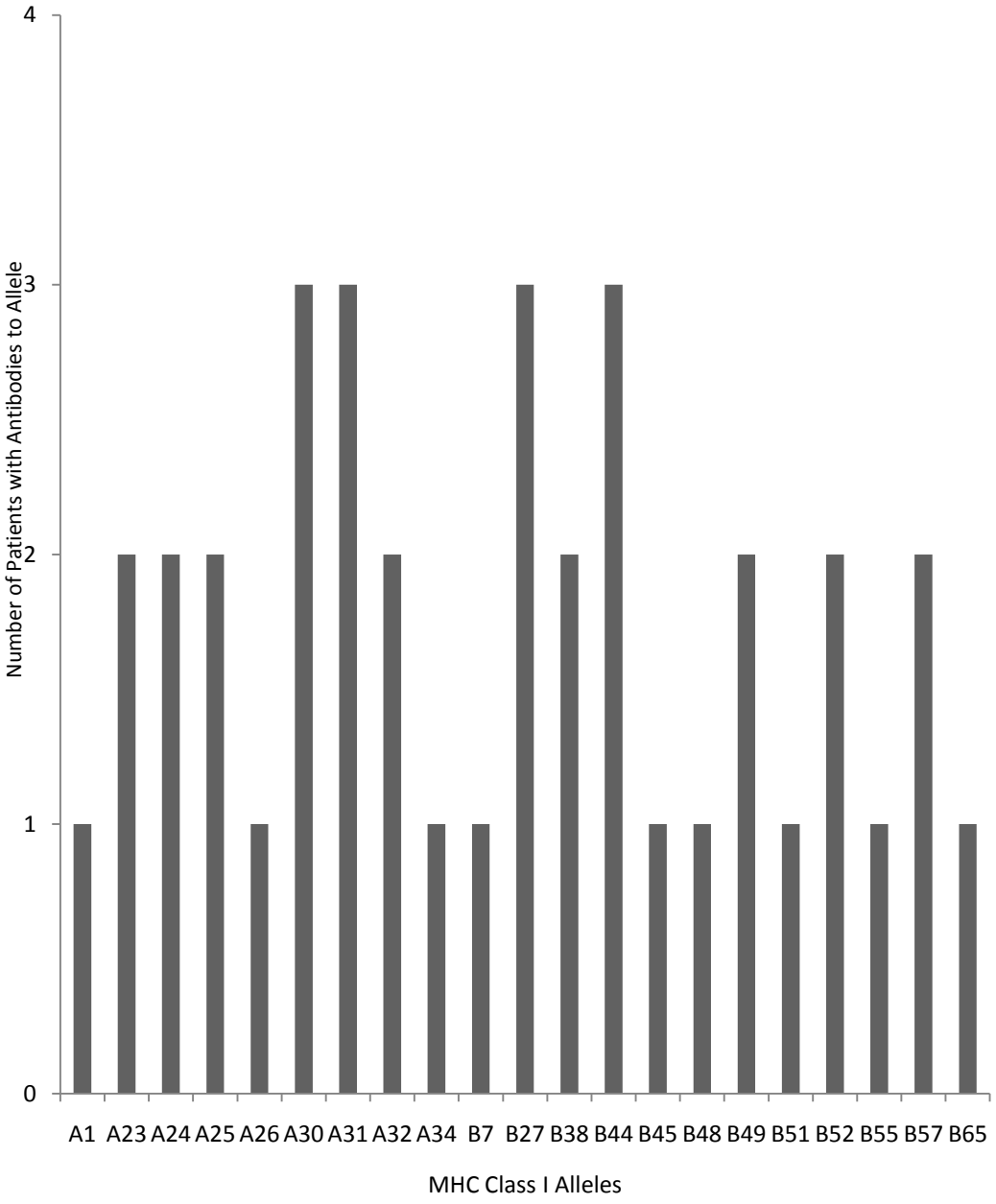

Figure 2. Number of Patients with Antibodies to Antigens in Various MHC Class I Alleles

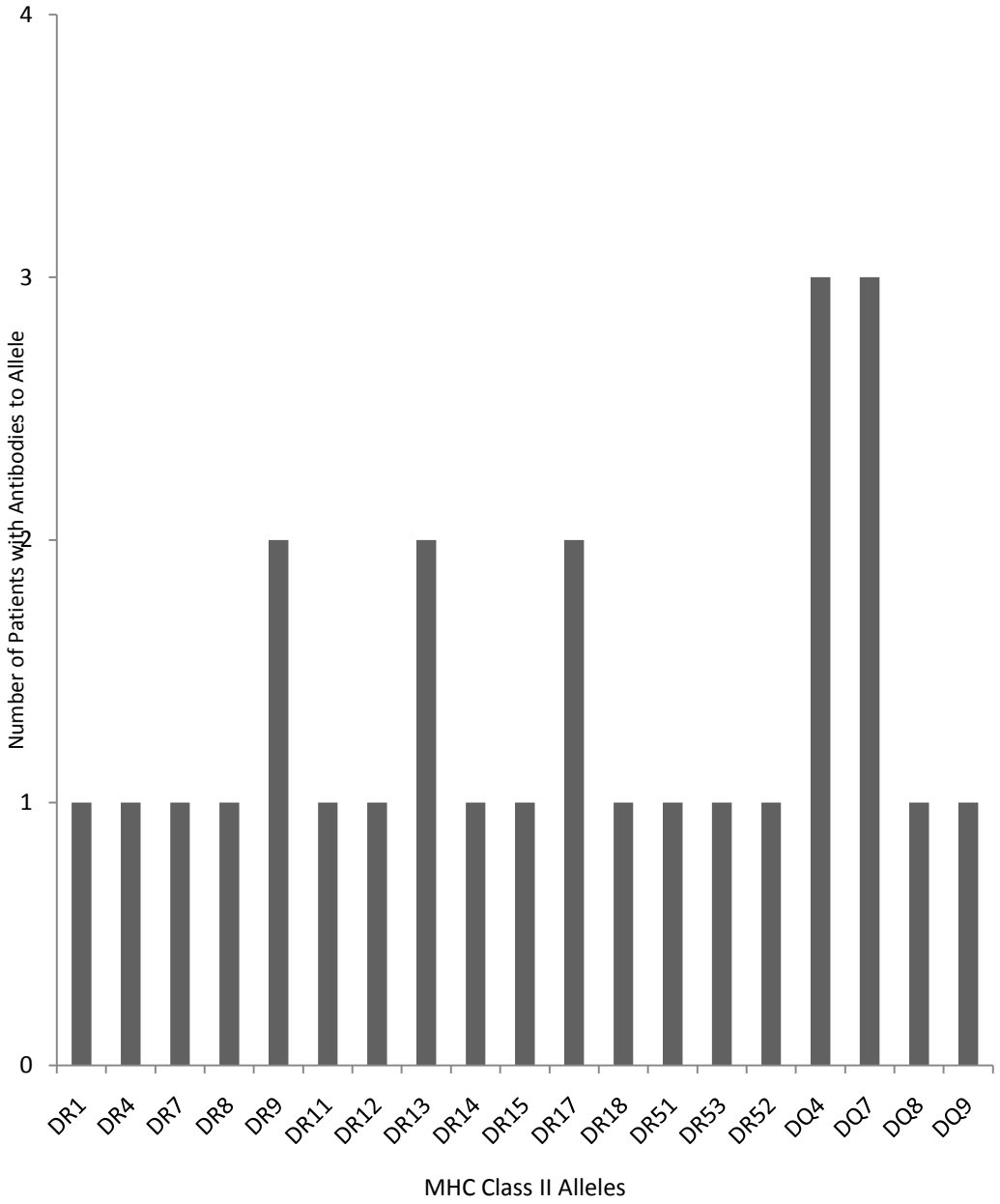

Figure 3. Number of Patients with Antibodies to Antigens in Various MHC Class II Alleles

Discussion

This study evaluated the ability of the annual influenza vaccine to induce the production of anti-HLA antibodies specific for donor HLA contained on the transplanted lung. The sera from sixty patients enrolled in a previous study at the University of Toronto investigating influenza booster immunizations in LTx recipients [15] were examined using flow cytometry for the presence of anti-HLA antibodies. The baseline sera and sera four or eight weeks following primary immunization were all examined. The previous study concluded that the influenza vaccine was effective at producing a protective antibody response in LT recipients as 63% of patients responded to the initial immunization [15]. However, the booster was found to be ineffective at increasing the antibody response to the influenza vaccine as only an additional 13.6% of LTx recipients responded [15]. While six cases of rejection did occur during this study, this was not above the normal published rates of rejection within the LTx population and therefore not considered an effect of the vaccine or booster immunization, indicating that influenza vaccination is likely safe in LTx recipients [15]. This conclusion of safety of the influenza vaccine has also been found in other cohorts of lung [16, 17], kidney [18-23] and heart [13, 14] transplant recipients. However, some published reports associate influenza vaccination with acute reversible rejection episodes in a small number of heart [24] and corneal [25] transplant recipients. No study has linked vaccination to the occurrence of BOS

although there are studies that link influenza infection to occurrence of the chronic rejection syndrome [27-31].

Presence of anti-HLA antibodies following influenza vaccination

Following FPRA screens and specificity testing, it was discovered that only 27.1% of patients had some form of anti-HLA antibody present in their serum (Table 4). In fact the majority (39/43) were negative following the initial FPRA screen (Table 2). We also did not find *de novo* anti-HLA antibodies following vaccination (Table 3). The lack of production of antibodies specific for donor HLA following influenza immunization was also found by Kimball *et al.* in a cohort of twenty-nine heart transplant recipients without previous influenza vaccination [13]. Interestingly a study conducted by Roddy *et al.* involving IGN301 immunization in patients with late-stage adenocarcinomas found that alloreactive T-cells and antibody did develop in some patients but were for the most part transient [32]. The second part of the study by Roddy *et al.* involved the vaccination of healthy persons over the age of thirty for hepatitis B and found that alloreactive T-cells were generated in some of the participants and were transient but no alloreactive antibodies were produced [32]. An interesting finding of the study was that alloreactive cells were found in the older participants and were not produced by the immunization [32].

While no DSA were produced, one patient did produce new anti-HLA antibodies following vaccination that were not directed against self-HLA alleles.

This patient was already producing anti-HLA antibodies prior to vaccination. After vaccination the number of different alleles that antibodies were directed against increased and in two cases the affinity of the antibody for its antigen increased from weak or moderate strength to moderate or strong following vaccination. The study by Kimball *et al.* had two patients who after vaccination did produce anti-HLA antibodies directed to minor alleles not present in the donor heart or the patient themselves [13].

The sixteen patients positive for anti-HLA antibodies were positive at both pre- and post-vaccination time points likely because of sensitization events that can lead to the production of anti-HLA antibodies. The events that have been shown to induce sensitization are blood transfusions, pregnancy and previous transplants [12, 33]. In the case of blood transfusions, it is the presence of HLA on the viable leukocytes in the packed red blood cells and platelets that result in the activation of the recipient's immune system. The probability of sensitization is also dependent on the number of transfusions and the volume per transfusion [33]. Previous transplants can, though not always, leave recipients with antibodies directed against any of the mismatched HLA alleles on the graft; the greater the mismatches, the more antibody specifities are produced leaving the recipient more sensitized [33]. During pregnancy the woman is exposed to the paternal HLA alleles in the fetus and this can result in the activation of the woman's immune system against these alleles [33]. However, it is not just the exposure to the paternal HLA alleles alone that results

in sensitization because with multiple pregnancies women produce antibodies to a wide panel of HLA alleles not just the paternal ones [33]. As only women can experience pregnancy, they have the potential for extra sensitization events and thus often make up the greater proportion of sensitized patients on a transplant waitlist. This greater proportion of females in the sensitized population was also seen in this cohort of LTx recipients, as 62.5% of patients positive for anti-HLA antibodies were female.

Twelve of the sixteen (75%) patients positive for anti-HLA antibodies were producing antibodies specific for MHC class I alleles opposed to class II (12.5%) or both classes (12.5%). This is most likely because MHC class I molecules are expressed on the surface of every cell in the body while class II are only expressed on a subset of immune cells whose function is antigen presentation [12]. With exposure on vast numbers of cells, the recipient's immune system has many chances to encounter foreign MHC class I alleles during sensitization events and mount an immune response. Encountering foreign MHC class II alleles during sensitization events would be rarer.

In this cohort of LTx patients, 27.1% (16/59) were positive for anti-HLA antibodies. In the past five years, the average percent of HLA allele sensitized LTx prospects screened at the University of Alberta Hospital Histocompatibility Laboratory was 40.8%. The percentage of sensitized patients in our study cohort is lower than that of the University of Alberta Hospital lung transplant program.

This could be because our cohort had sixty patients and the five-year average consisted of 375 patients. Another factor could be the transplant program locations, our cohort came from the University of Toronto, while the five-year average was from the gathered from University of Alberta.

This study of the production of anti-HLA antibodies by LTx recipients following influenza vaccination found that vaccination did not trigger production of *de novo* donor-specific antibodies. In addition, in all but one patient, influenza vaccination did not trigger the production of non-DSA *de novo* antibodies was. This confirms the conclusion from the previous study [15] that the influenza vaccine is safe for use in the LTx population and the findings of other studies involving the safety of influenza vaccination in transplant populations [13, 14, 16-23]. Therefore, recipients of lung transplants should continue to receive the annual influenza vaccine. It would be interesting to evaluate the cell-mediated immune response in the LTx population both in response to the influenza vaccine and for the development of anti-donor directed T-cells following influenza vaccination as further studies to define the interaction of the influenza vaccine in transplant recipients.

Study limitations

Our study had several limitations but the nature of these limitations does not compromise the conclusions that have been drawn from the results generated. One limitation of this study was that for nine of the patients positive

for anti-HLA antibodies there was no donor HLA typing available making the determination if the antibodies present were donor-specific antibodies or not impossible. However, because baseline and post-vaccination results could be compared it was still possible to determine that these patients did not produce any *de novo* antibody. Thus, if donor-specific antibody was present it would have been present prior to and not as a result of vaccination.

A second limitation of this study is the lack of an unvaccinated control group. This is difficult since the annual influenza vaccine is 'standard-of-care' at most institutions. The next time a similar study is undertaken to alleviate the limitation it could be completed outside the normal influenza infection season allowing for withholding the vaccine from some participants. While this would create a control group it is not the ideal time to investigate the effectiveness of the vaccine in preventing infection as natural infection as not likely to occur. An alternative to this would to look at the formation of anti-HLA antibodies in healthy persons vaccinated at the same time as the LTx recipients and compare the production rates.

The sample size of sixty patients is somewhat small but is reasonable given the size of the total lung transplant population at our center is approximately 250. The next time a similar study is undertaken the size could be increased to increase the statistical power of the conclusions drawn.

Study 2 Bibliography

1. CIHI, *Cumulative Report - 2008*, in *e-Statistics on Organ Transplants, Waiting Lists and Donors*. 2009: Ottawa.
2. UNOS, *2008 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 1998-2007.* . 2009.
3. Briggs, J.D., et al., *Viral infection and renal transplant rejection.* Br Med J, 1972. **4**(5839): p. 520-2.
4. Mauch, T.J., et al., *Influenza B virus infection in pediatric solid organ transplant recipients.* Pediatrics, 1994. **94**(2 Pt 1): p. 225-9.
5. Vilchez, R., et al., *Influenza and parainfluenza respiratory viral infection requiring admission in adult lung transplant recipients.* Transplantation, 2002. **73**(7): p. 1075-8.
6. Vilchez, R.A., et al., *Influenza virus infection in adult solid organ transplant recipients.* Am J Transplant, 2002. **2**(3): p. 287-91.
7. David, D.S., et al., *Viral syndromes and renal homograft rejection.* Ann Surg, 1972. **175**(2): p. 257-9.
8. Vilchez, R.A., J. Fung, and S. Kusne, *The pathogenesis and management of influenza virus infection in organ transplant recipients.* Transpl Infect Dis, 2002. **4**(4): p. 177-82.
9. Lopez-Medrano, F., et al., *Clinical implications of respiratory virus infections in solid organ transplant recipients: a prospective study.* Transplantation, 2007. **84**(7): p. 851-6.
10. Milstone, A.P., et al., *A single-season prospective study of respiratory viral infections in lung transplant recipients.* Eur Respir J, 2006. **28**(1): p. 131-7.
11. Ljungman, P., et al., *Influenza A in immunocompromised patients.* Clin Infect Dis, 1993. **17**(2): p. 244-7.
12. Richiard A. Goldsby, T.J.K., Barbara A. Osborne, Janis Kuby, *Immunology*. 5th ed. 2003: W.H. Freeman and Company.
13. Kimball, P., S. Verbeke, and D. Tolman, *Influenza vaccination among heart transplant recipients.* Transplant Proc, 2001. **33**(1-2): p. 1785-6.
14. Magnani, G., et al., *Safety and efficacy of two types of influenza vaccination in heart transplant recipients: a prospective randomised controlled study.* J Heart Lung Transplant, 2005. **24**(5): p. 588-92.
15. Manuel, O., et al., *Immunogenicity and safety of an intradermal boosting strategy for vaccination against influenza in lung transplant recipients.* Am J Transplant, 2007. **7**(11): p. 2567-72.
16. Mazzone, P.J., et al., *The humoral immune response to influenza vaccination in lung transplant patients.* Eur Respir J, 2001. **18**(6): p. 971-6.
17. Mazzone, P.J., et al., *Cell-mediated immune response to influenza vaccination in lung transplant recipients.* J Heart Lung Transplant, 2004. **23**(10): p. 1175-81.
18. Birdwell, K.A., et al., *Decreased Antibody Response to Influenza Vaccination in Kidney Transplant Recipients: A Prospective Cohort Study.* Am J Kidney Dis, 2009.
19. Briggs, W.A., et al., *Influenza vaccination in kidney transplant recipients: cellular and humoral immune responses.* Ann Intern Med, 1980. **92**(4): p. 471-7.

20. Keshtkar-Jahromi, M., et al., *Antibody response to influenza immunization in kidney transplant recipients receiving either azathioprine or mycophenolate: a controlled trial.* Am J Nephrol, 2008. **28**(4): p. 654-60.
21. Sanchez-Fructuoso, A.I., et al., *Influenza virus immunization effectivity in kidney transplant patients subjected to two different triple-drug therapy immunosuppression protocols: mycophenolate versus azathioprine.* Transplantation, 2000. **69**(3): p. 436-9.
22. Scharpe, J., et al., *Influenza vaccination is efficacious and safe in renal transplant recipients.* Am J Transplant, 2008. **8**(2): p. 332-7.
23. Smith, K.G., et al., *Suppression of the humoral immune response by mycophenolate mofetil.* Nephrol Dial Transplant, 1998. **13**(1): p. 160-4.
24. Blumberg, E.A., et al., *Safety of influenza vaccine in heart transplant recipients.* J Heart Lung Transplant, 1998. **17**(11): p. 1075-80.
25. Wertheim, M.S., et al., *Corneal transplant rejection following influenza vaccination.* Br J Ophthalmol, 2006. **90**(7): p. 925.
26. Arcasoy, S.M. and R.M. Kotloff, *Lung transplantation.* N Engl J Med, 1999. **340**(14): p. 1081-91.
27. Kumar, D., et al., *Clinical impact of community-acquired respiratory viruses on bronchiolitis obliterans after lung transplant.* Am J Transplant, 2005. **5**(8): p. 2031-6.
28. Khalifah, A.P., et al., *Respiratory viral infections are a distinct risk for bronchiolitis obliterans syndrome and death.* Am J Respir Crit Care Med, 2004. **170**(2): p. 181-7.
29. Billings, J.L., et al., *Respiratory viruses and chronic rejection in lung transplant recipients.* J Heart Lung Transplant, 2002. **21**(5): p. 559-66.
30. Garantziotis, S., et al., *Influenza pneumonia in lung transplant recipients: clinical features and association with bronchiolitis obliterans syndrome.* Chest, 2001. **119**(4): p. 1277-80.
31. Palmer, S.M., Jr., et al., *Community respiratory viral infection in adult lung transplant recipients.* Chest, 1998. **113**(4): p. 944-50.
32. Roddy, M., et al., *Heterogeneous alterations in human alloimmunity associated with immunization.* Transplantation, 2005. **80**(3): p. 297-302.
33. Claas, F.H. and J.J. van Rood, *The hyperimmunized patient: from sensitization toward transplantation.* Transpl Int, 1988. **1**(2): p. 53-7.

Summary

Summary of studies

The overall finding of both studies in the LTx and KTx recipients was that influenza vaccination is safe and effective in these populations. Vaccine safety was shown by the lack of rejection episodes in the KTx study and none associated with vaccination in the LTx study. Secondly, only one patient in the LTx study produced *de novo* donor-specific antibodies and there was absence of de novo non-DSA antibodies. The KTx study found that influenza vaccination was effective at stimulating a directed T-cell response and indeed leads to the creation of memory as evidenced by high baseline values in these patients. Efficacy of vaccination in the LTx group had been previous evaluated in the original enrolment study [1].

Influenza Vaccine Improvement

While the studies were able to show that the current vaccine preparation is effective at stimulating the immune response of transplant recipients it is only rated as being 50-80% effective in healthy persons and is unknown in transplant populations [2]; thus, the overall efficacy of the vaccine must be increased. This can be done through the use of adjuvants as previously discussed. Still, adjuvants do not completely eliminate the need for an annual vaccine as they do not address the antigenic drift and shift of the influenza virus. To address this particular problem the creation of a universal vaccine that can be administered

once or with a boosting or updating schedule of several years needs to be created. Such vaccines could be DNA vaccines encoding conserved proteins from influenza A and B strains that would then provide universal protection against all influenza strains and heterosubtypic immunity. One of these conserved proteins that could be the target of a universal vaccine is the M2 ion channel protein of influenza A viruses.

Recommendations

All transplant recipients should receive the influenza vaccine annually as this and other research has shown it is safe and effective especially in light of secondary complications including rejection that have been shown to occur with influenza infection. Research such as conducted in the current studies needs to be carried out in all transplant populations to gain a better understanding of the interaction of vaccination with the transplant recipient's immune system and the graft. This greater understanding can lead to better vaccine design and protocols for this population and greater understanding of the specific effects of immunosuppression on the various aspects of the immune system. Research also needs to continue in evaluating new methods of creating a more immunogenic vaccine with a longer period of protection.

Summary Bibliography

1. Manuel, O., et al., *Immunogenicity and safety of an intradermal boosting strategy for vaccination against influenza in lung transplant recipients.* Am J Transplant, 2007. **7**(11): p. 2567-72.
2. World Health Organization, *Weekly Epidemiological Report/Relevé épidémiologique hebdomadaire.* 2002. **77**(28): p. 229-240.

Appendix A: Initial Experimental Investigations & Optimisation of Protocol

Initial Experimental Investigation

In October 2007 the initial study into the cellular mediated immunity (CMI) response of kidney transplant (KTx) patients following annual influenza vaccination was undertaken. The protocol consisted two parts to assess the CMI response. The first assay was based on work by Dr. McElhaney and Catherine Ewen and used ELISA and colourimetric assays to determine the amount of secreted inflammatory cytokines and activation products [1-3]. The second assay employed flow cytometry to determine the percent of activated $CD8^+$ and $CD4^+$ T-cells in the population of lymphocytes using intracellular IFN-γ and re-internalized CD107a. Michael Betts *et al.* (2003) has shown that CD107a, a membrane glycoprotein that is normally found inside lysosomes but found on the cell surface after lysosomal degranulation of $CD8^+$ T-cells, can be detected with FACS to accurately mark activated T-cells [4].

Methods

Participant Enrolment

Thirteen adult KTx recipients were recruited from the University of Alberta Hospital's transplant outpatient clinics and HV were recruited from laboratory and hospital staff. The study was approved by the University of

Alberta Research Ethics Board and written informed consent was obtained from all participants. Persons with allergies to eggs, a previous life-threatening reaction to influenza vaccination, on anticoagulants, had febrile illness in the past two weeks, KTx patients currently receiving therapy for rejection or were less than six months post transplantation, HV on immunosuppressives or with underlying immunological disorders, were excluded.

Schedule of Blood Draws

Baseline venous blood samples were taken prior to vaccination in sodium heparin vacutainer tubes for the purpose of PBMC isolation and serology vacutainer tubes for antibody titre testing. Participants received the 2007-2008 influenza vaccine as the standard 0.5 ml dose intramuscularly in the deltoid muscle of the non-dominant arm. Vaccine was administered at influenza clinics held at the University of Alberta Hospital or at the kidney transplant clinic. At four weeks post vaccination, venous blood samples were again collected from patients.

Polymorphic Mononuclear Cell Isolation

Blood samples were processed on the same day they were drawn. Serology tubes were centrifuged at 2,200 rpm for 5 minutes. If after the initial spin blood remained in the serum or if samples were cooler than room temperature prior to centrifugation, the tubes were gently warmed in a hot water bath for ten minutes and respun. The resulting layer of serum was

removed and stored at -80°C for influenza antibody titre testing at a later time. Initially an adapted isolation protocol from Dr. McElhaney's laboratory was employed and was as follows. Fifteen millilitres of room temperature Histopaque-1077 (Histopaque; Sigma-Aldrich cat #1077-1) was pipetted into two 50mL conical tubes and half of the collected heparinised blood was gently overlaid the Histopaque layer. The ratio of blood to Histopaque was one to three parts blood to one part Histopaque. Tubes were then centrifuged for twenty minutes at 2,200 rpm with the brake turned off. After centrifugation the PBMCs should be located in the layer between the serum and Histopaque layers. However, it was difficult to observe such a layer and therefore, the entire Histopaque layer was removed using a 5mL pipette with minimal pipetteman suction slowly sweeping over the RBC pellet-Hisopaque interface all of the Histopaque and a small amount of serum next to the Histopaque layer was removed. The collected Histopaque layer was diluted with an equal volume of DPBS in a 50mL conical tube and centrifuged at 1700 rpm for fifteen minutes to pellet the PBMCs. Following centrifugation, the supernatant was discarded and 3mL of 1X Bio-Rad RBC lysis buffer (U of A Biochem Stores cat#732-6372) was added and the pellet vigorously resuspended by pipetting. After ten minutes, the 50mL conical tub was topped up with DPBS to a total 40mL and tubes were centrifuged for twelve minutes at 1200 rpm. If a significant number of RBCs still remained after centrifugation the lysis and wash steps were repeated. After centrifugation the supernatant was removed and the pellet resuspended in 1mL

of RPMI media containing 1% L-glutamine and 1% penicillin-streptomycin-antimycotic solution. To count cells, a 1:5 dilution of cells in DPBS was made and then used to make a 1:2 dilution in trypan blue which was then layer on a standard hemocytometer. The large outer four corner squares, made of 16 smaller squares, were counted and averaged to obtain a count number. Only cells that were completely round, with clear not blue cytoplasm and not of extremely small size were counted. To obtain the number of cells recovered the number of cells counted is multiplied by one million. As this protocol was not producing a good yield of recovered cells and was very time consuming, it was decided to switch to Dr. Hidalgo's protocol for the remainder of the study. Dr. Hidalgo's isolation protocol is as follows. The collected heparinised blood was pooled together and mixed 1:1 with DPBS in a 50mL conical tube. Ficoll-Paque™ PREMIUM (Ficoll) (GE Healthcare Life Sciences cat#17-5442-03) was used as the cell density gradient and was layered under the blood. To layer under the blood, 13.5 mL of Ficoll was drawn into a 10mL plastic pipette then the tip of the filled pipette was gently placed at the bottom of the blood containing conical tube. The pipetteman, motorised pipette filler, was removed allowing gravity to pull the Ficoll out of the pipette and under the blood resulting in a clean blood-Ficoll interface. To pellet the red blood cells and polymorphonuclear leukocytes and suspend the PBMCs blood-Ficoll tubes were centrifuged at room temperature for 20 minutes at 1200xg with the centrifuge rotor's brake turned off. After centrifugation the polymorphic mononuclear cells (PBMCs) should be located in

the layer between the serum and Ficoll layers. However, it was difficult to observe such a layer and therefore, the entire Ficoll layer was removed using a 5mL pipette with minimal pipetteman suction slowly sweeping over the red blood cell (RBC) pellet-Ficoll interface all of the Ficoll and a small amount of serum next to the Ficoll layer was removed. The removed Ficoll-PBMCs was transferred to a 50mL conical tube and DPBS was added to a final volume of 50mL and the cells resuspended. The tubes were then centrifuged for 600xg for ten minutes with the break off. After centrifugation, the supernatant was removed and if necessary 3mL of RBC lysis buffer was added for ten minutes. If RBC lysis buffer was added the tube was then again topped up with DPBS to 50mL and centrifuged at 1,200 rpm for 5 min. Following centrifugation, the supernatant was removed and the cell pellet was resuspended in 2mL of RPMI media containing 1% L-glutamine and 1% penicillin-streptomycin-antimycotic solution (media). To count cells, a 1:5 dilution of cells in DPBS was made and then used to make a 1:2 dilution in trypan blue which was then layered on a standard hemocytometer. The large outer four corner squares, made of 16 smaller squares, were counted and averaged to obtain a count number. Only cells that were completely round, with clear not blue cytoplasm and not of extremely small size were counted. To obtain the number of cells recovered the number of cells counted is multiplied by one million. This protocol did increase the yield of recovered PBMCs but not drastically.

Peripheral Blood Mononuclear Cell Stimulation

The stimulation protocol followed was modified from the one used by Dr. McElhaney's laboratory to allow for the cells to be used in FACS analysis and the supernatants in ELISA and colourimetric assays. For stimulation experiments Corning 48 well plates were used and cells were aliquoted at $1x10^6$ cells/well for CD107a detection and $2x10^6$ cells/well for IFN-γ detection. A total of ten different wells were involved in testing: Six wells were for CD107a detection and four were for the detection of intracellular IFN-γ. The CD107a detection wells consisted of a well for each of the following: isotype control, negative control, positive control) A/PR/8/34 (A/PR) (Charles River Laboratories cat#490710), B/Lee/40 (B/Lee) (Charles River Laboratories cat#490735) and A/Aichi/2/68 (A/Aichi) (Charles River Laboratories cat#490715). The IFN-γ detections wells consisted of a well for each of the following: isotype control, negative control, positive control) A/PR (Charles River Laboratories cat#490710). The isotype control and negative control wells only contained the cells and media. The isotype control cells were stained with a nonspecific isotype control antibody to assess the nonspecific binding level of the CD107a and IFN-γ detection antibodies. The negative cells were stained to assess the level of spontaneous activation due to the isolation and stimulation procedures. For the positive control wells, PBMCs from differing participants were mixed and 50 units of IL-2 added to induce an allogeneic reaction. Each of the viruses was used at a MOI of two. The MOI is a ratio of the number of virus particles to the number of target

cells to be infected. MOI was calculated as follows. First, for virus with hemagglutination units (HAU) per millilitre provided the conversion that 1 HAU was equal to $1x10^4$ virus particles (vp) was used. Then a dilution of the stock was calculated such that 20µL of the dilution would yield $4x10^6$vp. This would give an MOI of two for the IFN-γ wells and could be halved to give an MOI of two for the CD107a detection wells ($2x10^6$vp) by using 10µL instead of twenty. For viruses where the $CEID_{50}$ per millilitre was provided could be used directly in the dilution calculation as this is a measure of the number of virus particles.

Example calculation with HAU/mL provided:

Conversion: 1,310,720 HA $\boldsymbol{x}\ \frac{1x10^4 \text{vp}}{1\ \text{HAU}} = 1.310720x10^{10}$vp

Dilution: volume of stock virus $= \frac{(2x10^8 \text{vp})(1000\,\mu\text{L})}{1.310720\,x10^{10\,\text{vp}}} =$ 15.3µL

Therefore, diluting 15.3µL of the stock virus in 984.7µL of media will provide $4x10^6$vp in every 20µL.

Cells for the detection of CD107a were plated in 600µL of media and incubated at 37°C for twenty hours. Cells for the detection of IFN-γ were plated in 600µL of media and incubated at 37°C for sixteen hours at which monensin (eBioscience cat#00-4505) was added at a concentration of 1X. After the addition of monensin, the cells were left to incubate for 4 hours at 37°C resulting in a total twenty hour stimulation period.

Staining and Flow Cytometry

The staining and flow cytometry protocols were kindly provided by Dr. Luis Hidalgo and are as written. After the 20 hour stimulation, the supernatant of the cells for the detection of CD107a was transferred to the 12x75 mm tubes and centrifuged at 1,200 rpm for five minutes. After centrifugation the supernatant was transferred to tubes for storage at -20°C until ELISA and colourimetric testing. To the CD107a detection wells, after the removal of supernatant, 1mL of FACS wash (0.5% fetal bovine serum, 2mM ethylenediaminetetraacetic acid and 0.05% sodium azide) was added and 1mL of ICS wash (sterile phosphate buffered saline plus 1X monensin (eBioscience cat#00-4505), 0.5% fetal bovine serum, 2mM ethylenediaminetetraacetic acid and 0.05% sodium azide) was added to each IFN-γ dectection well for five minutes to help lift cells that had adhered to the bottom of the well, then a pipette tip was drawn gently over the bottom of the well surface to mechanically detach cells. Cells were transferred to 12x75 mm tubes, for the CD107a cells the same ones the supernatant had original been centrifuged in, and centrifuged at 1200xg for five minutes. After centrifugation the supernatant was poured off and 40µL of each of the surface markers, volume used based on manufacture specifications, was diluted in the remaining FACS/ICS wash, about 100µL, in each tube. Surface markers used were phycoerythrin-Cy7 tagged anti-human CD3 (eBioscience cat#25-0038), allophycoerythrin-alexa fluor 750 anti-human CD4 (eBioscience cat#27-0049) and allophycoerythrin anti-human CD8 (eBioscience

cat#17-0086) for all wells and phycoerythrin tagged anti-human CD107a (eBioscience cat#12-1079) added to the CD107a detection cells. Tubes were gently vortexed and incubated for 10 minutes in the dark at room temperature. After incubation 2mL of FACS (CD107a dectection) or ICS (IFN-γ detection) wash was added to each tube and the tubes centrifuged at 1200xg for 5 minutes. After centrifugation the supernatant was poured off and cell pellet resuspended in 500µL of fixation buffer (eBioscience cat#00-8222), vortexed gently and incubated for 20 minutes in the dark at room temperature. Following incubation 1mL of 1X permeabilization buffer (eBioscience cat#00-8333) was added to each tube and then centrifuged at 1200xg for 5 min. The supernatant was poured off all tubes and the CD107a detection cells where resuspended in 500µL of FACS wash in preparation for running on the flow cytometer and were store in the dark until staining of the IFN-γ detection cells was finished. To the IFN-γ detection cells, 1mL of 1X permeabilization buffer (eBioscience cat#00-8333) was added to each tube, gently vortexed and centrifuged at 1200xg for 5 min. The supernatant was poured off and the cell pellet was resuspended in the remaining buffer, about 100 µL, and 2.5µL, volume based on manufacturer's specifications, of phycoerythrin tagged anti-human IFN-γ (eBioscience cat#12-7319) was added and tubes gently vortexed. Tubes were incubated for 20 minutes in the dark at room temperature. After incubation 1mL of 1X permeabilization buffer (eBioscience cat#00-8333) was added to each tube and then centrifuged at 1200xg for 5 min. The supernatant was poured off and 500µL of FACS wash was

added, tubes gently vortexed. For reading on the BD FACSArray flow cytometer 250µL of each cell suspension, CD107a and IFN-γ detection, was transferred to a 96-well plate.

The FACSArray is a digital, not analogue, flow cytometer and was optimised for the detection of lymphocytes with the following laser voltage settings: forward scatter – 180V; side scatter – 305V; far red – 100V; yellow – 540V; near-infrared – 200V and red – 630V. During the testing up to 100,000 cells were counted for a single sample. The FACSArray flow cytometer is a four-colour, six-channel machine using two lasers: a green at 532 nm for the yellow and far red parameters and red at 635 nm for the red and near-infrared parameters. Weekly calibration was completed by Dr. Hidalgo on weeks in which the machine had been used and bi-weekly when the machine was not in use.

Generated flow cytometry data was analyzed with the program FCS Express (De Novo Software). Pre-vaccination negative control data from each subject was used to set gates and quadrants for that individual. The first gate set was to isolate the lymphocyte population on the side scatter vs. forward scatter plot which displays all PBMCs counted by the flow cytometer. These cells were then displayed on a dot plot of side scatter vs. $CD3^+$ in order to create a gate around only $CD3^+$ lymphocytes. Further dot plots were then created using either the gated lymphocyte population or the $CD3^+$ population to display $CD3^+$ vs. IFN-γ/CD107a, $CD8^+$ vs. IFN-γ/CD107a, $CD4^+$ vs. IFN-γ/CD107a and $CD8^+$ vs. $CD4^+$.

Quadrants were set on each of these graphs to define where the population of negative cells was located, negative quadrant. Any cells located in the quadrant right of the negative quadrant were considered positive for the marker in question. In the case of the negative control this displayed the percent of cells normally over-expressing the marker; in anti-CD3 and virus treatments displayed the percent of cells activated by the treatment. Using these gates and quadrants a layout was created for the patient, which was used for all treatments pre- and post-vaccination. The subject's layout was used to analyse the anti-CD3 and virus stimulated cells for evidence of activation based on change in the percentage of cells ($CD3^+$, $CD4^+$, and $CD8^+$) expressing surface CD107a or intracellular IFN-γ both pre- and post-vaccination.

ELISA detection of IFN-γ and IL-10 cytokines

ELISA kits were obtained from eBioscience, human IFN-γ cat#88-7316 and human IL-10 cat#88-7106, and contained all necessary standards, coating buffers, assay diluents and antibodies. The protocol was kindly supplied by Dr. McElhaney's laboratory and is identical for both cyokines. To create a standard curve with eight points, 1:2 serial dilutions, in 1X assay diluent, of the supplied top standards were performed starting with an initial concentration for the IL-10 of 300pg/mL and 500pg/mL for IFN-γ. In a Corning 9018 96 well plate, 1/250 dilution of the capture antibody in 1X coating buffer was plated 100µL per well. The plate was sealed and incubated for a minimum of overnight but not

exceeding 48 hours at 4°C. After coating the supernatant was removed by vacuum aspiration and washed three times with ≥300µL per well of wash buffer (1XPBS containing 0.05% Tween). Following washing the plate was inverted and knocked on absorbent to remove any residual buffer clinging on the well sides or bottom. One hundred microlitres per well of each the standard and samples were plated in duplicate after which the plate was sealed and incubated at room temperature for two hours. For the IFN-γ assay only supernatants were diluted 1:4 before plating. After incubation the supernatant was removed by vacuum aspiration and washed five times with ≥300µL per well of wash buffer. Following washing the plate was inverted and knocked on absorbent to remove any residual buffer clinging on the well sides or bottom. To each well 100µL of 1/250 dilution of detection antibody in 1X assay diluent was added. The plate was resealed and incubated for an hour at room temperature. After incubation the supernatant was removed by vacuum aspiration and washed five times with ≥300µL per well of wash buffer. Following washing the plate was inverted and knocked on absorbent to remove any residual buffer clinging on the well sides or bottom. To each well 100µL of 1/250 dilution of avidin-HRP in 1X assay diluent was added. The plate was resealed and incubated for thirty minutes at room temperature. After incubation the supernatant was removed by vacuum aspiration and washed seven times with ≥300µL per well of wash buffer. Following washing the plate was inverted and knocked on absorbent to remove any residual buffer clinging on the well sides or bottom. To each well 100µL of

TMB substrate solution was added, the plate sealed and incubated at room temperature in the dark for fifteen minutes. After the incubation 50µL of stop solution (1N H_2SO_4) was added to every well and the plate read at 450nm on microplate reader. The optical density (OD) readings generated for the standard concentrations were averaged, if more than one plate was created during the same run all sets of duplicates were averaged together, and were used to create a standard curve that could be then used to determine the concentration of cytokine in the samples from the average OD value of the duplicate wells. The calculations and graphs were completed using Microsoft Office Excel 2003.

Granzyme B colourimetric assay

The protocol for this assay was kindly provided by Dr. McElhaney's laboratory and is as follows. To create a standard curve with eight points, 1:2 serial dilutions, in cell lysis buffer (150mM NaCL, 15mM Tris, 1% Triton X-100, 2µg/µL bovine serum albumin, pH to 8.0 with HCl) using the Biomol Granzyme B units (Biomol, cat#SE-238) as the top standard were performed starting with an initial concentration of 20 units and 0 units for the final point. Throughout the plating portion of the protocol the plate and lysates were kept cold. In a Corning 9018 96 well plate 20µL of each the standard and samples were plated in duplicate to which 80µL of Master Mix was added. The Master Mix consists of the following multiplied by the number of total wells used: 50µL of 2X substrate reaction buffer (20% sucrose, 0.2% CHAPS, 100mM HEPES pH 7.5), 1µL (1M)

dithiothreitol, 2µL 2X Granzyme B substrate VIII, IEPDpNA (Calbiochem cat#368067) and 27µL of nanopure water. The plate was then read at 405nm on a microplate reader as time zero. The plate was then sealed and incubated for twenty hours in dark humidified chamber at 37°C. After the incubation the plate was again read at 405nm on a microplate reader. The OD readings generated for the standard concentrations were averaged, if more than one plate was created during the same run all sets of duplicates were averaged together, were used to create a standard curve that could be then used to determine the concentration of granzyme B in the samples from the average OD value of the duplicate wells. The calculations and graphs were completed using Microsoft Office Excel 2003.

Results

Collected data sets

Compete data sets consisting of serum, supernatant for ELISA and granzyme B assays and CD107a and intracellular IFN-γ flow cytometry were not gathered from every patient pre-vaccination because of difficulty in recovering adequate numbers of PBMCs. Therefore, because of this difficulty for each participant only what was collected pre-vaccination was collected post-vaccination. Many participants were lost to followup after vaccination leaving a total of five out of thirteen patients in the KTx group and twelve out of fifteen in the group of HV. In the KTx group for two patients only serum was collected, for two patients serum and supernatant were collected and for one patient serum,

supernatant and CD107a flow cytometry data were collected. In the HV group, serum only was collected from one participant, serum and supernatant collected from two participants, serum, supernatant and CD107a flow cytometry data from three participants and complete data sets from six participants. In data collection KTx recipients were designated with codes starting with a 'T' while a 'C' was used for HV.

ELISA detection of IFN-γ

For the detection of IFN-γ there were a total of fourteen participants eleven HV and three KTx recipients. The standard curves for both pre- and post-vaccination worked beautifully producing a linear line of points that lay essentially directly along the calculated regression line (Figures 1 and 2). The formula used to calculate the IFN-γ concentration in the samples was [IFN-γ]**=** (OD value**-**0.109)**/**0.002 for pre-vaccination and [IFN-γ]**=** (OD value**-**0.138)**/**0.002 for post-vaccination samples. Each formula was derived from the equation the regression line of the respective standard curves. Unfortunately for the majority pre- and post-vaccination the samples the OD values were roughly equal to or only slightly above that of the lowest point on the standard curve (Tables 1 and 2).

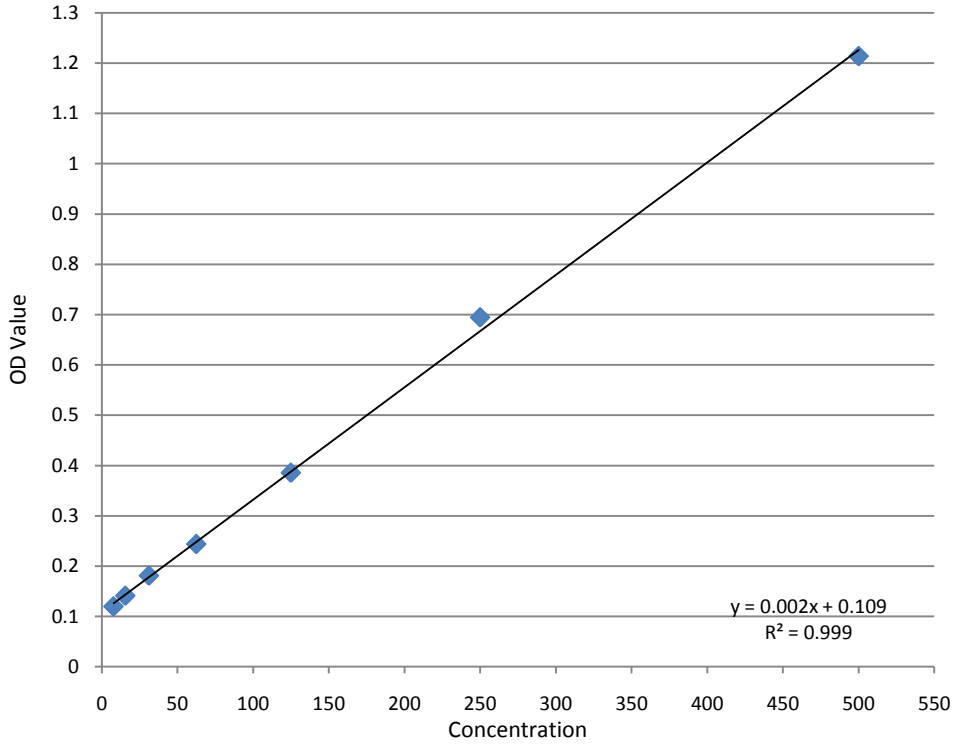

Figure 1. Pre-vaccination INF-γ standard curve

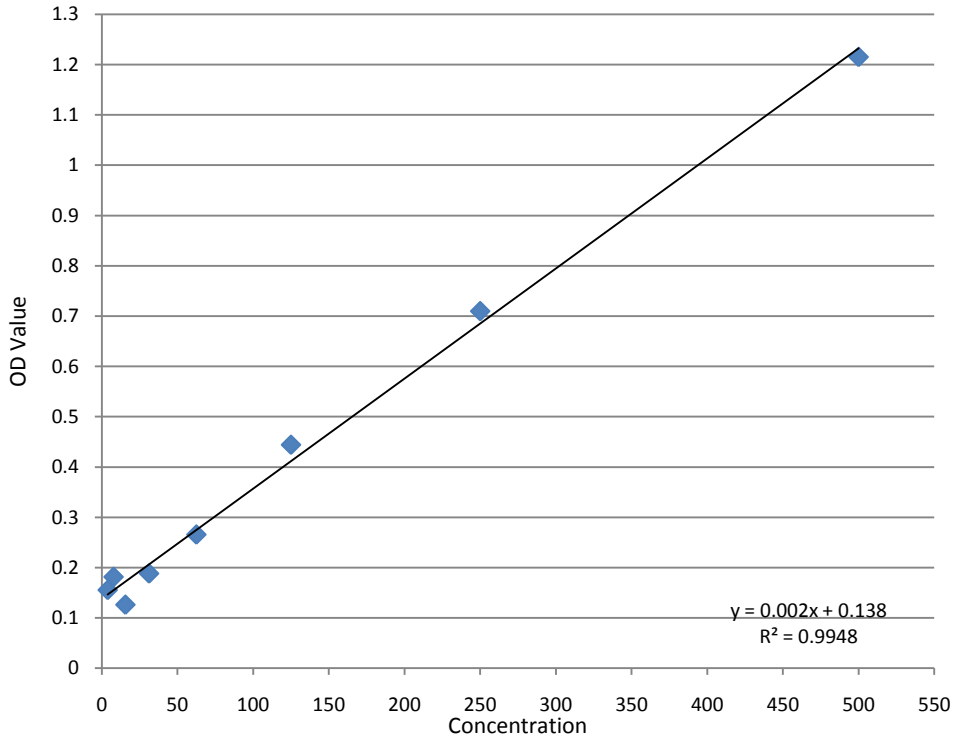

Figure 2. Post-vaccination INF-γ standard curve

Table 1. Pre-vaccination IFN-γ Concentrations

Sample	OD avg. Value	Plate [IFN-γ]	Total [IFN-γ]
C001-AH Negative Control	0.1260	8.5000	34.0000
C001-AH Positive Control	0.1305	10.7500	43.0000
C001-AH A/PR	0.1235	7.2500	29.0000
C001-AH A/Aichi	0.1400	15.5000	62.0000
C001-AH B/Lee	0.1285	9.7500	39.0000
C002-OM Negative Control	0.1195	5.2500	21.0000
C002-OM Positive Control	0.1020	-3.5000	-14.0000
C002-OM A/PR	0.0800	-14.5000	-58.0000
C002-OM A/Aichi	0.0885	-10.2500	-41.0000
C002-OM B/Lee	0.1000	-4.5000	-18.0000
C003-LP Negative Control	0.0850	-12.0000	-48.0000
C003-LP Positive Control	0.0915	-8.7500	-35.0000
C003-LP A/PR	0.1305	10.7500	43.0000
C003-LP A/Aichi	0.1030	-3.0000	-12.0000
C003-LP B/Lee	0.0960	-6.5000	-26.0000
C007-DF Negative Control	0.0800	-14.5000	-58.0000
C007-DF Positive Control	0.0725	-18.2500	-73.0000
C007-DF A/PR	0.0830	-13.0000	-52.0000
C007-DF A/Aichi	0.0649	-22.0500	-88.2000
C007-DF B/Lee	0.0945	-7.2500	-29.0000
C008-VL Negative Control	0.0980	-5.5000	-22.0000
C008-VL Positive Control	0.0945	-7.2500	-29.0000
C008-VL A/PR	0.1030	-3.0000	-12.0000
C008-VL A/Aichi	0.1125	1.7500	7.0000
C008-VL B/Lee	0.0805	-14.2500	-57.0000
C009-RP Negative Control	0.0790	-15.0000	-60.0000
C009-RP Positive Control	0.0785	-15.2500	-61.0000
C009-RP A/PR	0.0785	-15.2500	-61.0000
C009-RP A/Aichi	0.1085	-0.2500	-1.0000
C009-RP B/Lee	0.1010	-4.0000	-16.0000
C010-CF Negative Control	0.0940	-7.5000	-30.0000
C010-CF Positive Control	0.0925	-8.2500	-33.0000
C010-CF A/PR	0.0745	-17.2500	-69.0000
C010-CF A/Aichi	0.0980	-5.5000	-22.0000
C010-CF B/Lee	0.0800	-14.5000	-58.0000

Table 1. Pre-vaccination IFN-γ Concentrations (continued)

Sample	OD avg. Value	Plate [IFN-γ]	Total [IFN-γ]
C011-DK Negative Control	0.0955	-6.7500	-27.0000
C011-DK Positive Control	0.1170	4.0000	16.0000
C011-DK A/PR	0.0945	-7.2500	-29.0000
C011-DK A/Aichi	0.1700	30.5000	122.0000
C011-DK B/Lee	0.1495	20.2500	81.0000
C012-ML Negative Control	0.0980	-5.5000	-22.0000
C012-ML Positive Control	0.2085	49.7500	199.0000
C012-ML A/PR	0.2210	56.0000	224.0000
C012-ML A/Aichi	0.7625	326.7500	1307.0000
C012-ML B/Lee	0.1505	20.7500	83.0000
C013-KA Negative Control	0.0955	-6.7500	-27.0000
C013-KA Positive Control	0.1625	26.7500	107.0000
C013-KA A/PR	0.1750	33.0000	132.0000
C013-KA A/Aichi	0.2060	48.5000	194.0000
C013-KA B/Lee	0.1425	16.7500	67.0000
C015-LM Negative Control	0.0895	-9.7500	-39.0000
C015-LM Positive Control	0.1010	-4.0000	-16.0000
C015-LM A/PR	0.1585	24.7500	99.0000
C015-LM A/Aichi	0.3125	101.7500	407.0000
C015-LM B/Lee	0.2845	87.7500	351.0000
T003-AM Negative Control	0.1135	2.2500	9.0000
T003-AM Positive Control	0.0805	-14.2500	-57.0000
T003-AM A/PR	0.1040	-2.5000	-10.0000
T003-AM A/Aichi	0.1020	-3.5000	-14.0000
T003-AM B/Lee	0.0850	-12.0000	-48.0000
T005-BP Negative Control	0.0860	-11.5000	-46.0000
T005-BP Positive Control	0.0915	-8.7500	-35.0000
T005-BP A/PR	0.0900	-9.5000	-38.0000
T005-BP A/Aichi	0.0835	-12.7500	-51.0000
T005-BP B/Lee	0.0890	-10.0000	-40.0000
T009-EM Negative Control	0.0930	-8.0000	-32.0000
T009-EM Positive Control	0.0900	-9.5000	-38.0000
T009-EM A/PR	0.0955	-6.7500	-27.0000
T009-EM A/Aichi	0.1210	6.0000	24.0000
T009-EM B/Lee	0.0970	-6.0000	-24.0000

Table 2. Post-vaccination IFN-γ Concentrations

Sample	OD avg. Value	Plate [IFN-γ]	Total [IFN-γ]
C001-AH Negative Control	0.0990	-19.5000	-78.0000
C001-AH Positive Control	0.0860	-26.0000	-104.0000
C001-AH A/PR	0.0690	-34.5000	-138.0000
C001-AH A/Aichi	0.0690	-34.5000	-138.0000
C001-AH B/Lee	0.0835	-27.2500	-109.0000
C002-OM Negative Control	0.0870	-25.5000	-102.0000
C002-OM Positive Control	0.1185	-9.7500	-39.0000
C002-OM A/PR	0.0905	-23.7500	-95.0000
C002-OM A/Aichi	0.0880	-25.0000	-100.0000
C002-OM B/Lee	0.0675	-35.2500	-141.0000
C003-LP Negative Control	0.0740	-32.0000	-128.0000
C003-LP Positive Control	0.4340	148.0000	592.0000
C003-LP A/PR	0.0825	-27.7500	-111.0000
C003-LP A/Aichi	0.0720	-33.0000	-132.0000
C003-LP B/Lee	0.0720	-33.0000	-132.0000
C007-DF Negative Control	0.0825	-27.7500	-111.0000
C007-DF Positive Control	0.0910	-23.5000	-94.0000
C007-DF A/PR	0.0895	-24.2500	-97.0000
C007-DF A/Aichi	0.1265	-5.7500	-23.0000
C007-DF B/Lee	0.1030	-17.5000	-70.0000
C008-VL Negative Control	0.0955	-21.2500	-85.0000
C008-VL Positive Control	0.0990	-19.5000	-78.0000
C008-VL A/PR	0.1115	-13.2500	-53.0000
C008-VL A/Aichi	0.2130	37.5000	150.0000
C008-VL B/Lee	0.1200	-9.0000	-36.0000
C009-RP Negative Control	0.0855	-26.2500	-105.0000
C009-RP Positive Control	0.0890	-24.5000	-98.0000
C009-RP A/PR	0.0905	-23.7500	-95.0000
C009-RP A/Aichi	0.1255	-6.2500	-25.0000
C009-RP B/Lee	0.0950	-21.5000	-86.0000
C010-CF Negative Control	0.0965	-20.7500	-83.0000
C010-CF Positive Control	0.0970	-20.5000	-82.0000
C010-CF A/PR	0.0950	-21.5000	-86.0000
C010-CF A/Aichi	0.1625	12.2500	49.0000
C010-CF B/Lee	0.1330	-2.5000	-10.0000

Table 2. Post-vaccination IFN-γ Concentrations (continued)

Sample	OD avg. Value	Plate [IFN-γ]	Total [IFN-γ]
C011-DK Negative Control	0.1260	-6.0000	-24.0000
C011-DK Positive Control	0.1215	-8.2500	-33.0000
C011-DK A/PR	0.1115	-13.2500	-53.0000
C011-DK A/Aichi	0.1600	11.0000	44.0000
C011-DK B/Lee	0.1245	-6.7500	-27.0000
C012-ML Negative Control	0.1175	-10.2500	-41.0000
C012-ML Positive Control	0.0960	-21.0000	-84.0000
C012-ML A/PR	0.2665	64.2500	257.0000
C012-ML A/Aichi	1.1925	527.2500	2109.0000
C012-ML B/Lee	0.3755	118.7500	475.0000
C013-KA Negative Control	0.0840	-27.0000	-108.0000
C013-KA Positive Control	0.0960	-21.0000	-84.0000
C013-KA A/PR	0.1065	-15.7500	-63.0000
C013-KA A/Aichi	0.3740	118.0000	472.0000
C013-KA B/Lee	0.1300	-4.0000	-16.0000
C015-LM Negative Control	0.1055	-16.2500	-65.0000
C015-LM Positive Control	0.1035	-17.2500	-69.0000
C015-LM A/PR	0.1345	-1.7500	-7.0000
C015-LM A/Aichi	0.2785	70.2500	281.0000
C015-LM B/Lee	0.1565	9.2500	37.0000
T003-AM Negative Control	0.1015	-18.2500	-73.0000
T003-AM Positive Control	0.1090	-14.5000	-58.0000
T003-AM A/PR	0.1030	-17.5000	-70.0000
T003-AM A/Aichi	0.0920	-23.0000	-92.0000
T003-AM B/Lee	0.1125	-12.7500	-51.0000
T005-BP Negative Control	0.0950	-21.5000	-86.0000
T005-BP Positive Control	0.0975	-20.2500	-81.0000
T005-BP A/PR	0.1170	-10.5000	-42.0000
T005-BP A/Aichi	0.1815	21.7500	87.0000
T005-BP B/Lee	0.1515	6.7500	27.0000
T009-EM Negative Control	0.1260	-6.0000	-24.0000
T009-EM Positive Control	0.1040	-17.0000	-68.0000
T009-EM A/PR	0.1025	-17.7500	-71.0000
T009-EM A/Aichi	0.1305	-3.7500	-15.0000
T009-EM B/Lee	0.1005	-18.7500	-75.0000

ELISA detection of IL-10

For the detection of IL-10 there were a total of fourteen participants eleven HV and three KTx recipients. The standard curves for both pre- and post-vaccination worked beautifully producing a linear line of points that lay essentially directly along the calculated regression line (Figures 3 and 4). The formula used to calculate the IL-10 concentration in the samples was [IL-10]**=** (OD value**-**0.207)**/**0.003 for pre-vaccination and [IL-10]**=** (OD value**-**0.128)**/**0.007 for post-vaccination samples. Each formula was derived from the equation the regression line of the respective standard curves. Unfortunately for the majority pre- and post-vaccination the samples the OD values were roughly equal to or only slightly above that of the lowest point on the standard curve (Tables 3 and 4).

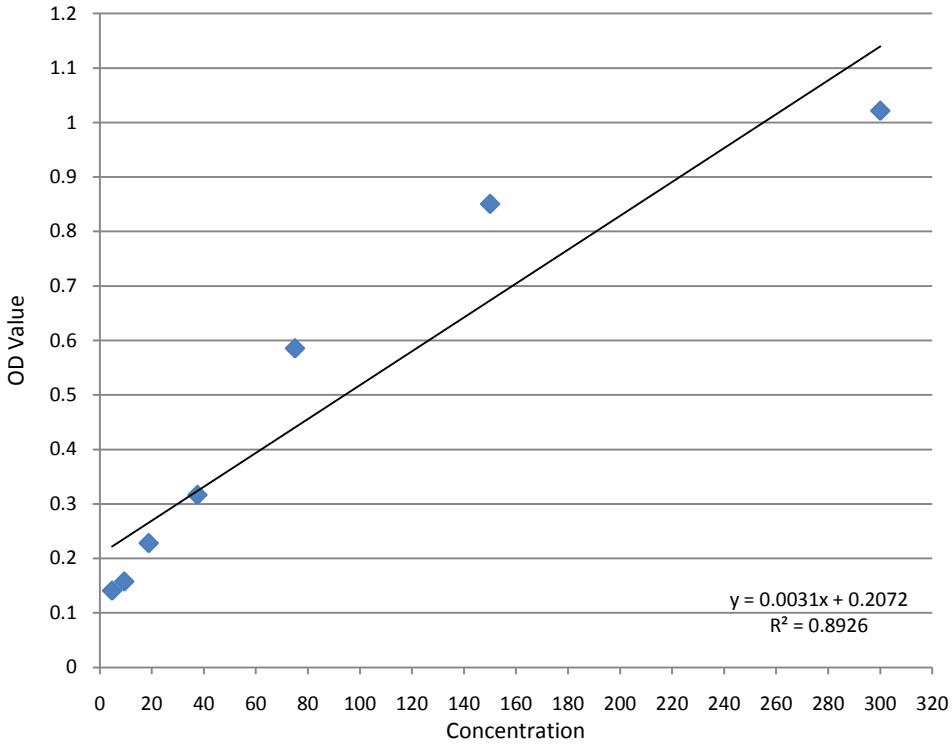

Figure 3. Pre-vaccination IL-10 standard curve

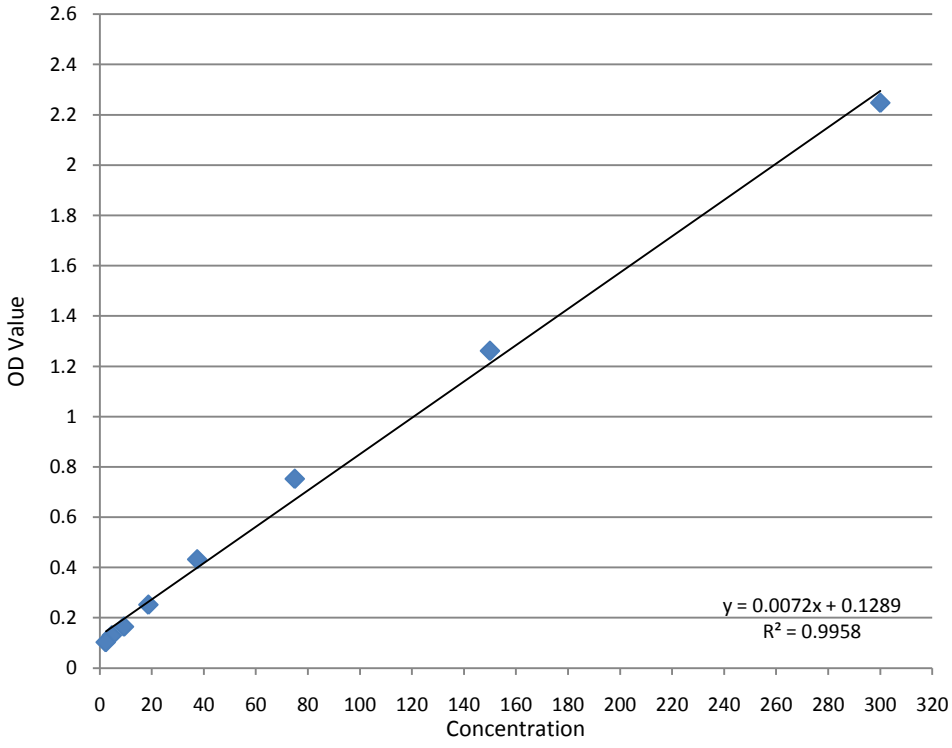

Figure 4. Post-vaccination IL-10 standard curve

Table 3. Pre-vaccination IL-10 Concentrations

Sample	OD avg. Value	Total [IL-10]
C001-AH Negative Control	0.2660	19.6667
C001-AH Positive Control	0.3725	55.1667
C001-AH A/PR	0.3620	51.6667
C001-AH A/Aichi	0.2085	0.5000
C001-AH B/Lee	0.1605	-15.5000
C002-OM Negative Control	0.1255	-27.1667
C002-OM Positive Control	0.1185	-29.5000
C002-OM A/PR	0.1015	-35.1667
C002-OM A/Aichi	0.1465	-20.1667
C002-OM B/Lee	0.2010	-2.0000
C003-LP Negative Control	0.2325	8.5000
C003-LP Positive Control	0.1385	-22.8333
C003-LP A/PR	0.1235	-27.8333
C003-LP A/Aichi	0.1055	-33.8333
C003-LP B/Lee	0.1040	-34.3333
C007-DF Negative Control	0.0925	-38.1667
C007-DF Positive Control	0.1205	-28.8333
C007-DF A/PR	0.1415	-21.8333
C007-DF A/Aichi	0.1510	-18.6667
C007-DF B/Lee	0.1090	-32.6667
C008-VL Negative Control	0.1095	-32.5000
C008-VL Positive Control	0.1030	-34.6667
C008-VL A/PR	0.0950	-37.3333
C008-VL A/Aichi	0.0890	-39.3333
C008-VL B/Lee	0.1525	-18.1667
C009-RP Negative Control	0.1270	-26.6667
C009-RP Positive Control	0.0955	-37.1667
C009-RP A/PR	0.1045	-34.1667
C009-RP A/Aichi	0.1030	-34.6667
C009-RP B/Lee	0.0870	-40.0000
C010-CF Negative Control	0.0965	-36.8333
C010-CF Positive Control	0.0905	-38.8333
C010-CF A/PR	0.1605	-15.5000
C010-CF A/Aichi	0.1705	-12.1667
C010-CF B/Lee	0.1415	-21.8333

Table 3. Pre-vaccination IL-10 Concentrations (continued)

Sample	OD avg. Value	Total [IL-10]
C011-DK Negative Control	0.1320	-25.0000
C011-DK Positive Control	0.1410	-22.0000
C011-DK A/PR	0.1070	-33.3333
C011-DK A/Aichi	0.1360	-23.6667
C011-DK B/Lee	0.1320	-25.0000
C012-ML Negative Control	0.0655	-47.1667
C012-ML Positive Control	0.0870	-40.0000
C012-ML A/PR	0.0930	-38.0000
C012-ML A/Aichi	0.1120	-31.6667
C012-ML B/Lee	0.0820	-41.6667
C013-KA Negative Control	0.0715	-45.1667
C013-KA Positive Control	0.0775	-43.1667
C013-KA A/PR	0.0750	-44.0000
C013-KA A/Aichi	0.1070	-33.3333
C013-KA B/Lee	0.0955	-37.1667
C015-LM Negative Control	0.1060	-33.6667
C015-LM Positive Control	0.0950	-37.3333
C015-LM A/PR	0.0900	-39.0000
C015-LM A/Aichi	0.1010	-35.3333
C015-LM B/Lee	0.1005	-35.5000
T003-AM Negative Control	0.0550	-50.6667
T003-AM Positive Control	0.0495	-52.5000
T003-AM A/PR	0.3095	34.1667
T003-AM A/Aichi	0.0655	-47.1667
T003-AM B/Lee	0.0660	-47.0000
T005-BP Negative Control	0.0560	-50.3333
T005-BP Positive Control	0.0550	-50.6667
T005-BP A/PR	0.0535	-51.1667
T005-BP A/Aichi	0.0565	-50.1667
T005-BP B/Lee	0.0660	-47.0000
T009-EM Negative Control	0.0695	-45.8333
T009-EM Positive Control	0.0795	-42.5000
T009-EM A/PR	0.0610	-48.6667
T009-EM A/Aichi	0.0665	-46.8333
T009-EM B/Lee	0.0575	-49.8333

Table 4. Post-vaccination IL-10 Concentrations

Sample	OD avg. Value	Total [IL-10]
C001-AH Negative Control	0.1215	-0.9286
C001-AH Positive Control	0.1135	-2.0714
C001-AH A/PR	0.0915	-5.2143
C001-AH A/Aichi	0.0760	-7.4286
C001-AH B/Lee	0.0820	-6.5714
C002-OM Negative Control	0.0640	-9.1429
C002-OM Positive Control	0.0730	-7.8571
C002-OM A/PR	0.0670	-8.7143
C002-OM A/Aichi	0.0680	-8.5714
C002-OM B/Lee	0.0820	-6.5714
C003-LP Negative Control	0.0725	-7.9286
C003-LP Positive Control	0.0620	-9.4286
C003-LP A/PR	0.0745	-7.6429
C003-LP A/Aichi	0.0615	-9.5000
C003-LP B/Lee	0.0695	-8.3571
C007-DF Negative Control	0.0540	-10.5714
C007-DF Positive Control	0.0610	-9.5714
C007-DF A/PR	0.0775	-7.2143
C007-DF A/Aichi	0.0735	-7.7857
C007-DF B/Lee	0.0650	-9.0000
C008-VL Negative Control	0.0685	-8.5000
C008-VL Positive Control	0.0585	-9.9286
C008-VL A/PR	0.0710	-8.1429
C008-VL A/Aichi	0.0745	-7.6429
C008-VL B/Lee	0.0825	-6.5000
C009-RP Negative Control	0.0885	-5.6429
C009-RP Positive Control	0.0715	-8.0714
C009-RP A/PR	0.1010	-3.8571
C009-RP A/Aichi	0.0975	-4.3571
C009-RP B/Lee	0.0710	-8.1429
C010-CF Negative Control	0.0650	-9.0000
C010-CF Positive Control	0.0590	-9.8571
C010-CF A/PR	0.0965	-4.5000
C010-CF A/Aichi	0.1450	2.4286
C010-CF B/Lee	0.1215	-0.9286

Table 4. Post-vaccination IL-10 Concentrations (continued)

Sample	OD avg. Value	Total [IL-10]
C011-DK Negative Control	0.1215	-0.9286
C011-DK Positive Control	0.1100	-2.5714
C011-DK A/PR	0.1125	-2.2143
C011-DK A/Aichi	0.1890	8.7143
C011-DK B/Lee	0.0980	-4.2857
C012-ML Negative Control	0.0750	-7.5714
C012-ML Positive Control	0.0720	-8.0000
C012-ML A/PR	0.1015	-3.7857
C012-ML A/Aichi	0.1280	0.0000
C012-ML B/Lee	0.0885	-5.6429
C013-KA Negative Control	0.0675	-8.6429
C013-KA Positive Control	0.0750	-7.5714
C013-KA A/PR	0.0790	-7.0000
C013-KA A/Aichi	0.0890	-5.5714
C013-KA B/Lee	0.0760	-7.4286
C015-LM Negative Control	0.0665	-8.7857
C015-LM Positive Control	0.0880	-5.7143
C015-LM A/PR	0.0795	-6.9286
C015-LM A/Aichi	0.3170	27.0000
C015-LM B/Lee	0.0780	-7.1429
T003-AM Negative Control	0.0650	-9.0000
T003-AM Positive Control	0.0655	-8.9286
T003-AM A/PR	0.0835	-6.3571
T003-AM A/Aichi	0.0775	-7.2143
T003-AM B/Lee	0.0750	-7.5714
T005-BP Negative Control	0.0795	-6.9286
T005-BP Positive Control	0.0620	-9.4286
T005-BP A/PR	0.0930	-5.0000
T005-BP A/Aichi	0.1105	-2.5000
T005-BP B/Lee	0.0920	-5.1429
T009-EM Negative Control	0.0750	-7.5714
T009-EM Positive Control	0.6850	79.5714
T009-EM A/PR	0.0775	-7.2143
T009-EM A/Aichi	0.0825	-6.5000
T009-EM B/Lee	0.0635	-9.2143

Granzyme B colourimetric assay

For the detection of granzyme B there were a total of thirteen participants eleven HV and two KTx recipients. The standard curves for both pre- and post-vaccination worked beautifully producing a quadratic line of points that lay essentially directly along the calculated regression line (Figures 5 and 6). The formula used to calculate the IL-10 concentration in the samples was [granzyme B]**=** (OD value**-**0.077)**/**0.019 for pre-vaccination and [granzyme B]**=** (OD value**-**0.092)**/**0.022 for post-vaccination samples. Each formula was derived from the equation the regression line of the respective standard curves. Unfortunately for the majority pre- and post-vaccination the samples the OD values were roughly equal to or only slightly above that of the blank standard curve wells (Tables 5 and 6).

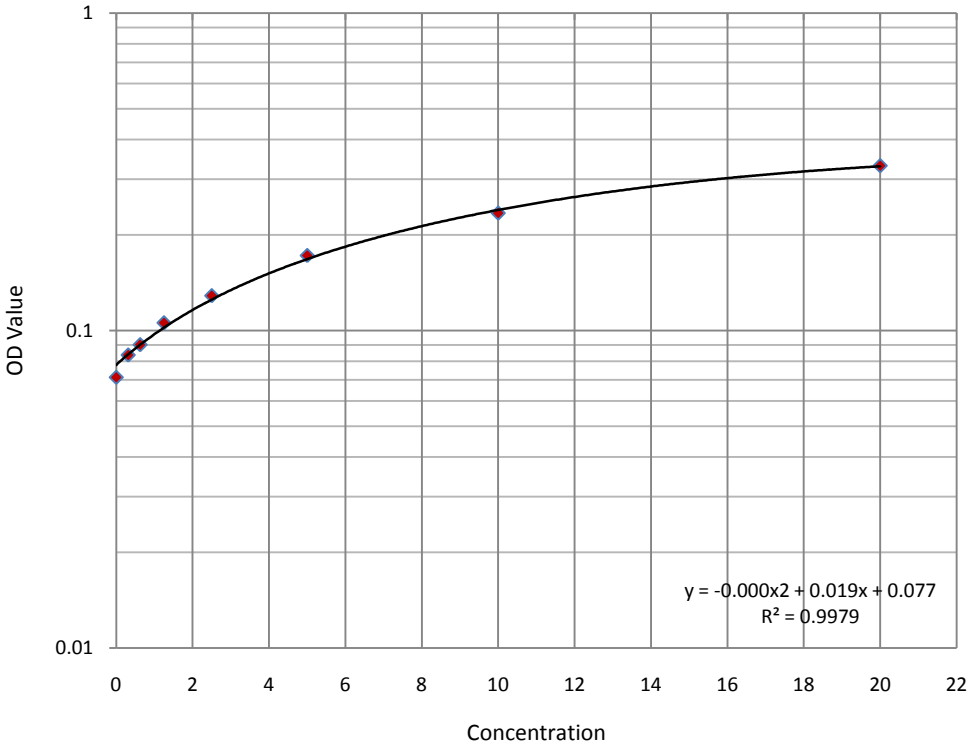

Figure 5. Pre-vaccination granzyme B standard curve

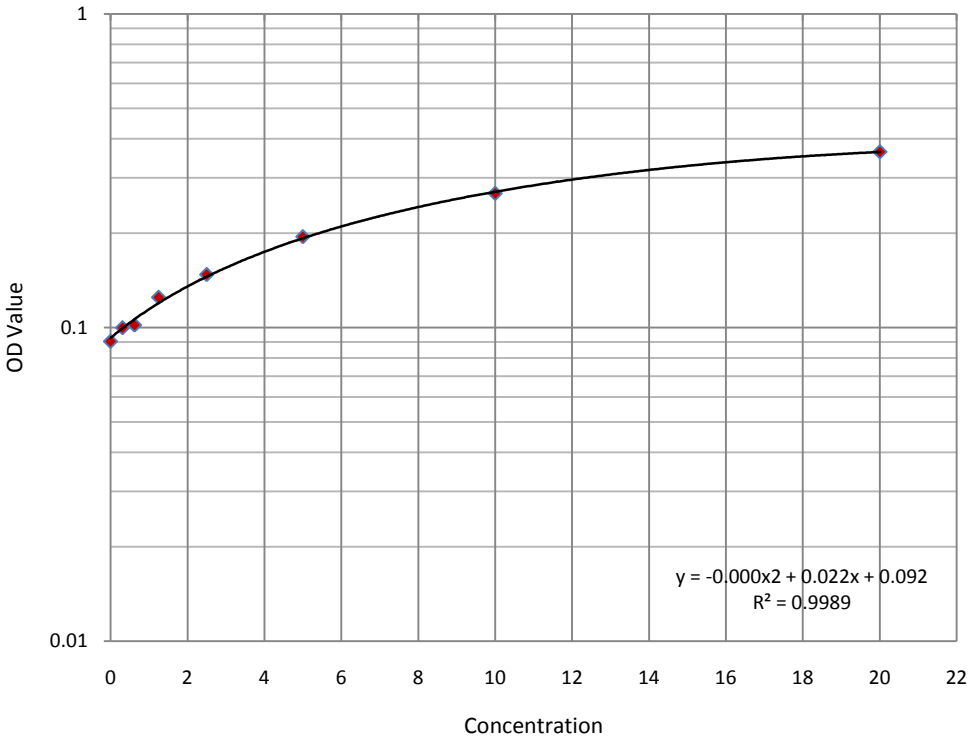

Figure 6. Post-vaccination granzyme B standard curve

Table 5. Pre-vaccination Granzyme B Concentrations

Sample	OD avg. Value	Total [Granzyme B]
C001-AH Negative Control	0.0635	-0.7105
C001-AH Positive Control	0.0710	-0.3158
C001-AH A/PR	0.0655	-0.6053
C001-AH A/Aichi	0.0660	-0.5789
C001-AH B/Lee	0.0700	-0.3684
C002-OM Negative Control	0.0625	-0.7632
C002-OM Positive Control	0.0690	-0.4211
C002-OM A/PR	0.0620	-0.7895
C002-OM A/Aichi	0.0640	-0.6842
C002-OM B/Lee	0.0710	-0.3158
C003-LP Negative Control	0.0655	-0.6053
C003-LP Positive Control	0.0670	-0.5263
C003-LP A/PR	0.0705	-0.3421
C003-LP A/Aichi	0.0640	-0.6842
C003-LP B/Lee	0.0730	-0.2105
C007-DF Negative Control	0.0620	-0.7895
C007-DF Positive Control	0.0655	-0.6053
C007-DF A/PR	0.0670	-0.5263
C007-DF A/Aichi	0.0625	-0.7632
C007-DF B/Lee	0.0675	-0.5000
C008-VL Negative Control	0.0715	-0.2895
C008-VL Positive Control	0.0640	-0.6842
C008-VL A/PR	0.0755	-0.0789
C008-VL A/Aichi	0.0625	-0.7632
C008-VL B/Lee	0.0635	-0.7105
C009-RP Negative Control	0.0705	-0.3421
C009-RP Positive Control	0.0645	-0.6579
C009-RP A/PR	0.0665	-0.5526
C009-RP A/Aichi	0.0695	-0.3947
C009-RP B/Lee	0.0655	-0.6053
C010-CF Negative Control	0.0720	-0.2632
C010-CF Positive Control	0.0610	-0.8421
C010-CF A/PR	0.0595	-0.9211
C010-CF A/Aichi	0.0650	-0.6316
C010-CF B/Lee	0.0615	-0.8158

Table 5. Pre-vaccination Granzyme B Concentrations (continued)

Sample	OD avg. Value	Total [Granzyme B]
C011-DK Negative Control	0.0650	-0.6316
C011-DK Positive Control	0.0700	-0.3684
C011-DK A/PR	0.0645	-0.6579
C011-DK A/Aichi	0.0740	-0.1579
C011-DK B/Lee	0.0595	-0.9211
C012-ML Negative Control	0.0635	-0.7105
C012-ML Positive Control	0.0680	-0.4737
C012-ML A/PR	0.0645	-0.6579
C012-ML A/Aichi	0.0660	-0.5789
C012-ML B/Lee	0.0725	-0.2368
C013-KA Negative Control	0.0655	-0.6053
C013-KA Positive Control	0.0725	-0.2368
C013-KA A/PR	0.0665	-0.5526
C013-KA A/Aichi	0.0630	-0.7368
C013-KA B/Lee	0.0725	-0.2368
C015-LM Negative Control	0.0630	-0.7368
C015-LM Positive Control	0.0665	-0.5526
C015-LM A/PR	0.0715	-0.2895
C015-LM A/Aichi	0.0665	-0.5526
C015-LM B/Lee	0.0730	-0.2105
T003-AM Negative Control	0.0620	-0.7895
T003-AM Positive Control	0.0625	-0.7632
T003-AM A/PR	0.0725	-0.2368
T003-AM A/Aichi	0.0640	-0.6842
T003-AM B/Lee	0.0685	-0.4474
T009-EM Negative Control	0.0705	-0.3421
T009-EM Positive Control	0.0655	-0.6053
T009-EM A/PR	0.0740	-0.1579
T009-EM A/Aichi	0.0590	-0.9474
T009-EM B/Lee	0.0625	-0.7632

Table 6. Post-vaccination Granzyme B Concentrations

Sample	OD avg. Value	Total [Granzyme B]
C001-AH Negative Control	0.0705	-0.9773
C001-AH Positive Control	0.0705	-0.9773
C001-AH A/PR	0.0700	-1.0000
C001-AH A/Aichi	0.0690	-1.0455
C001-AH B/Lee	0.0740	-0.8182
C002-OM Negative Control	0.0715	-0.9318
C002-OM Positive Control	0.0790	-0.5909
C002-OM A/PR	0.0680	-1.0909
C002-OM A/Aichi	0.0680	-1.0909
C002-OM B/Lee	0.0725	-0.8864
C003-LP Negative Control	0.0690	-1.0455
C003-LP Positive Control	0.0685	-1.0682
C003-LP A/PR	0.0735	-0.8409
C003-LP A/Aichi	0.0730	-0.8636
C003-LP B/Lee	0.0770	-0.6818
C007-DF Negative Control	0.0685	-1.0682
C007-DF Positive Control	0.0705	-0.9773
C007-DF A/PR	0.0740	-0.8182
C007-DF A/Aichi	0.0710	-0.9545
C007-DF B/Lee	0.0715	-0.9318
C008-VL Negative Control	0.0765	-0.7045
C008-VL Positive Control	0.0735	-0.8409
C008-VL A/PR	0.0775	-0.6591
C008-VL A/Aichi	0.0650	-1.2273
C008-VL B/Lee	0.0715	-0.9318
C009-RP Negative Control	0.0775	-0.6591
C009-RP Positive Control	0.0700	-1.0000
C009-RP A/PR	0.0725	-0.8864
C009-RP A/Aichi	0.0765	-0.7045
C009-RP B/Lee	0.0700	-1.0000
C010-CF Negative Control	0.0765	-0.7045
C010-CF Positive Control	0.0670	-1.1364
C010-CF A/PR	0.0695	-1.0227
C010-CF A/Aichi	0.0745	-0.7955
C010-CF B/Lee	0.0690	-1.0455

Table 6. Post-vaccination Granzyme B Concentrations (continued)

Sample	OD avg. Value	Total [Granzyme B]
C011-DK Negative Control	0.0715	-0.9318
C011-DK Positive Control	0.0755	-0.7500
C011-DK A/PR	0.0710	-0.9545
C011-DK A/Aichi	0.0785	-0.6136
C011-DK B/Lee	0.0655	-1.2045
C012-ML Negative Control	0.0655	-1.2045
C012-ML Positive Control	0.3825	13.2045
C012-ML A/PR	0.0685	-1.0682
C012-ML A/Aichi	0.0805	-0.5227
C012-ML B/Lee	0.0750	-0.7727
C013-KA Negative Control	0.0695	-1.0227
C013-KA Positive Control	0.0780	-0.6364
C013-KA A/PR	0.0720	-0.9091
C013-KA A/Aichi	0.0660	-1.1818
C013-KA B/Lee	0.0710	-0.9545
C015-LM Negative Control	0.0674	-1.1182
C015-LM Positive Control	0.0700	-1.0000
C015-LM A/PR	0.0750	-0.7727
C015-LM A/Aichi	0.0775	-0.6591
C015-LM B/Lee	0.0810	-0.5000
T003-AM Negative Control	0.0720	-0.9091
T003-AM Positive Control	0.0675	-1.1136
T003-AM A/PR	0.0725	-0.8864
T003-AM A/Aichi	0.0700	-1.0000
T003-AM B/Lee	0.0735	-0.8409
T009-EM Negative Control	0.0790	-0.5909
T009-EM Positive Control	0.0725	-0.8864
T009-EM A/PR	0.0800	-0.5455
T009-EM A/Aichi	0.0675	-1.1136
T009-EM B/Lee	0.0680	-1.0909

Flow cytometry

For the flow cytometry analysis there were a total of four participants with only CD107a staining; one KTx and three HV and there were a total of six participants with CD107a and IFN-γ staining, all were HV. This gave a total of ten participants examined for CD107a staining and six for IFN-γ staining. In both the CD107a and IFN-γ cells, mixing PBMCs from different donors to induce an alloresponse for use as a positive control failed to work consistently (Tables 7 and 8). In the majority of participants there was no difference between CD107a and IFN-γ expression between the negative and positive controls. In a few of the participants the positive control did work but not both pre- and post-vaccination and generally wasn't a large increase from the negative control value. When the gathered CD107a data on $CD8^+$ T-cells was examined for possible trends, it was found that this data was also not consistent. There were some participants with increases with all or some of the viruses, there were participants with decreases with all or some of the viruses, there were participants with both increases and decreases with viruses and in some participants there was no difference (Table 7). There was also no continuity with which viruses were increasing, decreasing or not responding between the participants. When the gathered IFN-γ data on $CD8^+$ and $CD4^+$ T-cells was examined for possible trends in only one of the six participants there was an increase; the other five all decreased (Table 8).

Table 7. Pre- and post-vaccination $CD8^+$ T-cell $CD107a^+$ values

Sample	Pre-vaccination $CD107a^+$ value	Post-vaccination $CD107a^+$ value
C001-AH Negative Control	1.39	2.55
C001-AH Positive Control	2.82	1.46
C001-AH A/PR	2.69	1.79
C001-AH A/Aichi	3.22	2.92
C001-AH B/Lee	2.91	2.59
C007-DF Negative Control	0.75	2.20
C007-DF Positive Control	1.52	2.53
C007-DF A/PR	2.55	3.54
C007-DF A/Aichi	3.32	3.32
C007-DF B/Lee	3.71	2.50
C008-VL Negative Control	0.49	0.38
C008-VL Positive Control	0.46	0.38
C008-VL A/PR	0.99	0.87
C008-VL A/Aichi	1.23	0.94
C008-VL B/Lee	1.05	1.16
C009-RP Negative Control	0.54	0.86
C009-RP Positive Control	0.89	0.97
C009-RP A/PR	1.54	2.09
C009-RP A/Aichi	1.17	2.21
C009-RP B/Lee	1.46	1.71
C010-CF Negative Control	0.68	0.48
C010-CF Positive Control	1.38	0.64
C010-CF A/PR	1.25	2.05
C010-CF A/Aichi	1.92	1.46
C010-CF B/Lee	1.27	1.98
C011-DK Negative Control	0.87	0.41
C011-DK Positive Control	0.94	0.32
C011-DK A/PR	1.19	0.81
C011-DK A/Aichi	1.88	0.92
C011-DK B/Lee	1.93	0.93
C012-ML Negative Control	0.72	0.66
C012-ML Positive Control	3.00	0.51
C012-ML A/PR	2.84	3.34
C012-ML A/Aichi	3.71	3.83
C012-ML B/Lee	2.57	3.47
C013-KA Negative Control	0.40	0.83
C013-KA Positive Control	0.55	0.30
C013-KA A/PR	1.27	0.71
C013-KA A/Aichi	2.28	1.38
C013-KA B/Lee	1.17	0.86
C015-LM Negative Control	0.61	0.49
C015-LM Positive Control	1.33	1.09
C015-LM A/PR	1.62	2.25
C015-LM A/Aichi	2.29	7.47
C015-LM B/Lee	1.43	3.91
T009-EM Negative Control	0.72	0.90
T009-EM Positive	0.75	1.52
T009-EM A/PR	1.12	1.08
T009-EM A/Aichi	4.84	1.79
T009-EM B/Lee	2.73	1.34

Table 8. Pre- and post-vaccination T-cell IFN-γ$^+$ values

Sample	Pre-vaccination IFN-γ$^+$ value	Post-vaccination IFN-γ$^+$ value
C001-AH Negative Control CD8$^+$	2.21	0.59
C001-AH Negative Control CD4$^+$	1.06	0.32
C001-AH Positive Control CD8$^+$	2.72	1.59
C001-AH Positve Control CD4$^+$	0.38	0.58
C001-AH A/PR CD8$^+$ T-Cell	1.87	1.20
C001-AH A/PR CD4$^+$ T-Cell	0.84	0.54
C007-DF Negative Control CD8$^+$	0.80	0.97
C007-DF Negative Control CD4$^+$	0.87	0.51
C007-DF Positive Control CD8$^+$	0.48	1.37
C007-DF Positve Control CD4$^+$	0.45	0.83
C007-DF A/PR CD8$^+$ T-Cell	0.88	0.98
C007-DF A/PR CD4$^+$ T-Cell	0.93	0.59
C008-VL Negative Control CD8$^+$	3.60	0.25
C008-VL Negative Control CD4$^+$	3.68	0.21
C008-VL Positive Control CD8$^+$	3.43	0.24
C008-VL Positve Control CD4$^+$	3.43	0.19
C008-VL A/PR CD8$^+$ T-Cell	3.22	0.51
C008-VL A/PR CD4$^+$ T-Cell	2.77	0.28
C011-DK Negative Control CD8$^+$	0.90	0.40
C011-DK Negative Control CD4$^+$	0.75	0.29
C011-DK Positive Control CD8$^+$	1.09	0.48
C011-DK Positve Control CD4$^+$	0.85	0.39
C011-DK A/PR CD8$^+$ T-Cell	0.96	0.52
C011-DK A/PR CD4$^+$ T-Cell	0.77	0.32
C012-ML Negative Control CD8$^+$	1.66	0.41
C012-ML Negative Control CD4$^+$	1.65	0.07
C012-ML Positive Control CD8$^+$	1.52	0.36
C012-ML Positve Control CD4$^+$	1.56	0.12
C012-ML A/PR CD8$^+$ T-Cell	2.58	0.63
C012-ML A/PR CD4$^+$ T-Cell	2.22	0.22
C013-KA Negative Control CD8$^+$	0.94	0.60
C013-KA Negative Control CD4$^+$	1.16	0.38
C013-KA Positive Control CD8$^+$	0.79	0.49
C013-KA Positve Control CD4$^+$	1.06	0.34
C013-KA A/PR CD8$^+$ T-Cell	1.12	0.95
C013-KA A/PR CD4$^+$ T-Cell	1.26	0.54

Discussion

With so few returning participants following vaccination, especially within the KTx group, and no real results generated from the ELISAs and colourimetric assays it was difficult to utilize the data for analysis.

IFN-γ , IL-10 and Granzyme B Assays

It is not known why exactly the ELISAs failed to detect any cytokines in the samples but as there was no problem with the standard curves it is not likely due to a failure of the kit or protocol. The most likely explanation is that 600µL was too large of a volume for the amount of cytokines released by one million cells resulting in the dilution cytokine concentrations to below the detection limits of the kits. Although the centrifugation of supernatant was done at low speed and for a short period of time it is possible, though unlikely, that some of the cytokines were spun down into the cell pellet and lost from the supernatant. Unfortunately the samples were not kept on ice and so it is possible but unlikely that the cytokines deformed enough to be no longer recognisable by the capture antibody. This may have been a particular problem for Granzyme B as granzyme B is very temperature sensitive and bovine serum albumin is added for stabilization during twenty hour incubation.

Flow cytometry

The lack of a working positive control, of only one returning KTx recipient and a general lack of flow cytometry data gathered it is not possible to draw any concrete conclusions from the ability of the vaccine to alter the T-cell response in HV or KTx recipients. The first step in solving this issue will be to find a consistent positive control. An issue with the CD107a staining is most likely due to the short period of time CD107a is present on the cell surface and adding the staining antibody during stimulation could correct this.

Optimisation of Protocol

With the problems encountered with the ELISAs and colourimetric assays combined with the difficulty isolating PBMCs it was decided that for the 2008-2009 only flow cytometry data would be collected. So that it was possible to obtain IFN-γ production information for stimulation by all three viruses cells would only be stained for intracellular IFN-γ. During this period of optimisation the switch to Dr. Wasilenko's PBMC isolation protocol occurred but as it was been described previously it will not be done so here. Also during this period, to increase the power of the flow cytometry the number of cells counted increased to a minimum of 100,000 and up to 200,000 per well. In addition it was discovered that there were nutrients missing from media used which were then added and used from that point on making the media consist of RPMI 1640 media supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin-

streptomycin-antimycotic solution, 1% nonessential amino acids and 1mM sodium pyruvate. The use of this media had a small but not significant effect on the production of IFN-γ.

Determination of positive control

In the IFN-γ flow cytometry data it was apparent that the use of the alloresponse through the mixing of the PBMCs from different participants was not consistent in providing a good positive control. A T-cell mitogen known as phytohemagglutinin A (PHA) (Sigma cat#L 4144) and anti-CD3 antibodies (kindly supplied by Dr. Hidalgo) were evaluated for their ability to stimulate T-cells for use as a positive control. Both are known to accentuate the stimulation T-cells by inactivated influenza virus increasing proliferation and production of IFN-γ [5] and are used individually in various T-cell studies as positive activation controls. The first two attempts at using the recommended concentrations, manufacture for PHA and Dr. Hidalgo for anti-CD3, of PHA and anti-CD3 did result in activation of the T-cells but not significantly above unstimulated cells. On the advice of Dr. Hidalgo fifty units of IL-2 was added to the anti-CD3 stimulation wells as an additional signal. The next trials of PHA and anti-CD3 showed a good activation of T-cells by the shift towards down regulation of surface expression CD3; however, the production of IFN-γ was still lower than expected. Drs. Hidalgo and Wasilenko suspected that the four hour monensin exposure was not enough time to prevent significant amounts of IFN-γ being exported out of the cell and

suggested that a sixteen hour period and a concentration of 2X might be more appropriate. With this trial and looking back to previous tests it was noted that the isotype control used was not working as it should be as it was producing a shift further right than the negative control cells stained for IFN-γ. A panel testing of all vials of isotype control was conducted and this problem was found in all of them; on the advice of Drs. Hidalgo and Wasilenko the use of an isotype was stopped in favour of unstained cells. Testing the sixteen hour monensin period and 1X versus 2X concentrations found that the increase in time made a significant difference in the amount of IFN-γ detected. The increase in monensin concentration increased the IFN-γ detection slightly but not significantly. Anti-CD3 was found to stimulate T-cells to produce IFN-γ. PHA on the other hand was activating T-cells as could be seen by the shift towards less surface CD3 but the production of IFN-γ was still very low. At this point three decisions in protocol were made; (i) a sixteen hour monensin period; (ii) 2X concentration of monensin used; and (iii) use of anti-CD3 antibodies as the positive control. While the recommended 10µg/mL of anti-CD3 antibody was producing reasonable results a panel of various concentrations was done to ensure that this was an optimal concentration for this protocol. Concentrations of 10µg/mL, 15µg/mL, 20µg/mL and 30µg/mL were tested and found that 10µg/mL was indeed a sufficient concentration with little increase seen in using greater concentrations.

Determination of optimal virus multiplicity of infection

The optimal MOI for each virus as the flow cytometry for the 2007-2008 influenza season virus stimulated IFN-γ production had been inconsistent. MOIs of 2, 3, 5 and 10 were tested several times on T-cells from more than one donor. It was found that MOIs of 2 and 3 produced the most IFN-γ while at MOIs of 5 and 10 production decreased likely due to the cytotoxic effect of the virus killing large numbers of infected PBMCs. The optimal MOI for both A/PR and A/Aichi viruses was 2; while, 3 was optimal for B/Lee virus.

Appendix A Bibliography

1. Ewen, C., et al., *A novel cytotoxicity assay to evaluate antigen-specific CTL responses using a colorimetric substrate for Granzyme B.* J Immunol Methods, 2003. **276**(1-2): p. 89-101.
2. McElhaney, J.E., et al., *Granzyme B: a marker of risk for influenza in institutionalized older adults.* Vaccine, 2001. **19**(27): p. 3744-51.
3. McElhaney, J.E., et al., *T cell responses are better correlates of vaccine protection in the elderly.* J Immunol, 2006. **176**(10): p. 6333-9.
4. Betts, M.R., et al., *Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation.* J Immunol Methods, 2003. **281**(1-2): p. 65-78.
5. Eisenthal, A., et al., *Costimulatory activity of inactivated influenza virus in response of human peripheral blood mononuclear cells to phytohemagglutinin A and anti-CD3 antibody.* Viral Immunol, 1998. **11**(3): p. 137-45.