Identification of Novel NK Cell-mediated Immunosurveillance Function: Immunogenicity Regulation by Monitoring Antigen Frequency

by

Jessica Dong

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree

MASTER OF SCIENCE

Department of Immunology
University of Manitoba
Winnipeg, Manitoba

Abstract

Computational analysis of total amino acid sequences indicate that select combinations that occur less frequently are correlated to increased immunogenicity in humans. Much evidence has been gathered *in silico*, but little is known about *in vivo* experimental validation. This concept can be applied to adjuvant research where increased immunogenicity is desirable and can aid in the potency and efficacy of vaccines. A rare peptide called 5mer4 was found to adjuvant influenza vaccines by increasing survival, humoral and cellular immune responses with a speculated NK cell mediated mechanism. Therefore we hypothesize that rare peptides are able to stimulate an increased immune response in comparison to common peptides through a NK-mediated fashion. The first aim of this study is to determine whether rare sequences are able to stimulate an increased immune response collectively in comparison to commonly occurring peptides. Mice vaccinated with rare, semi-common and common peptides indicate a trend of heightened cellular immune response from rare peptides. However, select rare peptide sequences based on high IFNγ responses do not always correlate directly to increased vaccine efficacy against H5N1-H05 influenza virus, indicating that additional immune parameters need to be taken into consideration. When compared against other adjuvants, 5mer4 performed better in both humoral and survival studies. Previous findings suggest NK cell involvement warranted the second aim of this thesis which is to further delineate the role of NK cells as rare peptide immune modulators. Macrophages were evaluated to determine the effect of peptide, but no increase in stimulation could be observed. NK cells incubated with rare peptides show

increased levels of early activation marker CD69 in comparison to common peptides. Microscopy data indicates that rare, but not common peptides are able to bind to NK cells. Depletion of NK abrogated adjuvant activity of 5mer4 peptide, suggesting the necessary role of NK cells for adjuvant effect. Taken together, rare peptides have shown the ability to modulate the immune response through NK cell activation verifying our hypothesis. These findings can be extrapolated towards multiple fields such as anti-tumor therapies and can lead to the development of immunomodulators with high efficacy at a lower cost.

Acknowledgements

Firstly, I wish to express my deepest gratitude to my supervisor Dr. Gary Kobinger for allowing me the privilege to join his research team. You have allowed me to have a high level of independence in my research, but never failing to provide guidance and direction when needed. My time here has been invaluable and I wish to thank you for the opportunity to be your student.

I would also like to thank my wise committee members Drs. Sam Kung and Xiaojian Yao. Your input, encouragement and enthusiasm have been an integral part of the completion and success of this project.

My deepest thanks to past and present members of the Kobinger Lab and also the entire Special Pathogens program, you have provided so much, not only in academic knowledge and teaching, but also, comical relief. Thanks for making science fun and for always being willing to lend a hand or answer questions. I am especially grateful to my mentor and friend Dr. Ami Patel – thanks for teaching me pretty much all that I know, without you this project would not have been possible!

I would also like to thank multiple departments and programs at the National Microbiology – everyone has been incredibly helpful and selfless and I have received input from departments across the building. I would especially like to thank Dr. Stephanie Booth and her laboratory team, especially – Anna Majer for your essential

contribution to the imaging in this project. Thank you for being so willing to help, and providing key assistance in many experiments.

I cannot forget the Department of Immunology, thank you for helping me become a better researcher – not only the quality of education, but your supportive nature has been greatly appreciated.

Last, but undoubtedly not least, I would like to express my appreciation to my friends and family. Your constant encouragement and prayers were indispensable during my time as a graduate student. Most of all, Tim, for your love and support that has carried me throughout.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AD	Agglutinating dose
ADCC	Antibody dependent cell-mediated cytotoxicity
AEC	3-amino-9-ethylcarbazole
AIDS	Acquired Immunodeficiency syndrome
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APC	Allophycocyanin
AS04	Adjuvant system 4
BMM	Bone marrow macrophage
BSA	Bovine serum albumin
CCL	Chemokine Ligand
CD	Cluster of differentiation
CHS	Contact hypersensitivity
CL4	Containment level 4
CS&T	Cytometer setup and tracking
Cy7	Cyanine dye 7
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid

ELISPOT	Enzyme-linked immunosorbent spot
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Hemagglutinin
HCV	Hepatitis C virus
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HI	Hemagglutination inhibition
HIV	Human Immunodeficiency virus
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
IM	Intramuscular
IN	Intranasal
IP	Intraperitoneal
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer immunoglobulin-like receptor
L-15	Leibovitz's medium
LB	Lysogeny broth
LD	Lethal dose
LIR	Leukocyte immunglobulin-like receptor
M-CSF	Macrophage colony-stimulating factor

MEM	Minimum essential medium
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NK	Natural killer cell
NLR	Nod-like receptors
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	R-Phycoerythrin
PFA	Paraformaldehyde
poly i:c	Polyinosinic-polycytidylic acid
PRR	Pattern recognizing receptor
PVDF	Polyvinylidene fluoride
Rag-2	Recombination activating gene-2
RDE	Receptor destroying enzyme
RLR	Rig-I like receptors
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SFU	Spot forming unit
T_H cell	T helper cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
T-soy	Trypic soy

USA	United States of America
VIOLIN	Vaccine investigation and online information network
VSV	Vesicular stomatitis virus
WHO	World Health Organization

1.0 Introduction

1.1 Immune System

The immune system is a large and diverse network of cells and processes that work synergistically to defend the host against pathogens such as bacteria, viruses and parasites. It is classically categorized into two arms called the innate and the adaptive immune response. The innate immune system is often referred to as the first line of defense. Their main functions include initial detection and immediate defense against a pathogen, activation and instruction of the adaptive immune response, regulation of inflammation, and maintenance of immunological homeostasis within the host. These functions are carried out by a network of diverse innate immune components such as complement, anti-microbial peptides, phagocytes and other cell types. The importance of the innate immune system is often emphasized by the fact that many bacterial species have a doubling time of minutes to hours, whereas the adaptive immune response requires days to weeks to establish fully. While the adaptive immune response is developing, the innate immune response is actively working to suppress and eliminate the pathogen. The adaptive immune response is often required for protection and mounts a fine-tuned, specific immune response towards invading pathogens as well as memory to prevent reoccurring infections. The interaction and the intricate balance between the innate and the adaptive allows for successful protection against invading pathogens (1).

1.1.1 Overview of immune clearance

The initial detection of pathogens is often mediated by pattern recognizing receptors (PRRs) on the surface of innate immune cells. PRRs can be broadly categorized into three types: toll-like receptors (TLRs), Nod-like receptors (NLRs) and Rig-I like receptors (RLRs). These PRRs recognize common components of pathogens known as PAMPs (pathogen-associated molecular patterns) such as lipopolysaccarides (LPS) of gram-negative bacteria, or double stranded RNA of viruses (2). These receptors are found on many innate cells such as antigen presenting cells (APCs). APCs are important for linking the adaptive and the innate immune responses. Examples of professional APCs are dendritic cells, macrophages and some subsets of B-cells (3). Once the PRRs are bound, this allows for a cascade of signaling events that lead to the activation of the APC. The APCs ingest and kill the target and begin to process the pathogen into smaller fragments called antigens. The APCs also secrete cytokines and chemokines to attract additional immune cells (4). The antigens are displayed on the surface of the APC in order to prime and activate additional immune cells. For pathogen-specific clearance, these APCs present the antigen to T helper (T_H) cells in order to develop the adaptive immune response. Once the T_H cells become primed they become classified as T_H1 or T_H2. T_H1 and T_H2 can now activate T and B cells respectively for pathogen specific cell-mediated or humoral immunity. Activated effector T cells carry out cell-mediated immunity by undergoing clonal expansion and releasing cytotoxic granules to induce apoptosis in pathogen infected cells (5). Antigen specific B cells bind can bind to matching antigen specific T_H cells and begin antibody production and proliferation. B cells can also act as APCs and can become activated in

a T-cell independent manner by the cross linking of B cell receptors to PAMPs. B cell secreted antibodies are specific to the pathogen and will bind to its surface which can neutralize the pathogen, or target the pathogen for phagocytosis or antibody-dependent cell-mediated cytotoxicity (ADCC) (6). ADCC can be carried out by a number of cells, but the most commonly noted cell is the natural killer (NK) cell. NK cells have receptors that recognize the Fc portion of the antibody, which then triggers the NK cells cytotoxic granules that promote cell death by apoptosis (7). NK cells have many diverse functions and applications and they are considered an important part of innate immunity.

1.2 Natural Killer Cells

NK cells play a major role in the innate immune response as well as anti-tumor function (8-10). NK cells were first identified in 1975 and named after their ability to "kill" targets without prior sensitization. Phenotypically, NK cells are large, granular and do not express T or B cell markers such as CD3 or CD19 although they originate from common lymphoid progenitor cells in the bone marrow (11). Once mature, NK cells are believed to be relatively short-lived, and comprise approximately 5-15% of human peripheral blood mononuclear cells (PBMCs) (12,13). Although limited in number, NK cells are able to influence and shape both the innate and adaptive immune response (14,15)

1.2.1 NK cell functions

NK are most notably known for two functions – cytotoxicity and cytokine release. NK cells are cytotoxic cells which when activated, migrate to a close proximity to its target and mediate release granzymes and perforin at immunological synapses. In humans, 5 different types of granzymes have been described: A, B, H, K and M. Perforin and granzymes work cooperatively to induce apoptosis. Granzymes are necessary for triggering apoptosis in target cells, but require delivery assistance by perforin (16). The exact mechanism of how perforin delivers granzymes into the target cell is still unknown and highly debated. Perforin is a pore-forming molecule that is capable of membrane permeabilization. Initial hypotheses suggested that these pores made by perforin acted entry points for granzymes. Some researchers have shifted away from this point of view, finding that these pores are too transient and small to allow granzymes to enter. A more recent arising hypothesis speculates that perforin creates these pores in order to destabilize the plasma membrane. Cytolytic molecules such as granzymes are capable of binding to the membrane surface, and in turn can be internalized into endosomes during the repair. Another recent model is the formation of a complex that includes granzymes and perforin. This complex can bind to membrane receptors such as the mannose-6-phosphate receptor and taken up into the cell by endocytosis. Once inside the cell, it induces caspase activation which allows a number of apoptosis pathways to commence, leading overall to DNA degradation and cell death (16,17). Perforin is considered essential for lytic activity as small decreases in perforin activity correlates with reduced total NK cytotoxicity (18).

An additional method of cytotoxicity by NK cells is mediated through a Fas-dependent mechanism. NK cells express Fas ligand which allows NK cells to have capability of killing target cells that express Fas antigen on their surface (19). NK cells can mediate tumor necrosis factor α (TNFα) cytotoxicity as well by TNFα inducing rapid apoptosis (20). As mentioned before, NK cells can carry out ADCC by recognizing antibodies that have already bound to target cells. The Fc portion of the antibodies cross link to an activating receptor (CD16) on NK cells. This engages NK cells and causes the release of cytokines and cytotoxic granules leading to death of the target cell (7).

In addition to cytotoxic effector functions, NK cells are also large producers of cytokines and chemokines. Two main cytokines produced by NK cells are interferon-γ (IFNγ) and TNFα. IFNγ can inhibit viral replication directly by making host cells less hospitable to the virus and can prevent infection in other neighbouring cells (9). Another major function of IFNγ is to stimulate and recruit additional innate and adaptive immune cells such as macrophages, CD4+ and CD8+ T lymphocytes. TNFα has been described with importance to anti-tumor functions. Unlike cytotoxic granules, secretion of cytokines occurs not only at synapses but all points on the cell surface (21). Other cytokines and chemokines secreted by NK cells include interleukin (IL) -10, Granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), IL-3, chemokine ligand (CCL)2, CCL3, CCL4, CCL5, chemokine ligand (XCL)1 (also known as lymphotactin), and IL-8 (22). The soluble factors released from NK cells are vital due to the downstream effects that can regulate

the adaptive immune response (23). IFNγ secretion can lead to the activation, differentiation and proliferation of macrophages and T-cells.

1.2.2 NK cell regulation

NK cells must be highly regulated. NK activation is dependent on the balance of activating and inhibitory receptors. If the inhibitory receptor signaling is more prominent, then NK cell activity will be inhibited. An increase in activating or decrease in inhibitory ligands on the aberrant cells are recognized by receptors on the NK cells, leading to the accumulation of activation stimuli. The increase in activation signals override the inhibitory signals and ultimately lead to the activation and of NK cells (Figure 1).

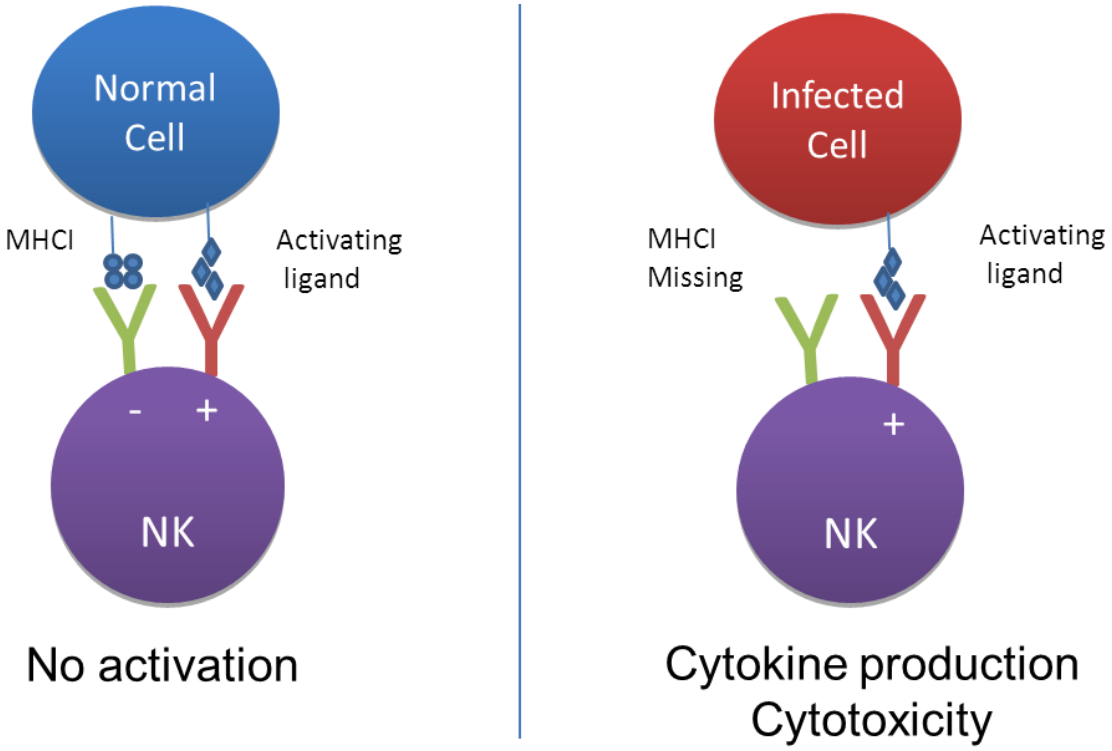


Figure 1: NK cell activation is regulated by the balance of signals from activation and inhibitory receptors.

Several activating receptors have been identified on NK cells such as natural cytotoxicity receptors (NCRs) which include NKp30, NKp44, and NKp46. Other well studied receptors include NKG2D, 2B4, NTB-A and CRACC (24). Ligands of these receptors are induced when the cell is stressed which can be a result from viral infection or other stress such as cancer or DNA damage. Evasion of NK cells has been shown with cancer cells by defective expression of the NKG2D and NCR ligands (25). Many activating receptors pair with immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasm such as DAP12. ITAMs are conserved sequences that become phosphorylated following the interaction of the receptor and ligand. This forms a docking site for other downstream signaling molecules (22,26). Several important

signaling molecules have been identified such as ZAP70 and Syk, but mice with genetic deletions of either still retain NK activity (27,28). This suggests that NK cells natural cytotoxicity signaling pathways are redundant and act synergistically.

Some families of inhibitory NK receptors include killer-cell immunoglobulin-like receptors (KIR) and leukocyte Immunoglobulin-like receptors (LIR). Most inhibitory signals are dominant over activating receptor signals but can be overridden when the accumulation of activation signaling becomes more prominent than the inhibitory signals, resulting in activation of NK cells. The inhibitory signaling pathway begins with the phosphorylation of immunoreceptor tyrosine-based inhibition motif (ITIM) sequences in the inhibitory receptor, which mainly leads to downstream de-phosphorylation of key components of the activating signaling pathway (22). One of the first recorded evidences of NK inhibitory signaling was conducted by Ljunggren and Karre after observing NK rejection of self H-2 deficient cancer cells over self H-2 expressing cells, suggesting that major histocompatibility complex (MHC) class I molecules protect cells from lysis by NK cells (29). In the absence of appropriate interaction between KIRs and MHC class I molecules, NK cells can exert their cytolytic function. Therefore, NK cells eliminate cells that present alterations or decreased expression of MHC class I molecules (30). An evolutionary trait of many viruses is to down regulate MHCI to prevent the presentation of foreign proteins to T cells but this renders the infected cell more susceptible to NK cell recognition and killing (31,32).

1.2.3 NK cells and dendritic cell interactions

Recently it has been shown that NK cells not only represent a significant role in innate immune response, but also participate in the complex network of cell to cell interaction that leads to the development of the adaptive immune response. In 1999, it was first discovered that activated dendritic cells (DCs) can stimulate NK cell effector functions by direct contact (22,33). Recently, more evidence has been found to show bidirectional crosstalk between DCs and NK cells and its importance in regulation of the early phases of innate immunity and the subsequent adaptive immune responses (22,34-40). Both immature and mature DCs use the NKp30 receptor as a pathway of NK stimulation. Once the receptor NKp30 is engaged by the DC, NK gain cytolytic activity and secrete IFNγ and TNFα (38). Activated NK cells facilitate "NK editing" which describes the ability to lyse immature DC even though MHCI are expressed but in rather low levels (37). As the DC matures, it becomes less susceptible to lysis due to the increase in MHCI expression (41). It is hypothesized to be advantageous to the host as it allows for the quality assessment of DCs undergoing maturation. DC maturation is often characterized by the acquisition of CCR7, up-regulation of MHC-class I/II and of co-stimulatory molecules. Expression of these molecules is known to be crucial for DC migration to secondary lymphoid organs and subsequently priming of T cells (36).

DCs as well as macrophages can prime NK cells by secretion of IL-2, IL-12, IL-18 and IFNα/β. IL-12 and IL-18 can synergistically work together to activate NK cells to enhance NK cytotoxicity and IFNγ secretion (8,42). Although soluble factors such as cytokines play a large role, direct cell to cell contact also has significant impact in NK

cross talk. For example, direct DC interaction appears to play a crucial role in the acquisition of CCR7, a chemokine receptor that enables NK cells to migrate towards lymph nodes in response to other chemokines. Trans-well separation of DCs and NKs has been shown to abrogate DC-dependent induction of NK cells cytotoxicity (33). DCs and NK cells are equipped with complementary sets of receptors that allow the recognition of various pathogenic agents, emphasizing the role of NK cell/DC crosstalk in the coordination of innate and adaptive immune responses (22). A summary of DC-NK cross talk can be found in Figure 2, showing the basic DC-NK interactions and how these interactions influence their immunological functions (36,42).

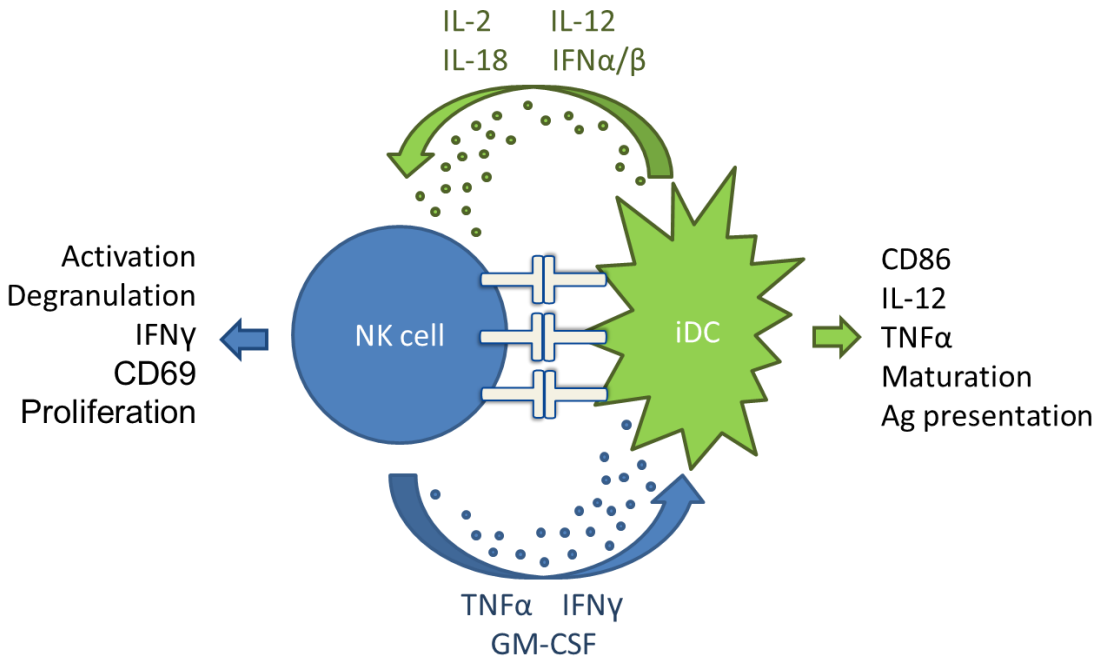


Figure 2: Crosstalk and immunological functions of NK cells and dendritic cells.

1.2.4 NK cells in response to disease and vaccines

Natural killer cells have been proven important in either the control of virus or shaping the adaptive immune response. The dysfunction of NK cells sheds insight on the impact of NK cells on the immune system as a whole. A number of studies have been conducted and have shown the importance of NK presence and activity against viruses, particularly cytomegaloviruses and herpes simplex viruses (43-45). For example, a patient was consistently afflicted with various viral diseases, in particular severe chicken pox varicella pneumonia, HSV infections, and human cytomegaloviral infections. Upon examination, this patient appeared to have a normally functioning adaptive immune system by in vitro B and T cell assays, but deficient in NK cells and NK-like activity (45). A number of studies have found the importance of NK cells and their functions (46,47) and have summarized as seen in Table 1.

Table 1: Examples of NK cell mediated immune response to viruses, parasites and bacteria

Pathogen		**Role of NK**	**References**
Viruses	MCMV	Promotes early CD8+ response, perforin and IFNγ from NK cells limits viral replication in the spleen and liver	Robbins, 2007; Tay, 1997; Loh, 2005
	Papillomaviruses	NK cell deficiency is correlated to reoccurring lesions and cervical cancers	Ballas, 1990
	HSV-1	Depletion of NK cells increases susceptibility	Habu, 1984
	Pichinde virus	Depletion of NK cells increases replication of susceptible strains	Welsh, 1990 and 1991
	Influenza A virus	Viral infection induces NK cell IFN-γ production, depletion increases morbidity and mortality	Siren, 2004; Stein-Streilein 1986;Mandelboi

			m 2001
	HIV	Neonatal NK cells suppress replication of some viruses	Bernstein, 2004
	Coxsackie virus	Induces cytotoxicity, depletion of NK cells result in increased viral replication	Godney 1987
Parasites	*P. falciparum*	Human NK cells produce IFN-γ in response to infected RBC	Artavanis-Tsakonas, 2002;
	L. major	Depletion of NK cells results in more severe parasitemia	Laskay 1993
	T. cruzi	NK cells are able to directly lyse *T.cruzi*	Lieke, 2004
Bacteria	*Shigella flexneri*	IFN-γ secretion from NK cells controls infection	Le-Barillec, 2005
	M. tuberculosis	NK cells kill infected monocytes	Vankayalapati, 2005

This table exemplifies a few of the known influences of how NK cells act as an initial defense against a vast diversity of pathogens and sheds insight on the unique role NK cells play in the immune response.

Recently, NK cells have been shown to mount an antigen specific memory response, which was previously only attributed to the adaptive immune response (48). This was first recorded evidence was found from a hapten-induced hypersensitivity model in a recombination activating gene-2 (Rag-2) deficient mice. These mice are known to be deficient in T and B cells since the gene for recombination has been deleted, but NK cells are unaffected. Hapten-induced contact hypersensitivity (CHS) was previously believed to be carried out by CD4+ T-cells, but CHS still found in the Rag-2 deficient mice. Moreover, these mice appeared to have hapten-specific memory

even one month after the chemical priming (49). Since then, NK memory has been described against viral pathogens as well, such as influenza, vesicular stomatitis virus (VSV) and human immunodeficiency virus-1 (HIV-1) (48,50). This concept is still relatively new, and the details of NK memory and how it enhances protection is still unknown and demonstrates the plasticity of the immune system.

1.3 Influenza virus

Influenza is a world-wide epidemic which results in an estimated 3-5 million yearly cases and approximately a quarter to half a million yearly deaths (51). It is widely distributed and can infect a number of different animal species including birds and mammals. Genetically, it is a negative sense, single stranded, segmented RNA virus of the family Orthomyxoviridae. It can be subtyped into 3 different categories: A, B or C. For influenza A viruses, it can be further subtyped according to its hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins of the viral envelope (52). Influenza vaccines generate immunity towards specific strains by targeting HA and NA, but influenza has the ability to undergo antigenic drift and shift, thus altering its major immunogenic determinants HA and NA. The immune system is very specific and can only recognized the matched vaccine strains, but not the new, mutated viruses (53). Despite use of influenza vaccines for more than 60 years, the ability of influenza to mutate renders previous vaccinations ineffective against new emerging strains and consequently yearly vaccine formulations must be administered (51).

1.3.1 Influenza mouse models

There are a number of animal models currently used for influenza vaccine research. Using ferrets as an animal model for influenza is the most widely accepted ideal model, however, hamsters, guinea pigs, mice and rats are acceptable influenza animal models. An ideal animal model should accurately mimic infection and immune response of humans. For decades, mice have been used extensively as an influenza animal model as they are readily available, relatively inexpensive, as well as the high availability of reagents and materials (54) and can be used as a stepping stone to more complex and expensive animal models such as ferrets. In this thesis, an influenza mouse animal model was used and has been previously tested using similar vaccines and adjuvants (55). Evaluation of influenza vaccine efficacy has been previously characterized as a hemagluttinin inhibition titre of 1:40 or greater. This antibody titre is the minimum required to prevent influenza infection in 50% of the human population. Neutralizing antibody titres as well as robust CD4+ and CD8+ responses have been correlated to protection against influenza, but the HI titre of 1:40 is the recognised benchmark by WHO (56).

1.4 Vaccines

Vaccinations have been described as one of the most successful methods of prevention against infectious diseases. The first known triumph of vaccines was against small pox in 1796. At the time, it was considered one of the most deadly pathogenic

diseases, but in 1977, World Health Organization (WHO) announced the eradication of small pox using a worldwide vaccination program. Since then, many vaccines are able to control diseases that were historically epidemic. It has been estimated that vaccinations have avoided 2.5 million deaths which would have been due to global health threats such as diphtheria, whooping cough and measles (57).

1.4.1 First generation vaccines

Early methods of vaccine generation include live attenuated virus and inactivated whole virus. Many of the most well-known vaccine successes have come from first generation type vaccines. Live, attenuated virus consists of virus that is still functional but less virulent than circulating strains. Viruses become attenuated by passaging the virus through another host such as feline or canine tissue culture. The original human virulent strain is added to foreign host tissue culture, and after several rounds passaging the virus evolves to replicate more efficiently in the new host. Over time the virus becomes adapts to the new host and becomes attenuated or less virulent in humans (58). This method of vaccine generation has been successfully used for viruses such as measles, mumps, yellow fever, polio, and rubella. The concept has also been applied to many non-viral diseases such as tuberculosis and typhoid fever.

Inactivated whole virus or killed virus vaccines consist of high tire virus generated in tissue culture or eggs, and then inactivated by heat or formaldehyde. This destroys the virus but the capsid and immunogenic antigens are still intact therefore still able to be recognized by the immune system. The immune system can generated

memory from these antigens, therefore allowing a strong response if the live virus is ever encountered. This method of vaccine preparation has been approved for use against pathogens such as polio, influenza, cholera and pertussis.

This approach to vaccine generation has been successful, but each strategy comes with strengths and weaknesses. Table 2 shows the advantages and disadvantages of both attenuated and inactivated vaccine types (58-60).

Table 2: Advantages and disadvantages of attenuated and inactivated vaccines

	Advantages	Disadvantages
Attenuated vaccine	Activates both humoral and cellular immune response	May revert to wild type virulence
	Long lasting immunity	Can be passed on to immunocompromised individuals
	Mimics actual infection	Temperature sensitive
		May cause mild disease
		Limited shelf life
		Not suitable for use in immunocompromised individuals
Inactivated vaccine	No reversion or disease pathogenesis	Improper inactivation leads to disease
	Will not spread to others	Booster shots may be required to maintain immunity
	More stable than attenuated virus	Weak cell mediated immunity
		May cause inflammation

Attenuated virus elicits a strong, long lasting humoral and cellular response with only one or two injections. However, it has a limited shelf life, temperature sensitive and the ability for the virus to revert back to wild type which leads to unwanted disease. Inactivated whole virus vaccines do not have the possibility of reversion, but produces

comparatively weaker cell-mediated immunity and may require multiple injections with greater amounts of antigen (60,61). Many vaccines are able to control diseases that were historically epidemic by using traditional vaccine preparation methods, but there are many other diseases such malaria and acquired immunodeficiency deficiency syndrome (AIDS) where inactivated and attenuated virus vaccine preparations are unpractical.

Many current vaccines could also benefit from improving their efficacy possibly with less invasive administration leading to better protection, public perception and therefore wider coverage. For example, the widely used trivalent inactivated yearly administered influenza vaccine, which has shown limited protection in a broad retrospective study reviewing data from 1967 to 2011 with a pooled efficacy of 59% in adults, aged 18 to 65 years (62). Better protection as well as the safety issues surrounding current vaccines highlights the current requirement for the development of new immunization strategies that are both efficacious and safe. The method of preparation of traditional vaccines may be ineffective.

1.4.2 Subunit vaccines

Subunit vaccines are generally composed of specific immunodominant epitope(s) instead of whole virus. This allows for increased safety and less antigenic competition, but in turn renders the vaccine less immunogenic than traditional formulations. The addition of adjuvants have aided in overcoming the decrease of immunogenicity of subunit vaccines (63). Currently, many licensed subunit vaccines such as Gardasil

(Human Papillomavirus) and Recombivax HB (Hepatitis B virus) require strong adjuvants such as aluminum hydroxide. DNA vaccines are a type of subunit vaccines that are generally composed of a plasmid that encodes an antigen of interest which expression is controlled by a strong eukaryotic promoter. In summary, the plasmid can be taken up by cells such as muscle cells or APCs such as dendritic cells. Cells and APCs can be directly transfected by the plasmid where it enters the nucleus and initiates gene transcription. The antigen is then produced in the cytoplasm and is translated using host machinery and presented on the cell surface or secreted. APCs can also be activated by capture of the secreted antigen or from antigen presentation of infected cells. Also, as the name implies, APCs are able to present the antigen to CD4+ T cells (via MHCII) or CD8+ T cells (via MHCI) in the lymph node. Activated CD4+ T cells can activate B cells to differentiate and produce antibodies against the target antigen, allowing a cellular and humoral immune response to the specific antigen expressed from the DNA plasmid. DNA vaccines have also been described to be safe, cost-efficient and highly stable (64-67). Despite their attributes, DNA vaccines are currently not approved for human use. Pre-clinical trials showed sufficient immune responses in animal models for a number of different diseases such as HIV but the results of these Phase I trials were disappointing (68-70). Although a humoral and cellular immune response against the transgene can be induced, the breadth of immune responses has been insufficient. A large number of factors have been attributed to this such as minimal production of proteins in the host cell, low transfection efficiency and ineffective delivery of the DNA vaccine (66,71-73). A main requirement of a successful vaccine is its immunogenicity, thus a vaccine that is unable to amount a strong immune

response will not be of use even if the vaccine is shown to be safe, stable, and cost efficient. However, the addition of an appropriate adjuvant can increase the immunogenic activity of vaccines and therefore aid in the development of safe and efficacious DNA vaccines.

1.5 Adjuvants

Immunologic adjuvants are agents that stimulate the immune system thus increasing the response induced by vaccines. Adjuvants that may be optimal or ideal in conjunction with one vaccine may not be as efficacious with another vaccine. Alum is the oldest and the most widely used adjuvant today. It has been used in conjunction with vaccines for more than a century. One of the first records of a substance able to adjuvant a vaccine was an observation of higher efficacy when the diphtheria toxin was purified using potash alum (Aluminum potassium sulfate), aluminum hydroxide or sulphate. The addition of potash causes precipitation with the toxin, and it was recorded that the precipitate contained a higher immunising value than the supernatant (74-76). Since then, aluminum based adjuvants have been incorporated into various licensed vaccine formulations, such as Pediarix (*Clostridium tetani* and Poliovirus) and Recombivax HB (Hepatitis B virus). In 2013, there were over 150 aluminum based adjuvant vaccines curated in VIOLIN (Vaccine Investigation and Online Information Network). Until recently, the only licensed adjuvants in the U.S.A include aluminum salts, such as aluminum hydroxide, aluminum phosphate, alum (aluminum sulfate), or a mixture of these compounds. Although aluminum based adjuvants are effective in certain vaccine formulations, they elicit mainly a T_H2 response, which can be

ineffective against pathogens which require a T_H1 dominant immune response for clearance (77). Also, a recent report by Chen *et al.* (2011) has suggested that the addition of adjuvants like alum to vaccine formulations can stimulate IL-10 production, leading to the suppression of a T_H1 response (78). Aluminum based vaccines may also cause unwanted side effects such as local reactions, augmentation of IgE antibody responses and granulomas (79,80). The relatively focused development of T_H2 stimulating adjuvants in relation to the current needs of cost efficient broad protective immune responses highlight the necessity to further develop new families of adjuvants. MF59 has been a licensed vaccine adjuvant mainly used in Influenza vaccines for over 13 years in Europe, but it is not yet approved in the USA (81). There are a number of clinical trials in the USA currently investigating the safety and immunogenicity of vaccines containing MF59 (clinicaltrials.gov). Both aluminum-based and oil-in-water based adjuvants have mechanisms of action that are still unknown.

1.5.1 Proposed Adjuvant Mechanisms

Different mechanisms have been proposed to explain the enhanced antigen specific immune response generated by adjuvants. Adjuvants such as alum have been hypothesized to work in three main mechanisms. First, the slow release of the antigen exposing it to the immune system for a longer period of time and consequently stimulating a stronger by increasing the likelihood of interaction between antigen presenting cells and antigen (60,82-84). Second, adjuvants assist delivery and uptake of the antigenic complex to antigen presenting cells such as macrophages and dendritic

cells which in turn can migrate to lymphoid organs and initiate a concerted response in interaction with T and B cells. An oil/water emulsion adjuvant, MF59, acts by interacts with macrophages which in turn can migrate to draining lymph nodes to increase the efficiency of antigen presentation (60,85). Third, immune cells including APCs can be directly activated by adjuvant and then initiate a faster and stronger immune response through the subsequent stimulation of T and B cells. Unmethylated CpG DNA is detected by the innate immune system via TLR9 which activates a cascade of events that lead to immune activation (86). In all, there are many unresolved postulates in the mechanism of action, and adjuvant effect may be exerted due to a combination rather than an individual mechanism.

1.5.2 Adjuvant limitations

To date, the majority of licensed adjuvants have been discovered by a trial-and-error type of approach, a highly inefficient method of discovery. The future of the adjuvant field would benefit from a systematic approach to vaccine-adjuvant development. Adjuvants strongly influence the breadth and quality of the immune response, which can be crucial to the effectiveness of a vaccine. Undefined inflammation and recruitment of immune cells may have benefits for increasing immunogenicity, but may lead to unwanted side effects such as hypersensitivity or local reactions due to over-stimulation (87,88). The most well-known and used adjuvant, aluminum based adjuvants, was added to diphtheria toxin vaccines not to adjuvant the vaccine, but to purify the toxin. It was only afterwards was it discovered that the

aluminum increased immunogenicity. The future of the adjuvant field would benefit from a systematic approach to adjuvant development. Adjuvants strongly influence the strength and quality of the immune response, which can be crucial to the overall effectiveness of a vaccine. Hypothesis-driven research is required to develop adjuvants that can target precise immune responses necessary for directed vaccine efficacy.

Recently, new adjuvants are undergoing clinical trials with vaccines with a more hypothesis-driven rationale. For example, Adjuvant system 04 (AS04) consists of aluminum hydroxide, but also a TLR 4 agonist, monophosphoryl lipid A which is adsorbed onto the aluminum salt (89,90). Understanding the mechanism of TLR stimulation and the downstream enhancement of the immune response has been essential in the development of a new generation of PRR targeting adjuvants (91). In 2005, AS04 became the second licensed adjuvant for human use in the United States. (92). There are many PRR-targeting adjuvant candidates such as polyICLC (induces TLR3 activation) currently being investigated for adjuvant use (93). Specificity and rational design based on extensive knowledge of the immune response can lead to the development of new, safe and highly efficacious adjuvants.

1.6. Short peptides as immunomodulators

Short peptides have been documented as having immunomodulating effects and have been investigated as a means of infection control. Antimicrobial peptides (AMP) have been noted not only in humans, but also in invertebrates, plants and fungi. AMPs are generally defined as proteins that contain fewer than 100 amino acids and show broad-spectrum antimicrobial activity (94). In humans, the most well studied types of AMPs are defensins and cathelicidins. Defensins are secreted by a number of different types of cells and are generally between 25- 45 amino acids in length. This group can be further categorized into two major subdivisions based on structure: α-defensins and β-defensins. Their functions include protecting epithelial surfaces such as the skin, as well as the lining of the GI tract, genitourinary tract, and nasal passages. They function by compromising the membrane of microbes by an unknown mechanism (95). Cathelcidins are mainly found in peroxidase-negative granules of human neutrophils, but can be expressed in various epithelia (96). One of the most well studied cathelicidins is LL-37. LL-37 is synthesized by macrophages, neutrophils and epithelial cells and it can modulate the immune response by recruitment and/or activation of immune cells which can lead to enhanced bacterial clearance (97). Humans express LL-37 as the inactive precursor hCAP18 is cleaved by proteases in order to be active (98). It is generally expressed, but can also be induced stimuli such as bacterial products (99).

Several researchers are testing the efficacy of short peptide-based therapeutics as immunomodulators. For example, EA-230 is a four amino acid long peptide that has been shown to elicit an anti-inflammatory effect. EA-230 is currently undergoing

clinical trials (Phase I-II) as a means of prevention of sepsis and protection against renal failure after cardiac surgery (88). Another short peptide of interest is Hp91. Hp91 is a short peptide portion of the high mobility group box 1 (HMGB 1) protein that has been discovered to act directly upon myeloid dendritic cells to potentiate both cellular and humoral immune responses to protein antigens *in vivo*.HMGB1 is released by monocytes and macrophages as potent pro-inflammatory cytokine in response to danger signals (such as lipopolysaccharides) or tissue damage (100). IMX-942 is a 5 amino acid long peptide that contains no antimicrobial activity, but show protective effects in antibiotic-resistant bacterial infections in mice. This peptide is currently under Phase II clinical trials as a means of prevention of infections in patients who are immunosuppressed due to chemotherapy (88).

1.6.1 Rare peptides as adjuvants

Short peptides can also be used to adjuvant vaccines. Currently, IC-31 is undergoing phase I clinical trials as a potent adjuvant in animal models. IC-31 is composed of an 11 amino acid long synthetic peptide in conjunction with oligonucleotides (ODN1a). IC-31 has been shown to elicit a humoral and cellular immune response and its mechanism of action has been revealed to be a TLR-9 receptor agonist, despite not containing any CpG motifs (101).

The low-similarity hypothesis has been defined as an immune unit containing little or no similarity to sequences within the host proteome. It predicts that the breadth

and amplitude of an immune response can be related to how frequently a specific amino acid sequence is found in nature (102). In the context of cancer, this reasoning can be used to illustrate the ability of tumors to be able to evade the immune system. Tumor escape may occur due to the lack of immunogenicity of tumor antigens via high similarity to host proteome and thus avoid immune destruction. The immune system can bypass tumor escape by the presence of low redundant peptides in self-antigens which can assist in breaking the immune tolerance (103). Multiple analysis of T and B cell epitopic motifs appear to fall predominantly in areas which have low-frequency. For example, major T-cell epitopes in the HIV gag protein have been shown to contain stretches of peptide sequences that are not found in known host proteins (104). Hepatitis C virus (HCV) E2 envelope derived B-cell immunogenic epitope sequences currently catalogued in the Immune Epitope Data Base were analyzed based on the number of occurrences that particular B-cell epitope appeared in the human proteome. Results indicated that B-cell epitopes correlated to sequences that were less common or non-existent in the human proteome (105).

Rare short peptide sequences that are not found or are extremely rare in nature have been shown to potentiate different vaccines-mediated immune responses relative to commonly encountered peptides. This development was following the initial hypothesis that rare or non-existent short amino acid sequences could act as immune modulators positively contributing to antigen-specific immune activation and adjuvant vaccines. This was highlighted recently in a paper by Patel, A. et al (2012), which showed that when a particular rare short peptide, 5mer4, was added to a suboptimal

dose of hemagglutinin (HA) DNA or commercial hepatitis B vaccine, substantially increased vaccine efficacy leading notably to improved protection and a dose-sparing effect. Both cellular and humoral immune responses were significantly higher in the presence of a short peptide adjuvant in mice and ferrets.

This paper has also speculated on the mechanism of immune modulation. Survival studies of mice vaccinated with only 5mer4 and no vaccine showed 40% survival in comparison to non-vaccinated mice that succumbed to the disease, therefore indicating a non-antigen specific or innate immune response (55). Firstly, TLRs and NLRs were screened as many current adjuvants use the activation of PRRs as a means of adjuvanticity (106,107). Screening results indicated that TLR2-5, 7-9 or NOD 1 and 2 were unresponsive in the presence of 5mer4. Dendritic cells (DCs) were also tested as studies have shown that many effective adjuvants are strong stimulators of DCs (108). Results show that NK cells and suggest that NK cells may mediate the modulation of immune responsiveness by peptide stimulation (55).

1.7 Hypothesis and Objectives

To summarize, Patel et al., demonstrated the ability of a rare peptide in conjunction with suboptimal dose of HA vaccine can provide an increased protection against influenza challenge possibly through NK cell activation (55). This is the first evidence of the phenomenon of infrequently occurring peptides modulating the immune

response *in vivo*. Thus, it is of importance to investigate whether this increased immune responsiveness is due to the rarity of the peptide sequence.

We hypothesized that infrequently occurring peptide sequences in the human proteome can stimulate natural killer cells leading to an increase in immune responsiveness.

The two main objectives in this study:

1) **To determine the correlation between infrequently occurring peptide sequences and an increase immune responsiveness**. This was accomplished by assessing survival and immune responses from mice vaccinated with sub-optimal doses of HA DNA vaccine in conjunction with three different degrees of peptide frequency

2) **To further investigate the effect of rare peptides on NK cells.** This was conducted by isolating individual immune cells from mice and measuring the effect of rare peptides by various immunological methods.

The completion of this study will advance the understanding of how peptide composition by frequency may affect the immune response and potentially lead to the development of safe and effective vaccine adjuvants.

2.0 Materials and Methods

2.1 *In silico* scanning of low frequency 5mers

All publically available proteomes were analysed using a combination of UNIX/LINIX shell scripts and Perl programs. The proteomes analysed were taken from the UniRef100 version of Uniprot as the UniRef100 eliminates duplicate and fragment entries. All sequences were scanned *in silico* to determine the frequency of each of the 20^5 possible combinations of 5 amino acids, which was input into a graph. From this graph, areas of semi-common, common and rare could be distinguished. After analysis of the graph, 200 of each category were chosen for synthesis (Genscript) at greater than 95% purity for experimental purposes.

2.2 Peptides

Peptide sequences were sent to commercial sources (Mimotope, Australia and Genscript, USA) for synthesis at greater than 95% purity. The peptides were received lyophilized, and re-suspended with dimethyl sulfoxide (DMSO, Sigma) at a concentration of 100μg/μl. These peptides were then aliquoted and stored at -20°C for long term storage. Peptides were diluted with phosphate buffered saline (PBS) prior to utilization.

2.3 A/Hanoi/30408/2005 H5N1 (H5N1-H05) DNA vaccine

A DNA vaccine expressing a codon-optimized H5N1-H05 HA antigen was generated from the homologous influenza virus. The H05 HA is expressed under the control of a chicken beta-actin promoter and has been previously shown to offer protection in mice against influenza challenges. Expression of HA has been previously characterized by western blot using a polyclonal mouse anti-HA antibody and goat-anti mouse secondary antibody conjugated to horseradish peroxidase (HRP) (109).

2.3.1 Propagation of H5N1-H05 DNA vaccine

The H5N1-HA DNA vaccine was propagated from the original construct by transformation of chemically competent cells following DNA plasmid extraction. Briefly, 1µg of H5N1-HA DNA plasmid was added to 100µl of thawed MAX Efficiency Stbl2 Competent Cells (Invitrogen). After thorough mixing, the cells were placed on ice for 2 minutes, and subsequently heat shocked for 40 seconds at 42°C. Cells were immediately transferred back to ice. After 15 minutes of on ice incubation, 400µl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose) was slowly added to cells. Cells were then incubated at 37°C for 10 minutes. Cells were spun down by centripetal force (13000 revolutions per minute or RPM) for 1 minute. The supernatant was discarded and 100µl of fresh SOC medium was added to re-suspend the cells. The cells were then plated on Tryptic soy (T-soy) plates with ampicillin as a selection marker for transformed cells. After 37°C overnight incubation, a single colony would be selected and placed into 3 ml of lysogeny broth

(LB) plus ampicillin for 6 hours at 37°C and then transferred into 500ml of LB broth plus ampicillin and incubated overnight at 37°C. The Endofree Plasmid Mega Kit (Qiagen) was used to obtain plasmid from culture. Procedure followed as described in protocol. Once plasmid was obtained, the concentration of plasmid was determined by spectrophotometer (Nanodrop) and adjusted to a concentration of 1μg/μl. To confirm identity, the H5N1-H05 DNA plasmid was sent for sequencing (DNA CORE, in house) using primers specifically designed for the H5N1-H05 HA gene.

2.3.2. Vaccination dosage

A suboptimal dose of HA was determined by vaccinating groups of 5 mice with 1μg, 5μg, 10μg or 25μg of H5N1-H05 HA DNA vaccine. Once the suboptimal dose was determined to be 1μg, this value was used for the rest of the survival studies. BALB/c mice were vaccinated under anesthesia (isoflurane, Baxter) with a sub-optimal dose of HA DNA vaccine (1μg) for all challenge studies and 50μg of HA DNA vaccine for enzyme-linked immunosorbent spot (ELISPOT) assays and PBS was added to a total of 100μl per mouse. All vaccinations were done intramuscularly (IM) in the right and left hind leg with 50μl in each leg to a total of 100μl per mouse using a 27 gauge needle. The addition of 50μg of 5mer was added directly into vaccine formulation prior to vaccination. Mice were monitored until full recovery from anaesthetics was achieved.

2.4 Animal Models

Survival, humoral or cell-mediated immune responses were measured from 6-8 week old BALB/c (Charles River) mice vaccinated with a sub-optimal dose of vaccine with or without 50μg of 5mers. Mice were challenged intranasally (IN) under anesthesia (isoflurane, Baxter) with a 100 times the lethal dose (100 LD_{50}) of A/Hanoi/30408/2005 H5N1 Influenza virus diluted in minimum essential medium (MEM, Gibco), with 0.1% bovine serum albumin (BSA, Gibco) in Containment Level 4 (CL4). Animals were monitored for signs of disease for 16-18 days post challenge. Animals were scored based on signs of disease on an approved humane endpoint scale. The clinical scoring ranged from zero to three.

Table 3: Clinical Scoring for mice

Score 0	No symptoms
Score 1	Ruffled fur, slowing activity, loss of body conditions
Score 2	Laboured breathing, hunched posture, bleeding
Score 3	Death

In addition to clinical scoring, weight loss was also observed. Mice were euthanized when combinations of these parameters were reached: a clinical score above two, showing continuous trembling, partial paralysis or weight loss of 35% or more of

their initial body weight. Mice were terminated if signs of disease progressed to humane endpoints or at day 18 post challenge.

2.5 Isolation of various cell types for ex-vivo evaluation

2.5.1 Splenocyte isolation

Spleens from naïve mice were removed for the isolation of NK cell isolation. Mice were euthanized first by overdose of anaesthesia (isoflurane) and physical dislocation as a secondary means. Spleens were removed and placed into a 60mm dish containing a fine metal mesh (50 mesh, Bellco Glass) and 5ml of Leibovitz's medium (L-15, Thermo Scientific). Spleens were crushed against the metal mesh using a syringe plunger until homogenous and filtered through a 40uM cell strainer (BD Biosciences) to isolate individual splenocytes. The dish was washed once with 10ml of L-15 and added to the filter. Splenocytes were washed and re-suspended in 10ml of L-15. Cells were then counted via haemocytometer by adding 10µl of cells to 100µl of ACK (ammonium-chloride-potassium) lysing buffer and 90µl of trypan blue. The purpose of the addition of ACK lysing buffer and trypan blue to the sample is to lyse red blood cells and to visualize live cells respectively.

2.5.2 Natural killer cell isolation

Splenocytes were obtained as previous described above (2.5.1) and NK cells were separated by negative selection magnetic bead cell separation (EasySep mouse NK cell enrichment kit, STEMCELL technologies and MACS NK cell isolation kit II, Miltenyi

Biotec). Procedure followed as described in protocol. Once NK enrichment was complete, cells were spun down and cultured in ELISPOT medium. The addition of 100 U/ml of recombinant mouse IL-2 (R&D system) was added to culture for maintenance if NK cells were to be cultured longer than 1-2 days. If expansion of NK cells were required, NK cells were plated at $5x10^5$ cells per well with 1000U of IL-2 in 1ml of NK expansion media (Roswell Park Memorial Institute (RPMI) 1640 medium, 10% fetal bovine serum (FBS), 0.1 mM beta-mercaptoethanol, 1% pen-strep). At day 3, cells were gently re-suspended by pipette and split 1:2 into a new well. Fresh NK expansion medium was added to bring the total volume back to 1ml. NK cells were used after day 5, but before day 10.

2.5.3 Macrophage isolation

The isolation of bone marrow derived macrophages (BMM) has been well established (110,111). Briefly, mice were euthanized by anesthesia (isoflurane) overdose, followed by physical dislocation as a secondary means. Legs of mice were severed and bone marrow progenitors were obtained by flushing bone marrow out of the femurs and tibia of mice using a 27 ½ gauge needle with a 1.0 ml syringe. Bone marrow was then homogenized by passing through the 27 ½ needle and washed using 10ml of L-15 media. These progenitors counted via haemocytometer and plated at a concentration of 1 x 10^6/ml and cultured using BMM medium (RPMI 1460 medium supplemented with 10% FBS, 1% Penicillin-Streptomycin and 50ng/ml of Macrophage colony stimulating factor (M-CSF)) in a humidified incubator with 5% CO_2 at 37°C.

After 2-3 days in culture, the medium was replaced with fresh BMM medium was added. At day 6, BMM are fully differentiated and ready for ex-vivo studies.

2.6 Enzyme-linked immunosorbent spot (ELISPOT) Assay

ELISPOT assays were conducted to determine IFNγ responses. Groups of 3 BALB/c mice were vaccinated under anesthesia (Isoflurane, Baxter) with 50μg of HA DNA vaccine with or without 5mers to a total of 50μl per leg as described in 2.3.2. Mice were sacrificed 10 days later to conduct the ELISPOT assay. One day prior to sacrifice, the 0.45um hydrophobic polyvinylidene fluoride (PVDF) plates (Millipore) were coated overnight with purified anti-mouse IFNγ (IFNγ capture antibody, BD Biosciences) diluted in PBS at 4°C. Mice were euthanized first by overdose of anaesthesia (isoflurane) and physical dislocation as a secondary means. Spleens were removed and crushed against a fine metal mesh (50 mesh, Bellco Glass) in Leibovitz's medium (L-15, Thermo Scientific) and filtered through a 40uM cell strainer (BD Biosciences) as described previously. Cells were washed, counted using a haemocytometer. The required amount of cells were taken, spun down and re-suspended in ELISPOT media (RPMI 1640 media, Thermo Scientific), supplemented with 10% FBS, 1% MEM non-essential amino acid, sodium pyruvate, hydroxyethylpiperazine ethane sulfonic acid (HEPES) buffer, L-glutamine, pen/strep, $5x10^{-3}$M beta-mercaptoethanol) and plated at $1x10^{6}$ cells per well. Splenocytes were re-stimulated with pools of overlapping peptides spanning the entire H5N1-H05 HA epitope. Splenocytes were incubated overnight at 37°C with 5% CO_2. IFNγ responses were evaluated the following day by using a biotinylated anti-mouse IFNγ antibody,

followed by horseradish peroxidase streptavidin (BD Biosciences). IFNγ visualization was obtained by incubation with 3-amino-9-ethylcarbazole (AEC) substrate (BD biosciences) for 15-30 minutes and washed thoroughly with water to prevent over-development. Plates were stored in the dark and read using an ELISPOT plate reader (AID ELISPOT reader).

2.7 Isolation of serum by saphenous bleed

To perform serology tests, blood was collected from BALB/c mice 25 days after vaccination. Mice were placed into 50ml conical tubes that have been cut at the tip to allow for steady airflow to the mouse while restrained in the conical tubes. The right hind leg was extended and immobilized by pinching the fold of skin around the knee joint. This not only prevents the mouse from movement, but also stretches the skin allowing for simple shaving of fur on the side of the leg using a Wahl peanut clipper. A minimal amount of Vaseline was applied to the shaved area allowing for better visualization of the saphenous vein, prevention of blood from seeping into the fur, as well as allowing the blood to collect as a round drop on the skin for easier collection. A 22G needle (BD) is used to puncture the vein at a 90° angle. Blood is then collected using a microtainer tube with serum separator additive (BD biosciences) to approximately 100µl. If required, the foot was exercised to increase blood flow. Once collected, cotton gauze was placed over the puncture with gentle pressure for approximately 30 seconds or until the bleeding halted. Mice were monitored to ensure hemostasis. Tubes were capped and spun at 13000RPM for 1 minute to separate serum.

The serum is transferred to a fresh eppendorf tube and heat inactivated at 56°C for 45 minutes.

2.8 Hemagglutinin Inhibition (HI) Assay

2.8.1 Serum treatment for HI Assay

Prior to the assay, serum was treated with receptor destroying enzyme (RDE) to allow for the cleavage of HA. This allows infection of red blood cells by the influenza virus. RDE was added in a ratio of 1 part serum to 3 parts RDE. The serum was then placed at 37°C for 18-20 hours followed by 45 minutes to 1 hour incubation at 56°C to inactivate compliment. 2-fold serial dilutions were performed on RDE-treated mouse sera starting with a 1:10 dilution.

2.8.2 HI Assay

50µl of serially diluted sera were added to each well in a 96 well V-bottom microtitre plate (Corning). Four agglutinating doses (AD) of the Hanoi 2005 virus were calculated and added to each well. The plate was then incubated at room temperature for 1 hour. After the incubation, 50µl of 0.5% turkey, 0.5% horse, or 0.5% guinea pig red blood cells were added to each well and the assay was incubated at room temperature for up to 1 hour. The HI titre was scored as the highest dilution where red

blood cell agglutination did not occur and the data were reported as the reciprocal of this dilution.

2.9 Flow cytometry

2.9.1 Natural killer cell activation

NK populations were isolated from whole splenocyte populations as described above and plated into 96 well round bottom plates at a concentration of $1x10^6$ cells per ml. Four different groups were evaluated: DMSO (negative control), 50μg of 5mer 4, 97, and 248 peptide was added to different groups respectively. To stimulate NK cells, 1000 U/ml of recombinant mouse IL-2 (ebiosciences) was added to positive control wells in triplicate. After 2, 4, 8, 18 hours, cells were collected and washed. After two PBS washes, NK cells were stained with anti-mouse CD69 conjugated to fluorescein isothiocyanate (FITC), anti-mouse CD3e conjugated to allophycocyanin coupled to cyanine dye Cy7 (APC-Cy7) and CD49b (DX5) conjugated to R-phycoerythrin (PE) for 1 hour at 4°C. Cells are washed with PBS twice and fixed with paraformaldehyde (PFA) to a final concentration of 1%. Cells are kept at 4°C until flow cytometry analysis and analysed no longer than 24 hours after fixation. NK cell activation was determined by percentage of CD3e-CD49b+CD69+ cells.

2.9.2 Macrophage activation

For activation studies, 50μg of 5mer, 97, 248 peptide, DMSO or polyinosinic:polycytidylic acid (poly i:c, Sigma) was added and after a 24 hour

incubation, macrophages were harvested for flow cytometry analysis. Macrophages were separated from cell culture dish (Co-star) by placing plates containing macrophages on ice and flushing with ice cold PBS with 1mM of ethylenediaminetetraacetic acid (EDTA, Teknova). After vigourous pipetting, cells were washed with PBS and spun down at 1500RPM for 5 minutes. After repeating the above wash, cells were stained with anti-CD11b conjugated to PE (Invitrogen), F4/80 conjugated to brilliant violet 421 (Biolegend) and anti-CD86 conjugated to Peridinin Chlorophyll Protein Complex conjugated to cyanine dye Cy5.5 (PerCP-Cy5.5) for 1 hour at 4°C. Cells are washed with PBS twice and fixed with PFA to a final concentration of 1%. Cells are kept at 4°C until flow cytometry analysis and were analysed no longer than 5 days after fixation. Cells were gated on CD11b+ and F4/80+ and activation was monitored by percent of CD86+ cells.

6.9.3 Flow Cytometry Analysis

The LSRII (BD) flow cytometer was used along with FACSDiva software (BD) to analyze the NK cells and macrophages for activation. Rainbow beads (Spherotech) and cytometer setup and tracking (CST) beads (BD) were used prior to each experiment to ensure proper performance of the LSRII. Compensation beads (CompBeads, BD) were used for multicolour compensation. Positively stimulated samples were also stained with each individual fluorochrome used in respective experiments to aid in distinguishing positive populations in data analysis.

2.10 NK depletion

BALB/c mice were depleted of NK cells by administration of rabbit anti-mouse asialo antibody (Wako). Mice received 50µl intraperitoneal (IP) injections (0.1mg in PBS) at two days and one day prior to vaccination. One day after the last injection, saphenous bleeds were performed on mice to confirm depletion. Depletion was confirmed by flow cytometry evaluating the percent of CD49b+ cells. Groups of 8 mice were vaccinated with 1µg of HA-DNA with or without 50µg of 5mer4. A lethal dose of $100LD_{50}$ of H5N1-H05 was used to challenge mice 28 days post immunization and monitored for weight loss, clinical score and survival.

2.11 Fluorescent Microscopy

NK cells were harvested as described above and plated in 24 well dishes at $5x10^5$ cells per well. 50µg of 5mer4 and 248 conjugated to biotin were added into respective wells at 2 hours, 6 hours and 18 hours. Cells were spun down at 1200RPM for 5 minutes and the supernatant was carefully aspirated. Cells were then washed with PBS in the same fashion. An anti-biotin PE antibody diluted 1:100 in PBS was added for 30 minutes at room temperature. Cells were spun down and washed twice with PBS and then fixed with 4% PFA to a total concentration of 1%. Visualization was performed on a Zeiss Axiovert 200M inverted fluorescent microscope at 32x magnifications where

representative pictures were taken in phase contrast, Cy3 filter and an overlay image of both.

2.12 Confocal microscopy

NK cells were harvested as described above in 24 well dishes at a concentration of $5x10^5$ cells per well. 50µg of 5mer4, 248 and 97 conjugated to biotin were added into respective wells at 4 hours. Cells were harvested in 5ml falcon tubes (BD) and spun down at 1200RPM for 5 minutes. Supernatant was removed and cells were washed with PBS. Cells were stained with anti-biotin PE antibody diluted 1:100 in PBS at room temperature for 30 minutes. Cells were washed twice in PBS and fixed with 4% PFA to a final concentration of 1%. Microscope slides (Fisherbrand) were submerged in diluted poly-D-lysine (Millipore) solution overnight. Slides were left to dry in a biosafety cabinet and 10µl of each NK cell group was placed drop-wise onto the slide as dispersed as possible. To cure the slides, 10µl of prolong gold anti-fade reagent with DAPI (Invitrogen) was added at the top edge of the microscope slide. The 1.5 coverslips (Fisherbrand) were added at an angle, starting at the top of the microscope slide to prevent air bubble formations. The slides were visualized using the LCM700 Axio Observer microscope (Zeiss) at 63x oil and 20x objectives. The two fluorescent channels used to visualize DAPI and PE stains were 405nm and 555nm respectively. Pictures were processed using the ZEN lite digital imaging software (Zeiss).

3.0 Results

3.1 Identification of rare, semi-common and common peptides

To determine the immune responsiveness of infrequently occurring peptides, the identification of rare, semi-common and common peptides needed to be established. After *in silco* scanning of UNIPROT database, the information obtained was translated into a graphical presentation (Figure 3). This allows for the selection of common (At occurrence count of 604 on a logarithmic scale), semi-common (At occurrence count of 68) and rare (At an occurrence count of 1). Once the occurrence count was determined, a list of all 5-mers with the same count was generated. For these studies, three frequency groups were created for testing purposes – rare, semi-common and common. From this scan, a list of 1632 semi-common, 3939 common and 417 rare 5-mers were generated. From each list, 200 representatives of each population were chosen by random for experimental purposes.

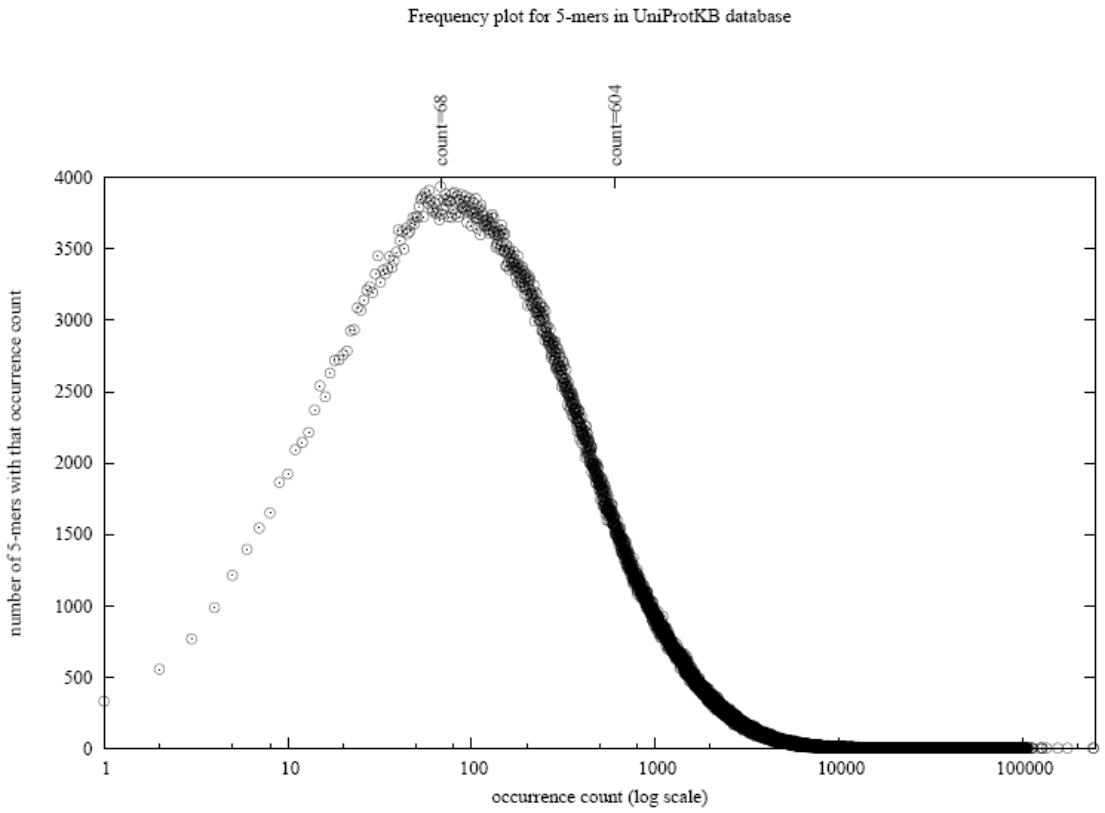


Figure 3: Frequency of 5-mers in the Universal Proteome. A graphical distribution comparing number of 5-mer peptides and occurrence in the UniProtKB database.

3.2 Determining suboptimal dose of H5N1-HA DNA vaccine

In order to test the effect of an adjuvant in conjunction of a vaccine, a suboptimal dose of the vaccine must be determined in order to effectively judge its efficacy. Groups of 4 BALB/c mice were vaccinated with 1μg, 5μg, 10μg, or 25μg of H5N1-HA DNA vaccine, and on day 28 post vaccination, mice were challenged with a $100LD_{50}$ of homologous H5N1-H05 influenza virus. Mice were monitored for a 16 days for survival (Figure 4A) and weight loss (Figure 4B). Mice receiving the 25μg and 10μg doses all survived challenge, whereas four of the mice in the group receiving the 5μg

dose survived. The 1μg dose group proved to be the optimal suboptimal group with 1/4 mice surviving. The control group and weight loss behaved as expected. All control mice that were not vaccinated succumbed to disease by day 9, while surviving mice show initial weight loss with disease progression, but recover quickly.

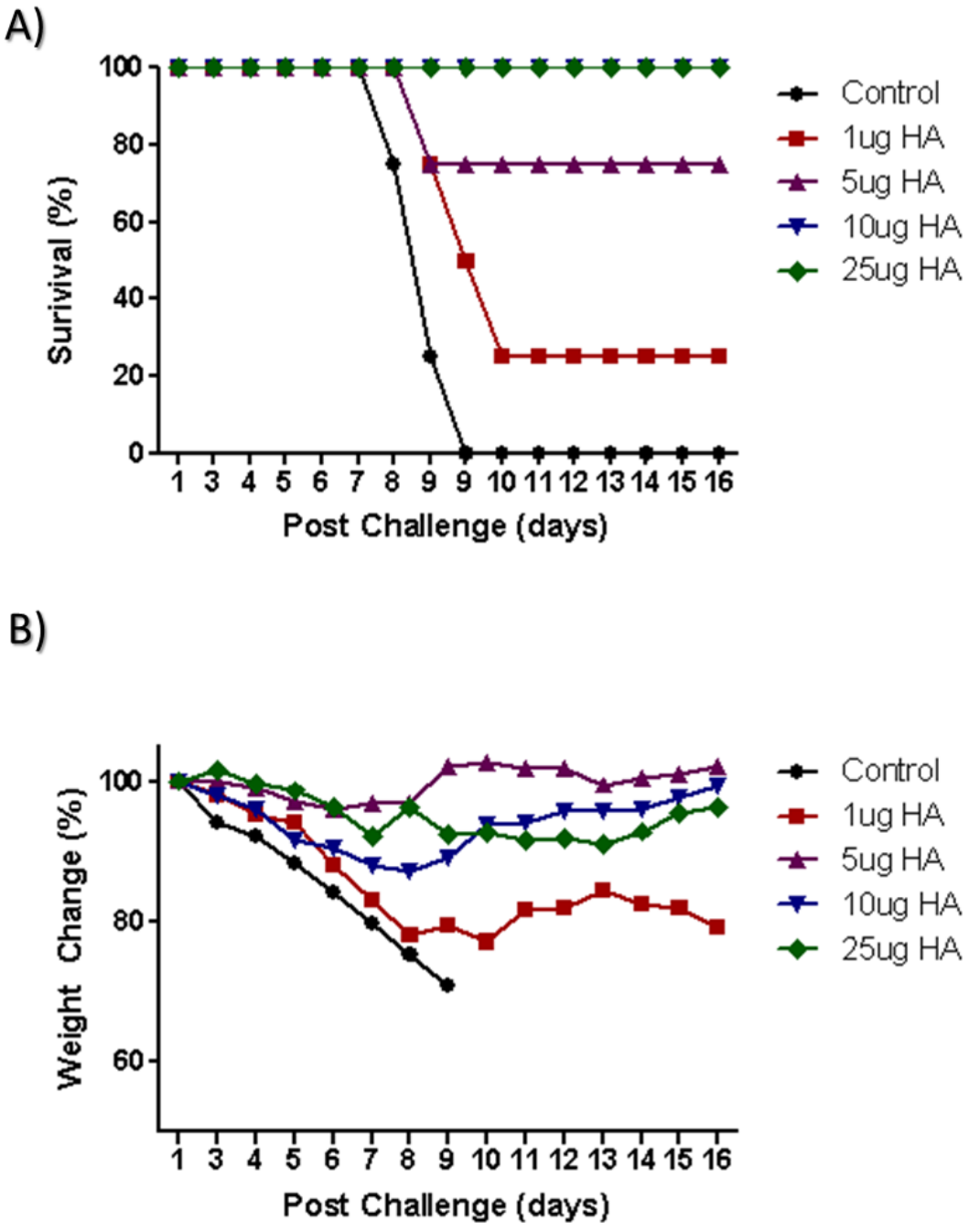


Figure 4: Dose titration of H5N1-HA DNA vaccine. Four (1µg, 5µg, 10µg, 25µg) doses of the H5N1-H05 DNA vaccine were administered to groups of four BALB/c mice. (A) Survival and (B) weight loss was monitored following homologous (Hanoi05) challenge.

3.3 Evaluation of cellular immunomodulation by rare, semi-common and common frequency peptides

For each of the three frequency categories, 200 peptide sequences were grouped into pools of ten peptides, resulting in a total of 20 groups of ten peptides to be tested per frequency category. For each group of ten peptides, three BALB/c mice were vaccinated with 50μg of pooled peptide and 50μg of H5N1-HA DNA vaccine, as well as control groups with 50μg of H5N1-HA DNA vaccine alone. This results in a total of 60 groups, with a total of 180 BALB/c mice. After ten days post vaccination, mice were sacrificed and splenocytes were isolated and re-stimulated with antigen peptides. IFNγ response was measured by spot forming units (SFU) and normalized to H5N1-HA DNA vaccine alone to measure either an increased or decreased response (Figure 5A). When averaging all 20 groups of each frequency category, a significant increase was found between common and semi-common and rare peptides (Figure 5B).

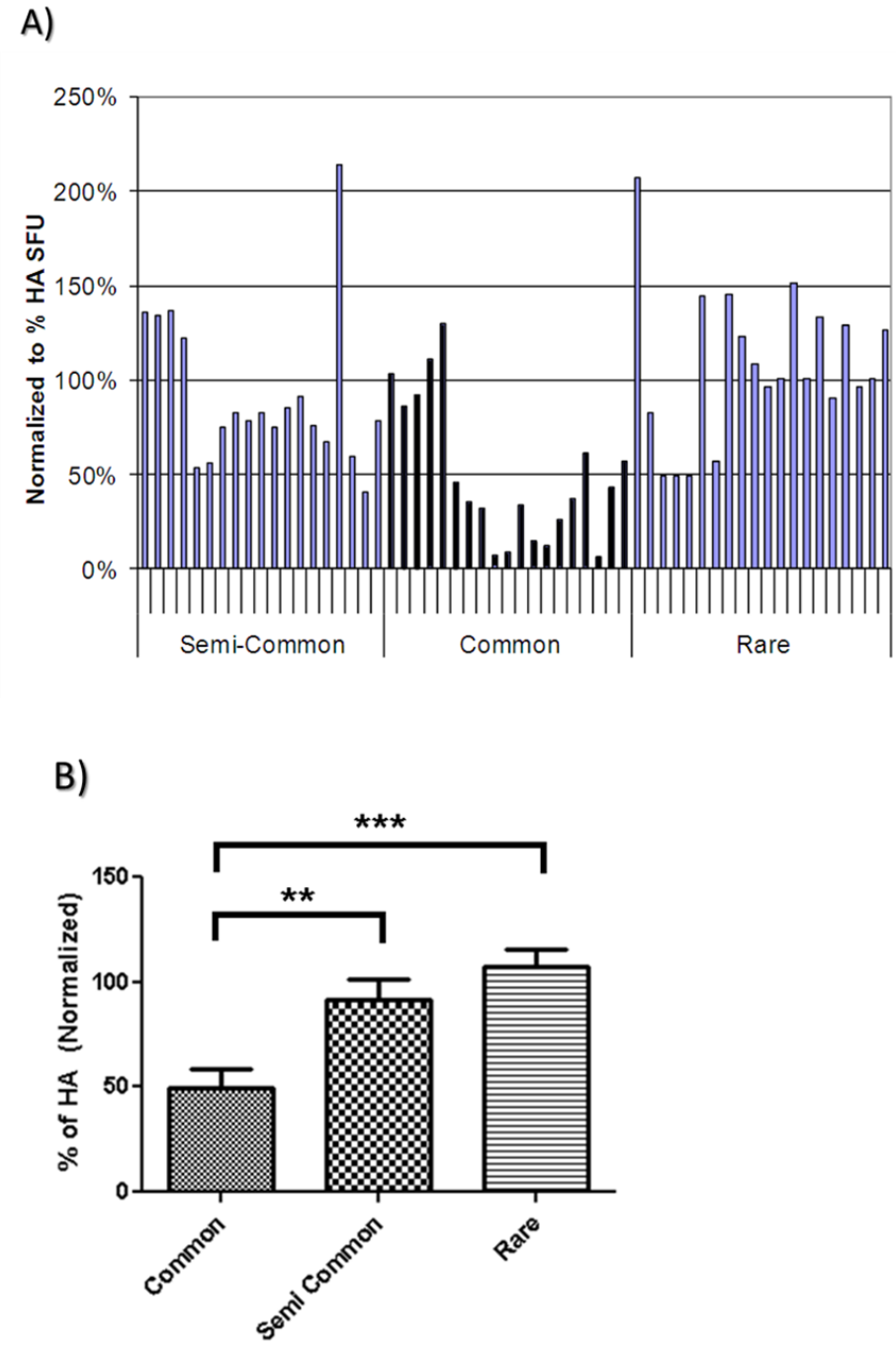


Figure 5: Evaluation of cellular immune responses by Interferon-γ secretion ELISPOT assay. (A) Individual bars representing spot forming units (SFU) counted for each of the 20 groups per frequency category (B) Average SFU of all 20 groups in each frequency category. *** P-value< .0001 ** p-value = 0.0024

3.4 Evaluation of rare, semi-common and common frequency groups to modulate survival against H5N1-H05 influenza virus

Groups of eight mice were vaccinated 1μg of H5N1-HA DNA vaccine with a total of 50μg of peptides from a stock pool of all 200 common, semi-common or rare peptide frequency groups. After 28 days post vaccination, mice were intranasally challenged with a $100LD_{50}$ of H5N1-H05 influenza virus. Of the common frequency group, 1/8 mice survived, 2/8 mice of the semi-common frequency group survived and 3/8 of the rare frequency group survived (figure 6A). None of the 5 mice in the control (non-vaccinated) group survived, and 1/5 of the H5N1-H05 HA alone group survived, which was expected. Weight loss averages indicate mice became were infected and loss weight due to illness, but recovered over time (Figure 6B).

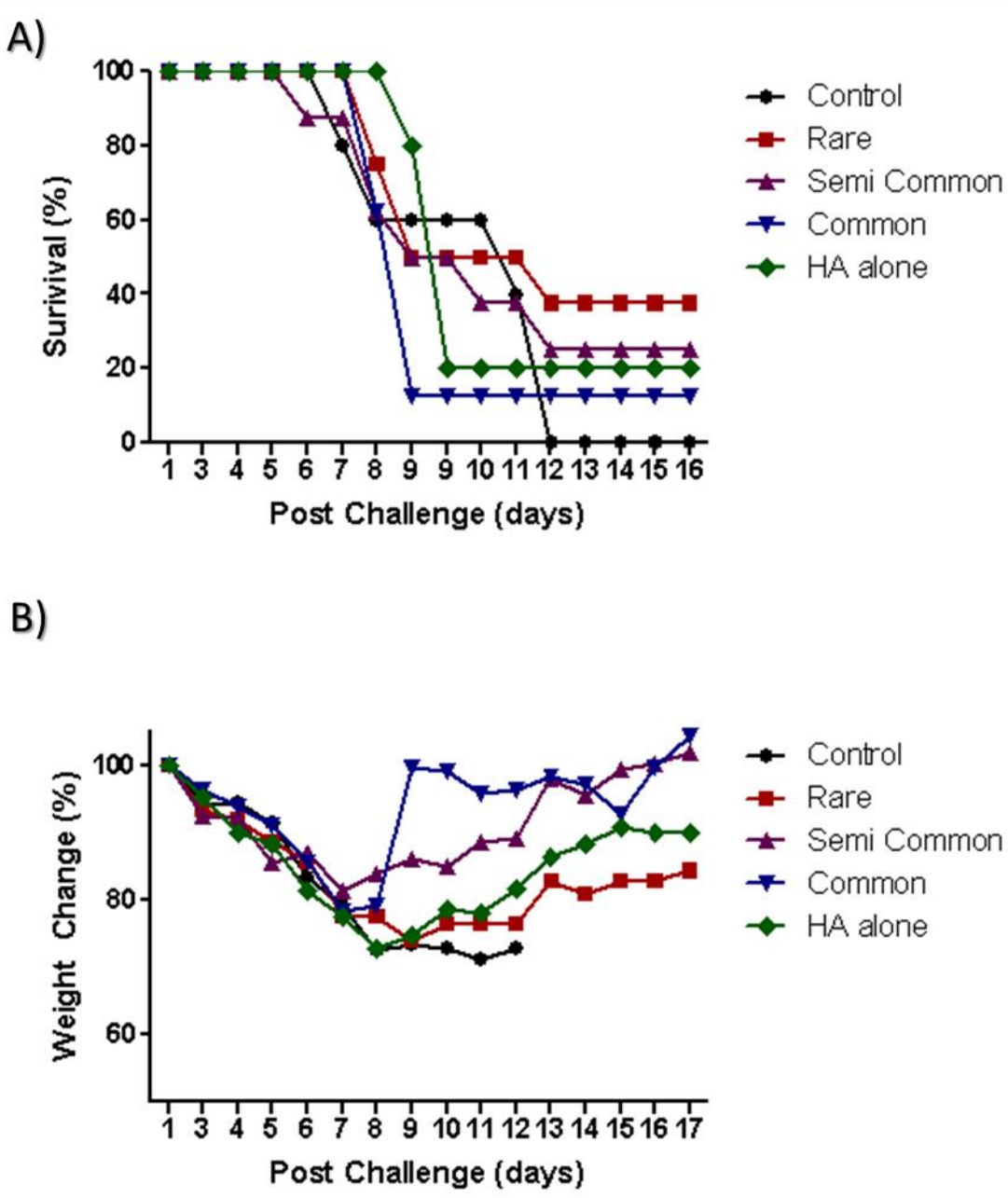


Figure 6: Percent survival of pooled peptide frequency groups challenged against H5N1-H05 influenza virus. Eight BALB/c mice were vaccinated with 50μg from a pool of either 200 rare, semi-common or common frequency groups. Mice were challenged with $100LD_{50}$of H5N1-H05 influenza virus and monitored for (A) survival and (B) weight loss for 16 days.

3.5 Individual evaluation of high IFNγ stimulating peptides in cellular immune responses and survival

The groups from Figure 5A that exhibited the highest IFNγ response were chosen to test each peptide to see the effects of each individual peptide. The best pools broken into individual peptides are peptides 161-170 (semi-common peptides), peptides 241-250 (common peptides) and peptides 91-100 (rare peptides).Similarly, three BALB/c mice were vaccinated with 50μg of 5-mer along with 1μg of H5N1-HA DNA vaccine. Splenocytes were harvested ten days post vaccination and re-stimulated with HA antigen peptides. Peptide 97 shows the highest IFNγ release with an increase of 373% over H5N1-HA DNA vaccine alone (Figure 7).

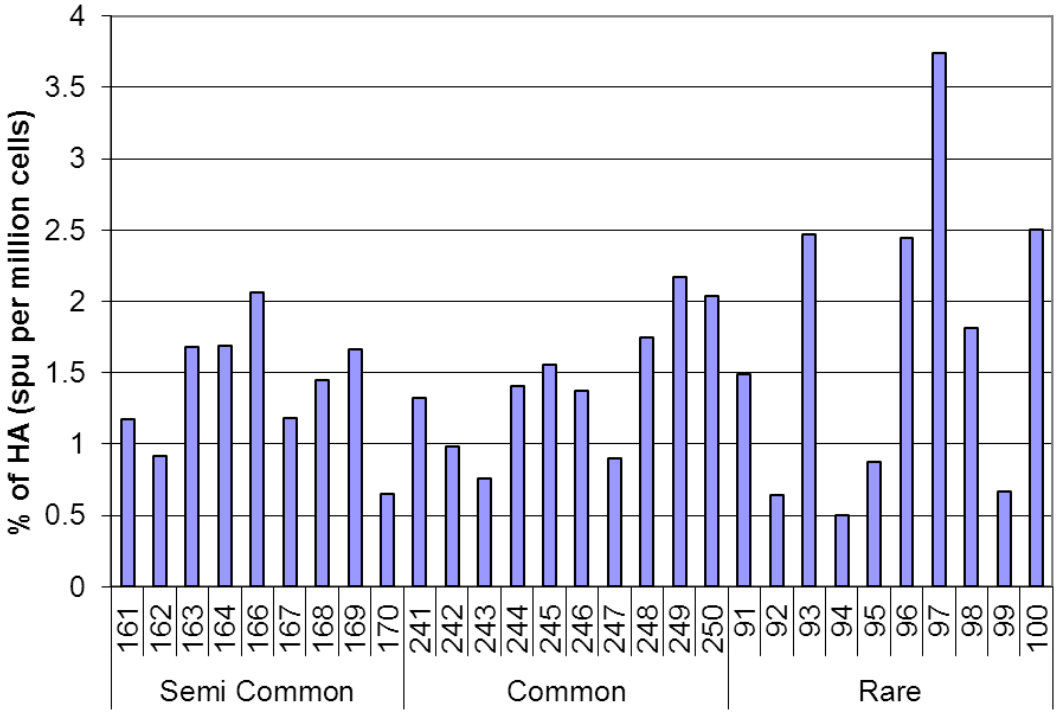


Figure 7: Identification and characterization of 5-mer with highest IFN-γ secretion. Highest IFN-γ secreting group of each frequency group were individually tested for individual 5-mers abilities to stimulate IFN-γ.

3.6 Individual evaluation of high IFNγ stimulating peptide 97 in antibody titre and survival challenge

Groups of eight mice were immunized with 1μg of H5N1-H05 HA DNA vaccine with or without 50μg of 97 as well as 5mer4 for comparison, or 1μg of HA alone. The same mice were bled 25 days post vaccination for evaluation of antibody production (Figure 8A) and were then lethally challenged 28 days post vaccination with $100LD_{50}$ of H5N1-H05 virus. Mice monitored for survival (Figure 8B) and weight loss (Figure 8C) for 16 days post challenge. Out of the peptide 97 + HA experimental groups, 1/8

mouse survived challenge, whereas 4/8 mice survived from the 5mer4 + HA vaccinated groups. From the H5N1-H05 HA DNA vaccine control group, 1/5 mice survived, the expected level of protection in these conditions leading to partial protection in order to observe increased efficacy from adjuvant effect. Weight loss indicates that mice became ill and survivors recovered over time.

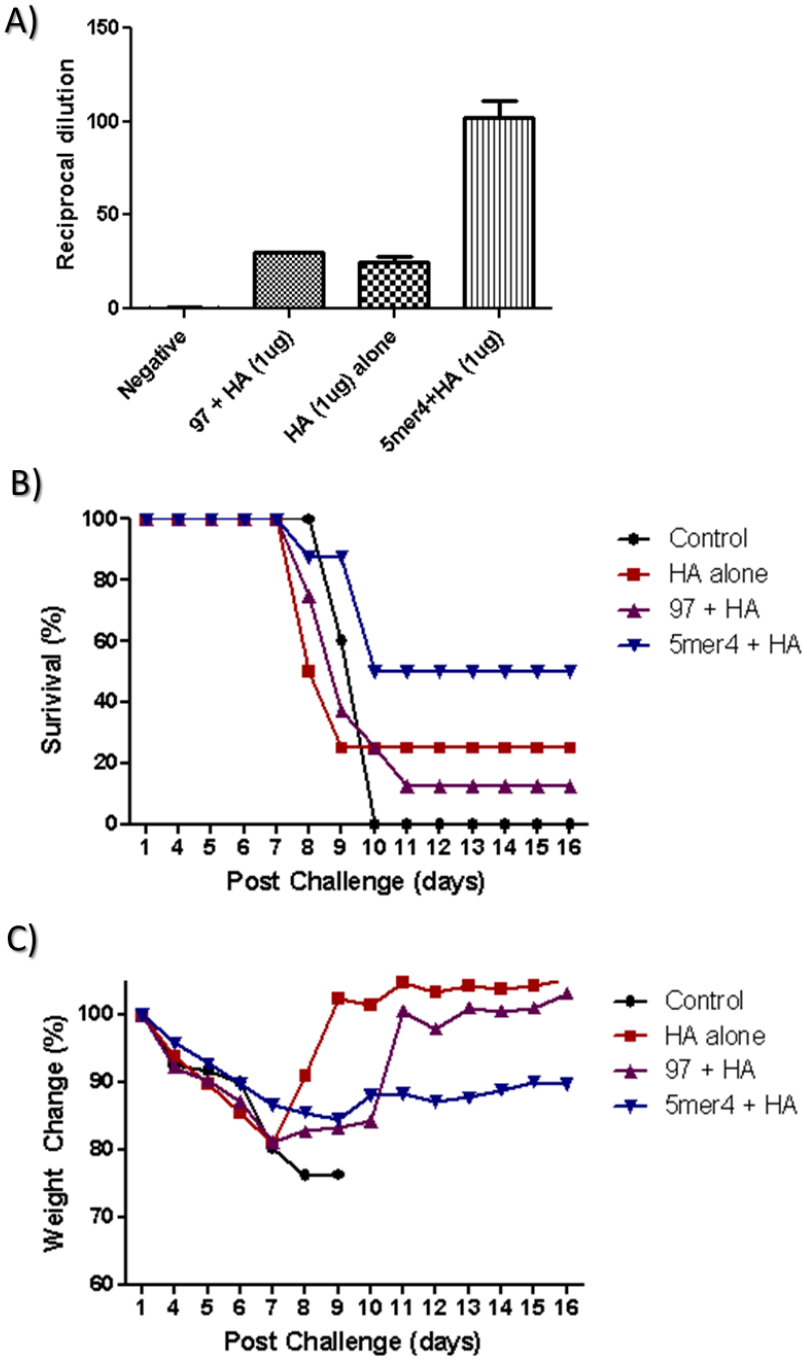


Figure 8: Evaluation of Peptide 97 as adjuvant. Groups of 8 were immunized with 1µg of H5N1-H05 HA DNA vaccine, as well as 50 of 97 or 5mer4 + HA or with 1µg HA alone.(A) Antibody production evaluated by hemagglutinin inhibition assay. (B) Survival challenge and (C) weight loss against lethal H5N1-H05 virus.

3.7 Comparison of 5mer4 against additional commercially available adjuvants

Previously, a comparison of cellular immune responses of 5mer4 to additional commercially available adjuvants showed equal or increased IFNγ secretion (55). To further evaluate the efficacy of rare peptides as an adjuvant, groups of 9-10 mice were vaccinated with 1μg H5N1-HA and with or without adjuvants in order to determine antibody response by HAI and survival against lethal challenge. The adjuvants used in comparison were as follows: Alhydrogel (450μg), CpG ODN (10μg), and 5mer4 (50μg). The mice were bled by saphenous bleed on day 25 post vaccination for hemagglutinin inhibition assays (Figure 9A), and then lethally challenged with $100LD_{50}$ of H5N1-H05 virus on day 28 post vaccination and monitored for 15 days (Figure 9B). The 5mer4+ H5N1-HA group had the highest percent survival at 60% (6/10 mice), which provided a slightly better level of protection in comparison to CpG ODN (4/9 mice). There were no survivors (0/10) in the Alhydrogel + H5N1-HA group. The control group (0/5 mice) and the H5N1-HA alone group (1/5 mice) results were as expected as well as weight loss over time with surviving mice recovering back towards original weight (Figure 9C).

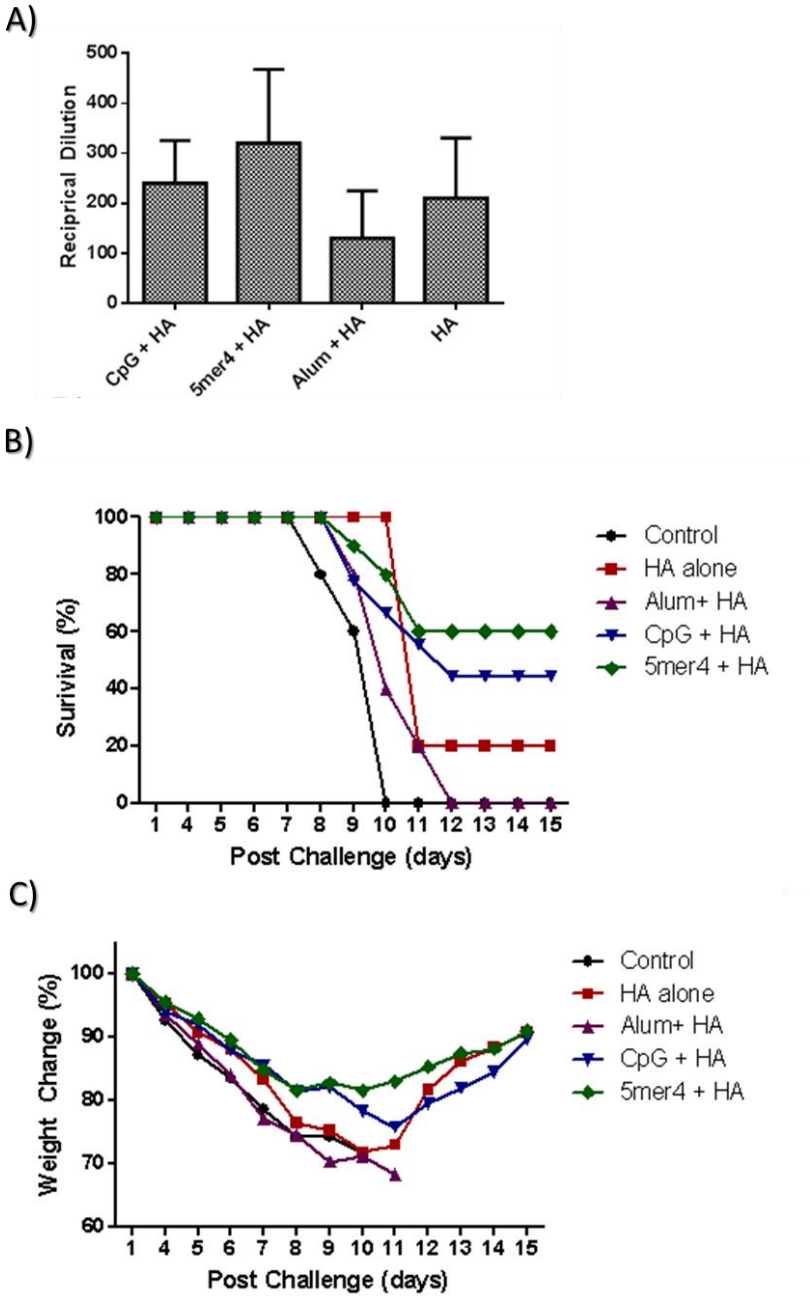
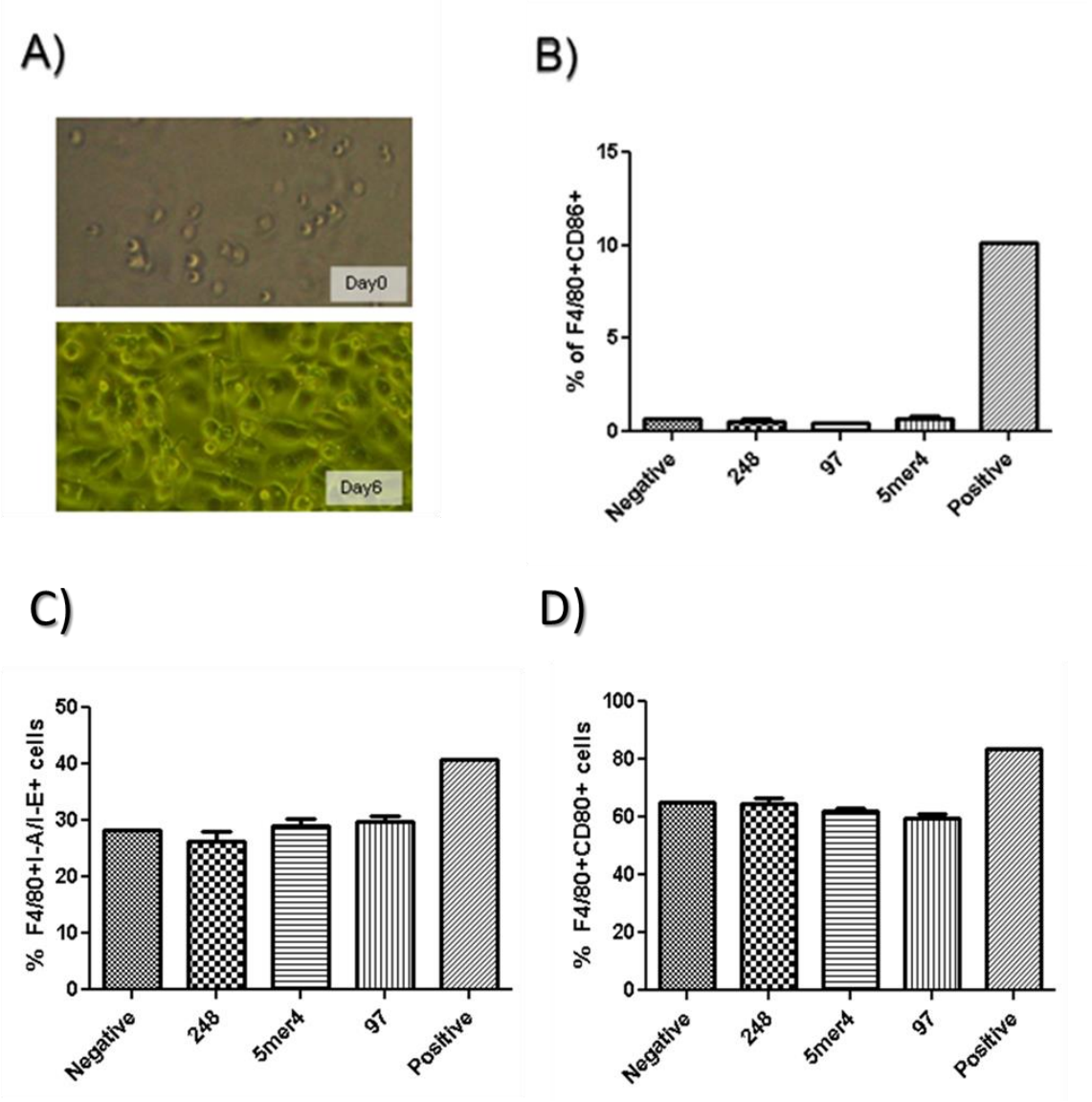


Figure 9: Comparison of 5-mer4 with other commercial adjuvants. Groups of 9-10 BALB/c mice were immunized with H5N1-HA (1 µg) alone, H5N1-HA (1 µg) + 5mer4 (50 µg), H5N1-HA (50 µg) + CpG (10 µg) or H5N1-HA (50 µg) adsorbed to Alhydrogel (450 µg). (A) Mice were bled 25 days prior to challenge to measure antibody response. Mice were then challenged with a lethal dose ($100LD_{50}$) of H5N1-H05 virus 28 days after vaccination and were monitored for (B) survival and (C) weight loss for 15 days post challenge

3.8 The effect of rare peptides on macrophage

Immune activation by rare peptides has been suggested to be linked the innate immune response. Previously dendritic cells and natural killer cells have been evaluated, but the effect of rare peptides on macrophages has yet to be explored. Macrophages play a key role in the innate immune response, so the role of rare peptides on macrophages needed to be evaluated. Macrophages were derived from the bone marrow of BALB/c mice and differentiated by the addition of M-CSF. After 6 days, cells were visualized via inverted microscope to ensure differentiation (Figure 10A). The macrophages were then incubated with 50μg of 5mer4, DMSO (negative) or poly I:C (positive). After 24 hours of stimulation, cells were harvested and stained with macrophage markers F4/80, as well as activation markers CD86 (Figure 10B), I-A/I-E (Figure 10C) and CD80 (Figure 10D). Macrophages treated with 5mer4 appear to have the same level of activation as the negative control.


A)
Day0
Day6
B)
% of F4/80+CD86+
15
10
5
0
Negative
248
97
5mer4
Positive
C)
% F4/80+I-A/I-E+ cells
50
40
30
20
10
0
Negative
248
5mer4
97
Positive
D)
% F4/80+CD80+ cells
100
80
60
40
20
0
Negative
248
5mer4
97
Positive

E)

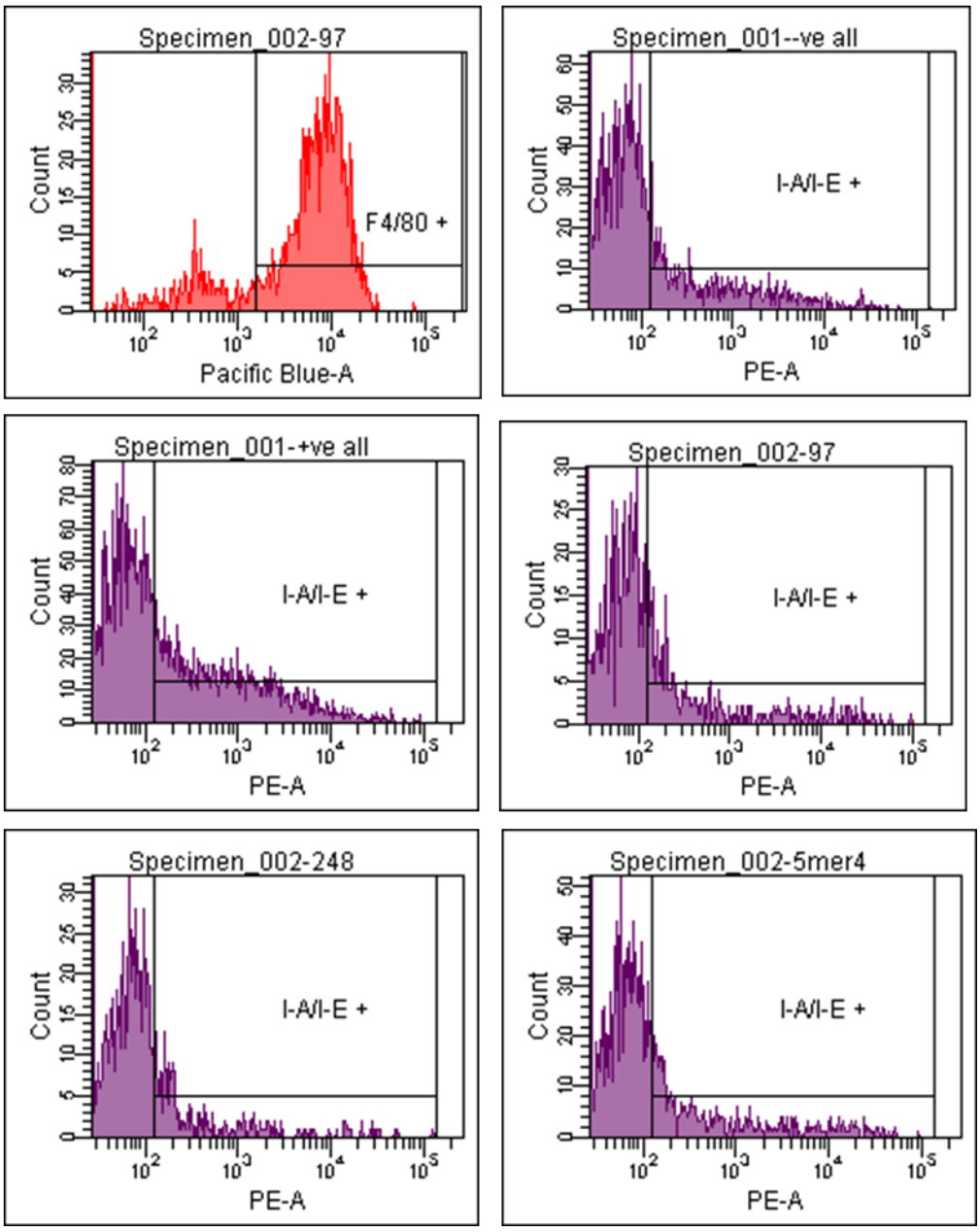


Figure 10: Macrophage isolation and effect of rare peptide on macrophage activation

(A) Bone marrow was isolated from naïve BALB/c mice and cultured in M-CSF for 6 days to differentiate cells into macrophages. Cells were visualised under 32 x magnifications. The macrophages were incubated with 5mer4, 248, 97, DMSO (negative) or poly i:c (positive). After 24 hours of incubation, cells were harvested and analysed by flow cytometry for expression of F4/80+ and (B) CD86, (C) I-A/I-E or (D)

CD80. E) Representative MFI plots showing gating on F4/80+ cells and I-A/I-E+ cells when incubated with DMSO (-ve), poly I:C (+ve), peptide 97, 248 and 5mer4.

3.9 The effect of rare peptides on natural killer cells

3.9.1. NK CD69 activation

Our previous findings indicate that natural killer cells can become activated with 24 hours incubation of rare peptides. To further this study, an activation time course was performed in order to determine the peak activation time point. Natural killer cells were obtained by isolating splenocytes from naïve BALB/c mice and enriched for natural killer cells by negative selection. The cells were then incubated with 50µg of 5mer4, 50µg of 97 peptide, 50µg of irrelevant peptide, and DMSO (negative control) for 2, 4, 8 or 18 hours. Cells were harvested and stained with natural killer cell markers DX5 (CD49b). An early activation marker CD69 was used to determine activation levels (Figure 11A). Peptide 97 appears to have the highest level of activation. 5mer4 shows significantly higher levels of CD69 activation in comparison to the common peptide. It appears that at 4 hours after the addition of peptide, NK cells were at the highest level of activation.

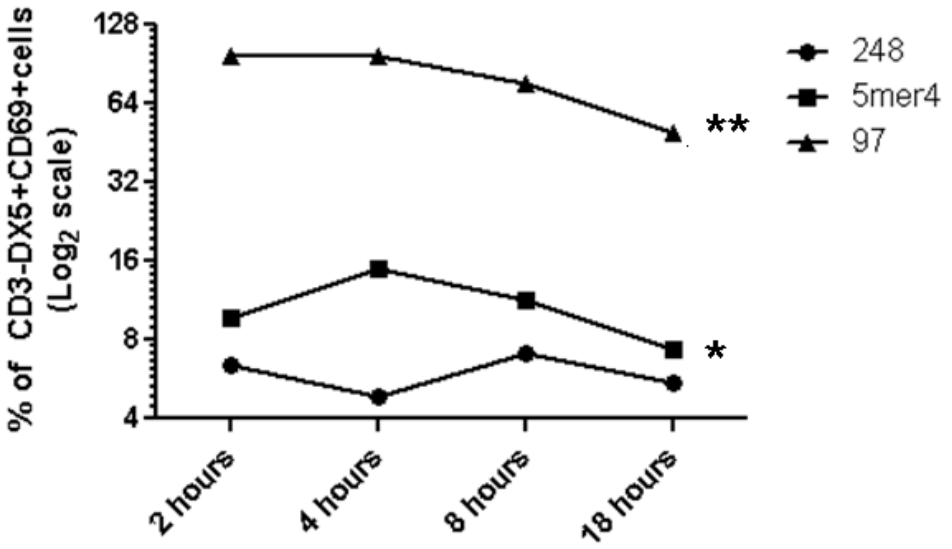


Figure 11: Natural killer cell CD69 activation time course
Natural killer cells were obtained by isolating splenocytes from naïve BALB/c mice and enriching NK population by negative selection. NK cells were incubated with 5mer4, 97 peptide, common peptide, and DMSO (negative) and incubated for 2, 4, 8, or 18 hours. **p value = 0.001, *p value = 0.04

3.9.2 NK depleted vaccinated mice against H5N1 Hanoi05 challenge

With the evidence that rare peptides may activate NK cells, we tested to see if the depletion of NK cells would affect the ability of 5mer4 to act as an adjuvant. Two groups of depleted mice were vaccinated with 1μg of H5N1-H05 HA DNA vaccine with or without 50μg of 5mer4. An additional 2 groups were also vaccinated with 1μg of H5N1-H05 HA DNA vaccine with or without 50μg of 5mer4, but the NK cell population remained non-depleted. After a lethal challenge with H5N1-H05 virus, there was no difference between the depleted groups whether peptide was added or not. On the other hand, the non-depleted mice, mice who were vaccinated with the peptide survived lethal challenge whereas their counterpart of H5N1-HA DNA vaccine alone

was not protected against the challenge (1/5 mice survived). Unvaccinated control mice fully succumbed to the disease.

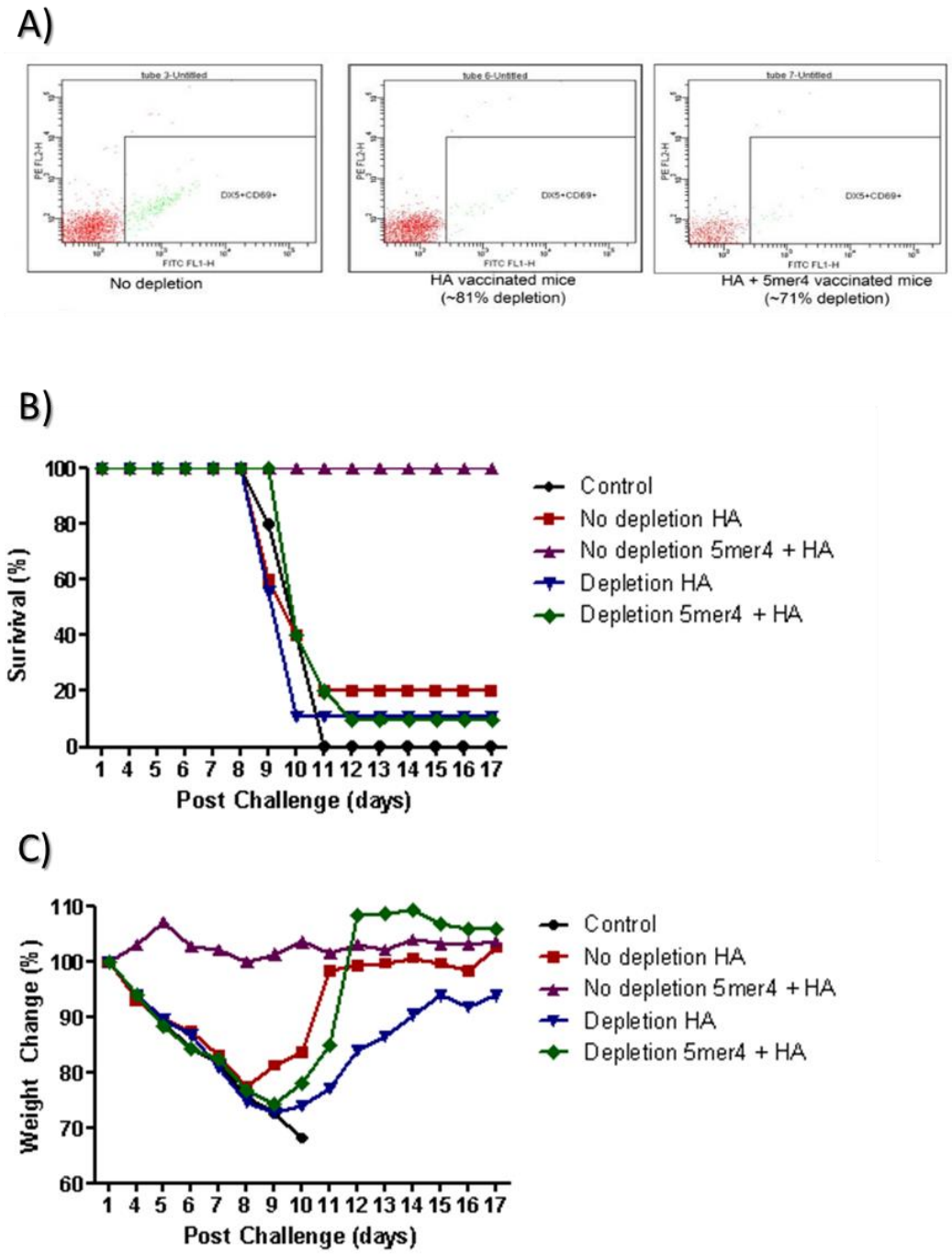
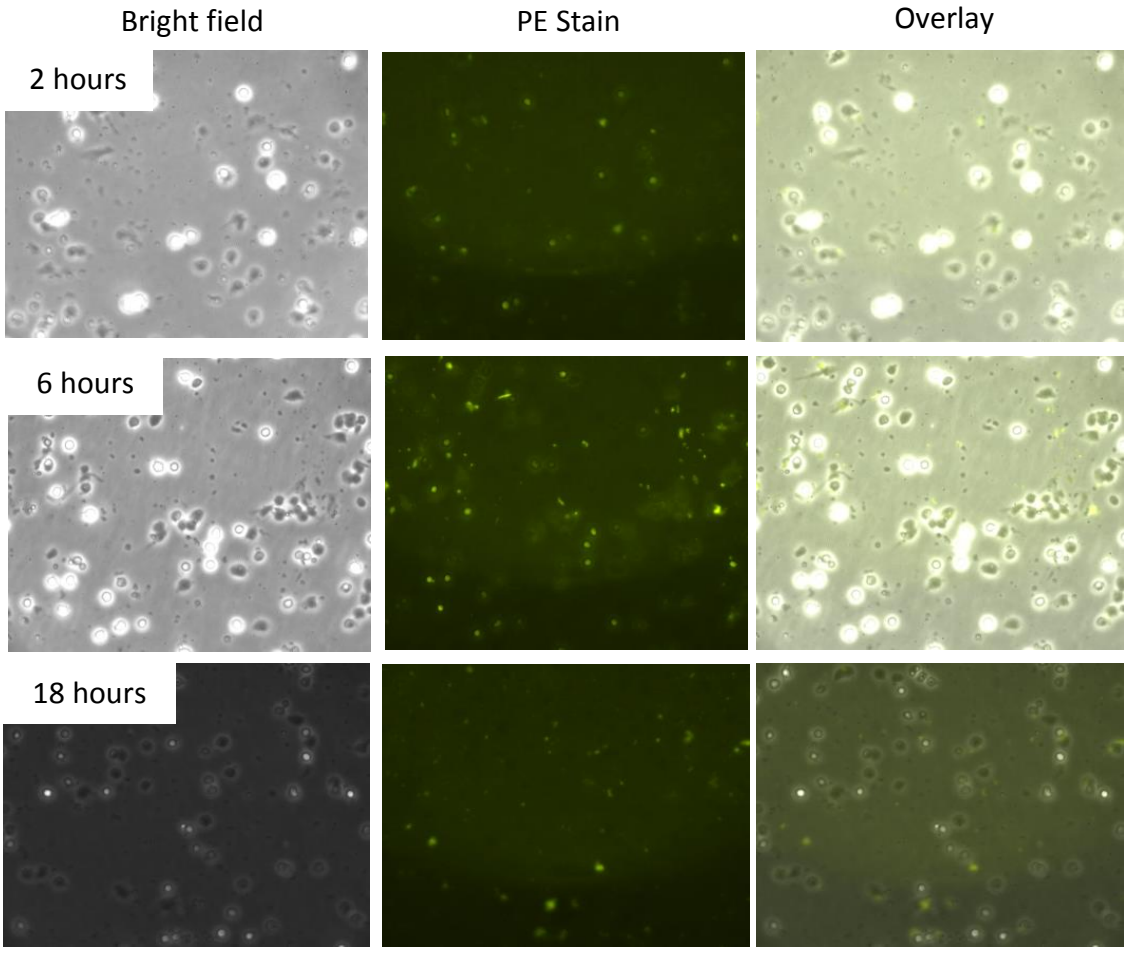


Figure 12: The adjuvant effect of 5mer4 on NK depleted mice against lethal H5N1-H05 challenge. Groups of 5 BALB/c mice were depleted of NK cells by intraperitoneal injection of anti-asialo and vaccinated with 1µg H5N1-H05 HA DNA vaccine with or without 50µg of 5mer4. (A) NK depletion was confirmed by flow cytometry. (B) Mice were challenged 28 days after vaccination with $100LD_{50}$of H5N1-H05 virus and monitored for (C) weight loss.

3.10 Fluorescent microscopy and confocal imaging of 5mer 4 and common peptide

To address whether the rare peptides directly interact with NK cells, 5mer4 and 248 peptides were conjugated to biotin. These peptides were added onto NK cells and allowed to bind for 2, 6 or 18 hours. Cells were washed and incubated with a PE anti-biotin antibody. After two washes, cells were visualized under the fluorescent microscope. The NK cells treated with the 248 peptide do not appear to have a strong interaction as no fluorescence can be seen at any of the time points (Figure 12B), whereas fluorescence corresponding with NK cells indicates that 5mer4 peptides are able to bind well (Figure 12A).

A)



B)

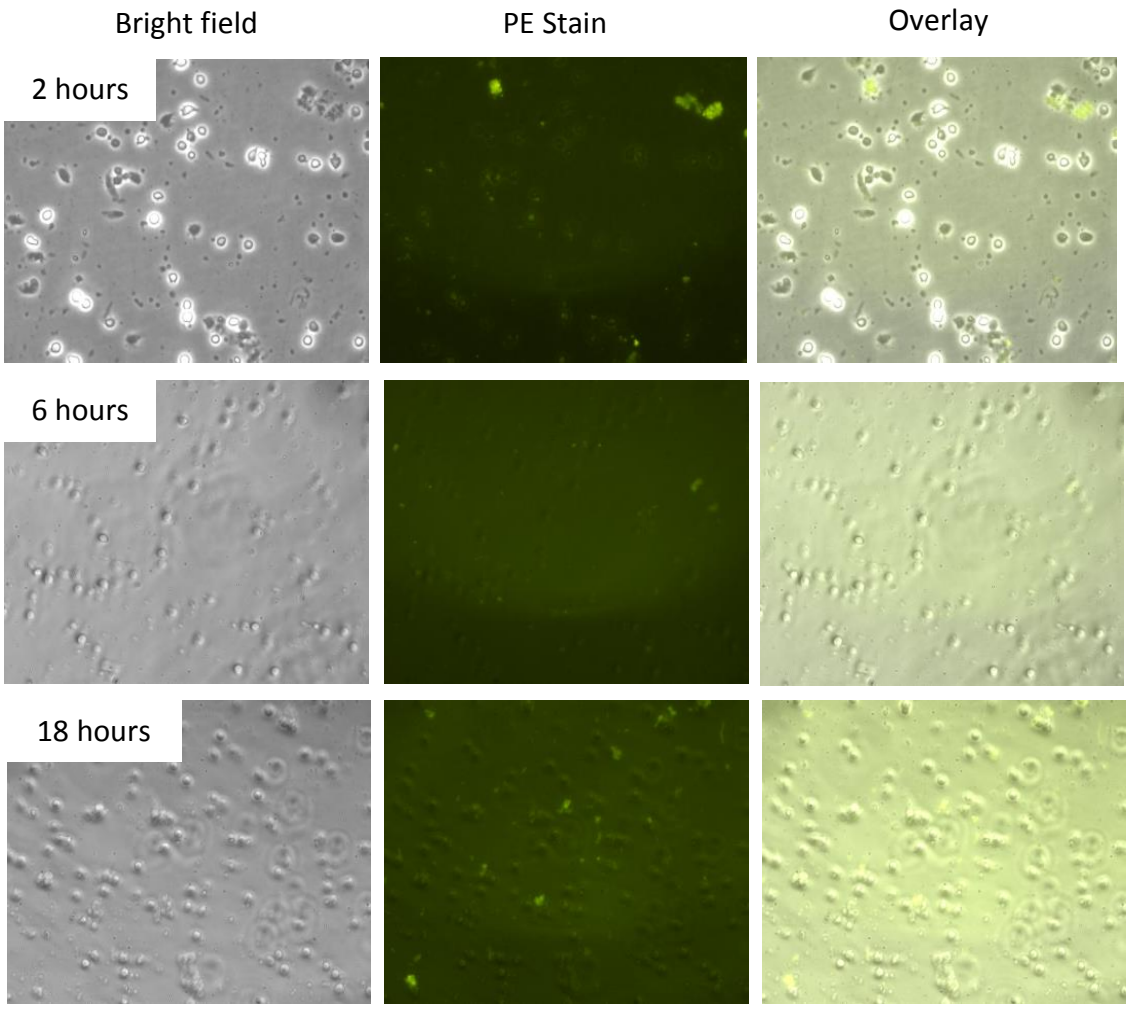
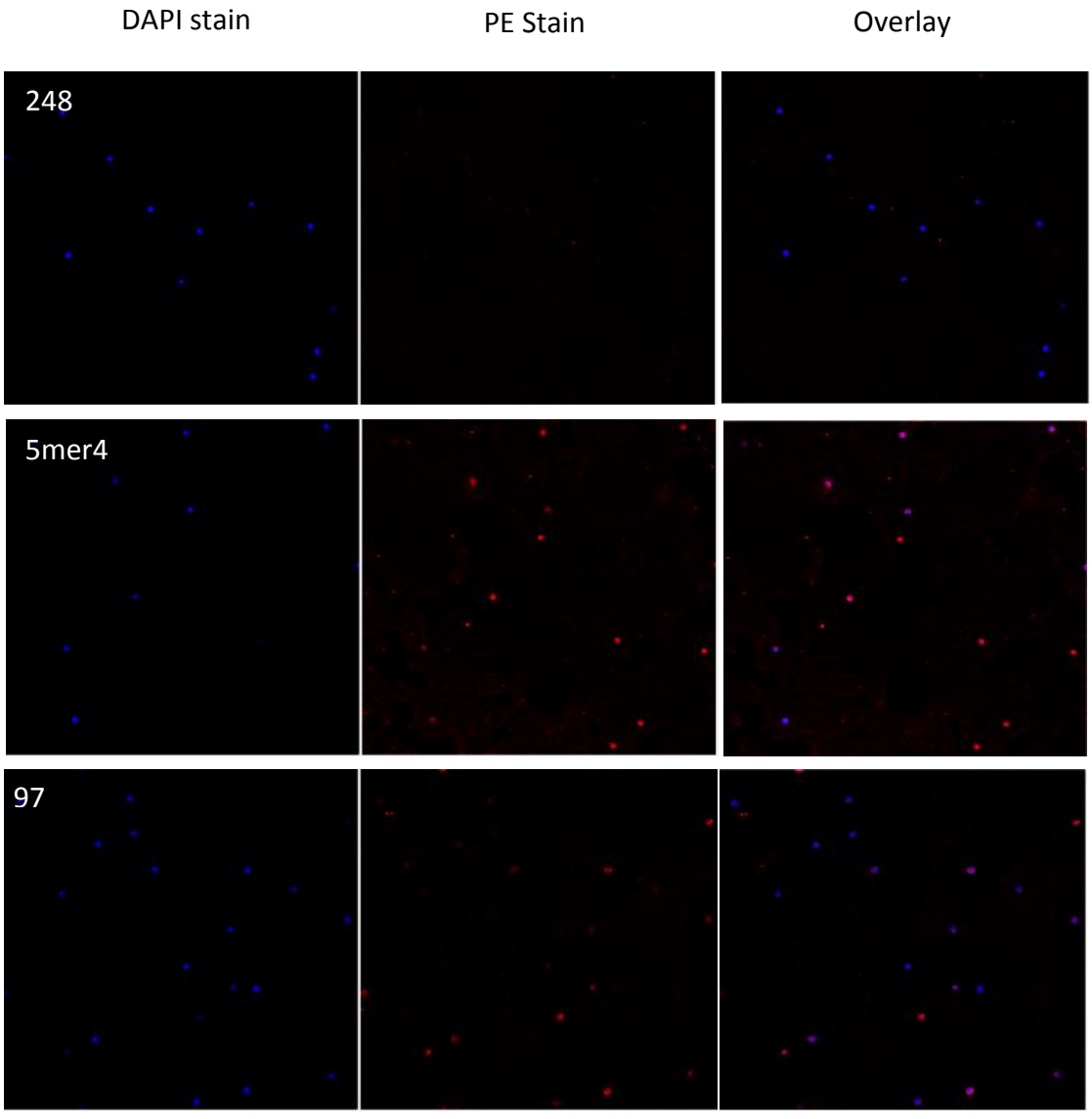


Figure 13: Fluorescent microscopy and confocal imaging of 5mer 4 and common peptide. Natural killer cells were incubated with (A) 50μg of biotin conjugated 5mer4 or (B) biotin conjugated common peptide for 2, 6 or 18 hours. PE anti-biotin secondary antibody was used for visualization via fluorescent microscopy.

To further investigate the interaction of rare peptide with NK cells, 5mer4, 97 and 248 conjugated to biotin were added at 4 hours. This time point was chosen since the most fluorescence from Figure 13 can be seen between time points 2 and 6 as well as

the NK activation time course (Figure 11) indicate that 4 hours was the peak level of NK cell activation due to rare peptides. Cells were stained with DAPI to visualize the cell nucleus (blue) and with PE anti-biotin (red) to visualize bound peptide. NK cells incubated with common peptide do not show the peptide overlapping the nucleus indicating that these peptides do not bind to NK cells. The rare peptides, 5mer4 and 97, are shown to be overlapping the nucleus, indicating that these peptides are able to bind to NK cells (Figure 14A). The NK cells were also visualized at a 63x magnification (Figure 14B). These images suggest that common peptides do not bind NK cells, whereas rare peptides such as 5mer4 and 97 are able to bind abundantly to the NK cells.

A)



B)

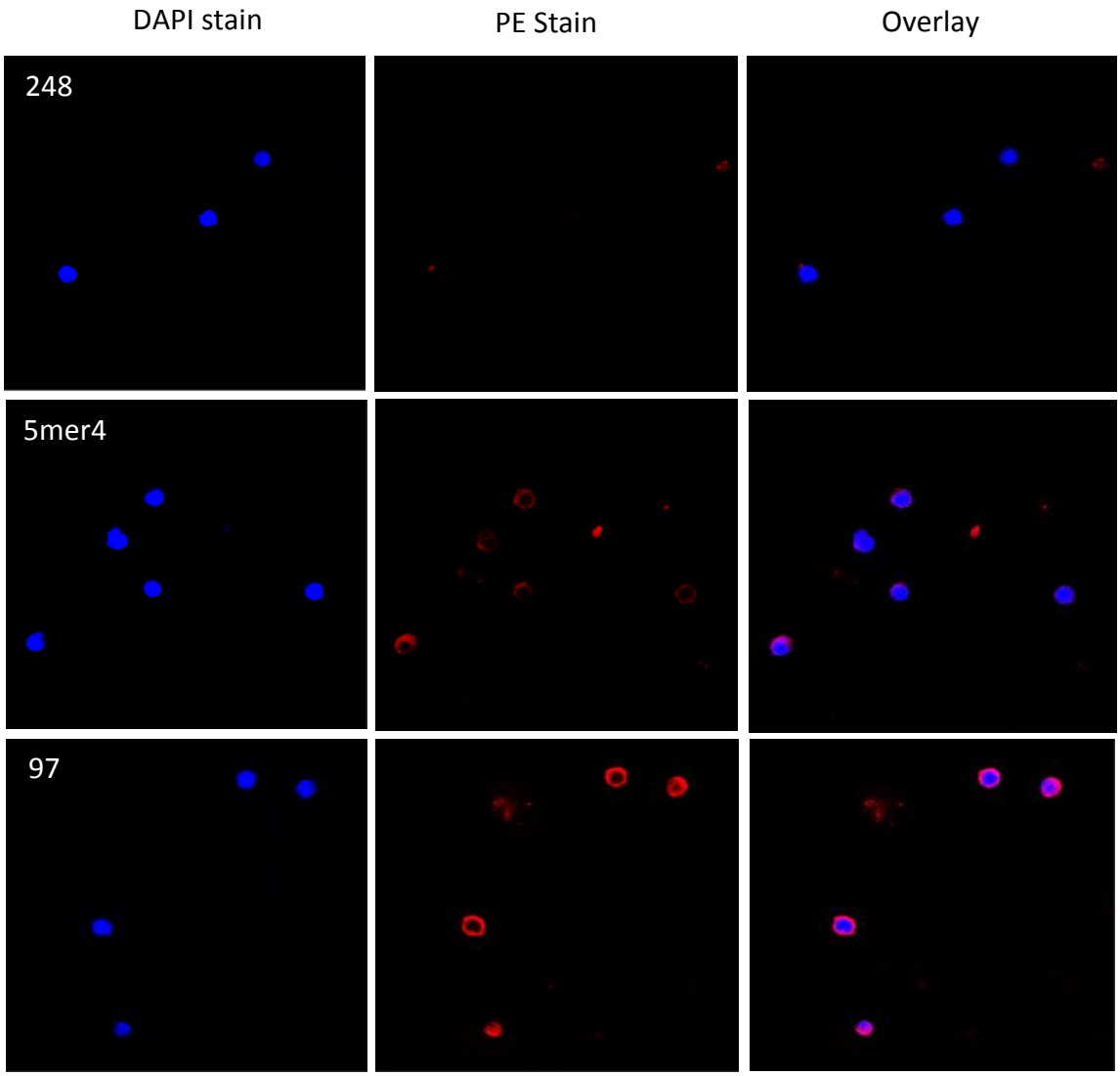


Figure 14: Confocal microscopy image of NK cells incubated for 4 hours with biotin conjugated common peptide (248), biotin conjugated 5mer4 or biotin conjugated peptide 97 at (A) 20x magnification or (B) 63x magnification. PE anti-biotin secondary antibody was used for visualization via confocal microscopy.

4.0 Discussion

4.1 Determining correlation between peptide frequency and immune responsiveness

The immune response is a diverse network that is required to distinguish what is safe and what is a threat. An overactive immune response to a non-threat such as self-antigens can lead to autoimmunity, whereas an underactive immune response can lead to pathogenic illness, both actions which can consequently lead to death. This is a delicate area that requires specificity. The immune system is trained not to attack self as seen in lymphocyte education and tolerance. Overall, this study suggests a general trend of increased immune responsiveness when rare peptides are administered in comparison to semi-common and common peptides.

A non-existent peptide is defined in this study as a peptide sequence not naturally present in the human proteome. Initially, the 5mers selected for this study did not appear in any of the proteomes within the UNIPROT database, but this database is versatile, new discoveries can add, subtract and change the database. Pentapeptides were chosen as they have been defined as a minimal requirement for immune recognition (112), as well as data indicating that there appears to be a bias in the appearance of these peptides in nature. Mathematically speaking, the probability of total 5mer amino acid combinations is expected to be found in many of the known proteomes, but interestingly, analysis of all known proteomes at that time indicate that a

large number of pentapeptides are non-existing. There are a number of speculations of why particular sequences do not occur in nature. For example, one possibility includes the simple fact of adaptation to the environment and availability. Another possibility is that many of the non-occurring peptide sequences contain unique biochemical properties such as tryptophans (W) which is a large, aromatic and hydrophobic or cysteines (C) with the ability to form disulfide bridges. Peptides containing amino acids with different properties within close proximity may disrupt the folding of proteins. A simple amino acid completely change can alter the protein folding and therefore function (113). Taken together, it appears that rare peptides are able to modulate an IFNγ response in comparison to common peptides. This trend is much more apparent when looking collectively (Figure 5B), but the variations between groups at times can be large as seen in Figure 5A. There are many different possibilities for this variation, one being that the definition of "rare" are consistently changing, as mentioned previously, as well as the degree of rarity of each peptide differs. The basis of this entire project is based on the definition of rare, which in itself is a fluid concept.

The highest responding groups from Figure 5A were tested individually to determine the highest IFNγ responder. From this, the 97 peptide was identified as the highest stimulator of IFNγ (Figure 7A). Mice were then vaccinated with 50μg of peptide 97 in conjunction with suboptimal H5N1-HA DNA vaccine (1μg) and challenged 28 days after with homologous H5N1 influenza virus. Although 97 peptide was able to elicit a strong IFNγ response, the 97 peptide was unable to adjuvant the H5N1-HA DNA vaccine resulting in no difference in protection in comparison to

H5N1-HA DNA vaccine alone (Figure 8B). This data suggests that additional factors other than high IFNγ stimulation may play a major role in adjuvanting H5N1-HA DNA vaccines for protection against homologous influenza challenge. The correlates of protection against influenza challenge have not been fully elucidated although the current standard of protection is determined by antibody titre (114). Although a high IFNγ response was obtained, the 97 peptide elicited a low antibody response after vaccination (Figure 8A). Past literature has shown that T_H1 cytokines such as IFNγ down regulate T_H2 responses and up-regulate T_H1 responses (115,116), which may explain why peptide 97 did not mount a strong antibody response and decreased survival. Many papers have been published arguing whether a cell-mediated or antibody mediated response is necessary, but the immune system is complex and interconnected and perhaps multiple immune functions are required for viral clearance. In addition, the effectiveness of an adjuvant in one vaccine may not directly correlate to effectiveness in other vaccines. For example, alum has been proven to be highly effective in multiple vaccines, but there are studies of other vaccines where no significant differences could be observed between alum adjuvanted and non-adjuvanted groups (117). Although data collected using peptide 97 as an adjuvant for H5N1 influenza vaccines were not as promising as expected, peptide 97 still shows strong potential in alternative diseases and applications such as Hepatitis B vaccines, where high IFNγ stimulation can be beneficial for immunity (118,119).

The 5mer4 rare peptide is quite comparable to other adjuvants currently used. Alum is the oldest and most widely used adjuvant currently in North America. It has

been noted previously that there is low efficacy when used in conjunction with DNA vaccines (66) which we also saw in our studies. This highlights the need of adjuvant development as optimal adjuvanticity varies between different vaccine platforms and antigens. Although 97+HA elicited a high level of IFNγ response, it did not correlate with increased protection. Although IFNγ is known to activate and stimulate a cell-mediated immune response, cytotoxic T-cells should be analysed for functional performance such as cytotoxicity and secretion of additional cytokines.

4.2 The effect of rare peptides on natural killer cells

It has been previously suggested that rare peptides have a stimulatory effect on natural killer cells, and not dendritic cells (55). One major cell type that has not been investigated is macrophages. Macrophages are important players in the innate immune response and are one of the professional APCs. APCs are essential in presenting antigens to T and B cells for activation and expansion which leads to clearance of invading pathogens. Studies have shown the importance of macrophages results in the immune response against various pathogens (120-122). Macrophages undoubtedly play an important role in the development of adaptive immune responses; therefore the effect of rare peptides on these cell types must be established. Bone marrow from mice was incubated with M-CSF into a more branched, macrophage morphology (Figure 10A) appeared. After 6-7 days, peptides 97, 248 and 5mer4 were added. A number of activation markers were examined. CD80 and CD86 are co-stimulation markers that are expressed on multiple cell types, including activated macrophages (123). MHCII (or I-

A/I-E on mice models) are also expressed in higher concentrations on activated macrophages to allow further activation of T-cells (124) and therefore also examined by flow cytometry. Although activation of macrophages can be hard to determine based on cell activation markers, when examining multiple markers and comparing the response to controls, it suggests that macrophages are not activated with the addition of rare peptides (Figure 10). These data suggests that macrophages do not play a major role in the rare peptide induced increased immune responsiveness.

Natural killer cells have been previously suggested to have an increase in immune responsiveness after the addition of rare peptides after 24 hours. CD69 is one of the first activation marker expressed by lymphocytes that have been activated (125). NK cells incubated with peptide 97 show the greatest amount of CD69 expression, but both 5mer4 and 97 elicit an increase in CD69 expression on NK cells in comparison to peptide 248 (common) and to the negative (DMSO) control. There is no statistically significance difference when comparing CD69 expression of 248 peptide and negative control. The peak of peptide activated CD69+ NK cells can also be seen at approximately 4 hours after addition (Figure 11). Although overall high levels of IFNγ could be detected by ELISPOT assays, these levels are generally produced mainly by CD8+ T-cells. The evaluation of the functionality of NK cells stimulated by rare peptides, such as the cytotoxicity and IFNγ secretion of NK cells would shed more insight on the effect of rare peptides on NK cells, and should be added in the future directions of this project.

The addition of 5mer4 to vaccine did not alter the survival rate of mice depleted of NK cells as seen in Figure 12, yet 5mer4 adjuvant activity remained in non-depleted mice, suggesting that NK cells play an important role in 5mer4 adjuvant capacity. One day following depletion, mice were bled by the saphenous vein to determine whether NK cells were in fact depleted by flow cytometry as seen in Figure 12A to show that NK cells were 70-80% depleted in comparison to mice that did not receive anti-asialo-GM1 treatment. One must note that the selectivity of NK cell depletion with asialo-GM1 antibodies is not solely restricted to NK cells as asialo GM1 can be expressed by several different cell types such as myeloid cells, basophils and select T cell subsets. Nonetheless, NK depletion mediated by anti-asialo GM1 treatment still remains a powerful and useful tool to evaluate *in vivo* functions of NK cells (126).

To visualize the peptide after the addition to NK cells, a biotin tag was attached to peptide 97, 5mer4 and peptide 248. This tag was chosen as it is one of the smallest tags available. Although small (244.31g/mol), it is still plausible that the tag may inhibit peptide function. Studies which include the effect of biotinylated peptides to IFNγ secretion or CD69 up-regulation in comparison to non-biotinylated peptides should be conducted to ensure that the tag is not altering the results. Rare and common peptides was added to a culture of enriched NK cells and viewed under two different types of microscopy. The 5mer4 and 248 peptides were added to the enriched NK culture 18 hours, 6 hours and 2 hours prior to fluorescent visualization. All samples were viewed at the same time (Figure 13 A and B). From the confocal and fluorescent microscopy

data, it appears that rare peptides bind more strongly to NK cells in comparison to common peptides (Figure 14 A and B).

A possible mechanism of rare peptide induced increased immunogenicity can only be speculated at this point as further characterization is required. One possibility includes activated NK cells are able to secrete soluble factors such as IFNγ and TNFα which are able to recruit additional immune cells such as DC to the area where the DNA vaccine and cells presenting the vaccine are available. Another possibility is that the rare peptides are able to bind to activating receptors of NK cells, therefore overriding the inhibitory receptors and leading to their activation. Activated NK cells then influence an immune response bias towards a T_H1 response by IFNγ secretion and DC cross talk, which may explain why a strong T-cell mediated IFNγ response can be elicited by rare peptides (127) A potential synergistic effect by combining various adjuvants may stimulate different parts of the immune response, allowing them to orchestrate into increased immune responsiveness.

The variability between different rare 5mers could possibly be due to a number of reasons, such as particular biochemical attributes, or how each 5mer stimulates the immune response. 5mer4 appears to be able to elicit an increased antibody and cellular mediated response, whereas peptide 97 can induce only a robust cellular mediated response. In this thesis, we see no increase in survival when mice are vaccinated with peptide 97 and HA DNA vaccine compared to DNA vaccine alone. This could be due to the fact that mice vaccinated with peptide 97 and HA DNA vaccine elicited low HI

antibody titres. An HI titre of only 1:30 was achieved with peptide 97, whereas a titre of 1:40 is considered to be seroprotective of 50% of animals against influenza (128,129). This concept can be contrasted to mice vaccinated with 5mer4 and HA DNA vaccine, where a titre of 1:100 was measured with over 60% survival against influenza challenge. Since peptide 97 cannot elicit a protective antibody response, there is a strong possibility that this peptide is stimulating the "wrong" arm of the immune response against influenza – a cell-mediated response rather than an antibody response. For example, in the 1960's, a vaccine developed for infants against respiratory syncytial virus (RSV) was tested and found to develop a strong antibody response. Unfortunately, the high antibody titres were non-protective whereas natural infections of RSV were cleared by a cell-mediated response. Vaccinated children provided no protection against RSV, and control groups (vaccinated with parainfluenza, or unvaccinated children) experienced milder cases of RSV and provided low levels of antibody titres against RSV. Many researchers hypothesize that the vaccine skewed the immune response towards a non-protective antibody response against RSV (130). Peptide 97 is still a strong candidate as an adjuvant but the antigen and disease model should be examined carefully as the proper immune response needs to be developed for protection.

4.3 Rare peptides as novel adjuvants

An ideal adjuvant should induce a stronger immune response, show vaccine compatibility, safe, stable and cost-effective (79). Select rare peptides may be able to

address all these points. The advantages of using rare short peptides as an adjuvant are listed in the Table 4.

Table 4: Advantages and disadvantages of rare short peptide based adjuvants

Advantages	Disadvantages
Select peptide based on desired immune response (humoral and/or cellular immune responses)	Preliminary animal model only
No visible side effects	Formal toxicity profiling required
Cost-efficient production	Partial mechanism of action elucidated
Ease in transportation and storage	
Ease in administration	
Low cross-reactivity	
No additional instruments required	
No new techniques for administration	

Rare short peptides have a large potential in customization of adjuvants. Rare peptide 5mer4 has been shown to adequately stimulate a higher humoral and cellular immune response. Peptide 97, although survival against influenza challenge could not be obtained, can be used in formats where a high levels of NK activation or high levels of cell-mediated responses are required. These two rare short peptides have been studied the most extensively, therefore the opportunity of other beneficial rare peptides need to be evaluated. Investigation and evaluation of these rare peptides as adjuvants are worthwhile as these peptides can quickly be mass synthesized in a fashion that is relatively inexpensive because of their short length. This reduces the cost per vaccine and allows more vaccine doses to be available for use due to dose sparing, which is

especially crucial during an epidemic or pandemic situation. The dose sparing effect allows vaccine doses to be synthesized quicker (less vaccine is required) as well as cost-efficient. Short five amino acid long peptides can be produced at a large scale production quite easily and cost efficiently. Peptides can be added to vaccines for as low as eight cents per human dose making their use affordable for large vaccination campaigns. Lyophilisation of peptides allows for stable transport and storage at non ideal temperatures without losing potency. Administration of the vaccine with peptide is no different than vaccines without peptide adjuvant; there are no additional instruments to purchase or training required.

A current disadvantage of rare peptide adjuvants is the lack of knowledge regarding efficacy with various vaccine platforms, sustainability of immune potentiation, toxicity/safety profile, efficacy in larger animal species (e.g. humans), among others, overall all relating to incomplete investigation and development. This is a novel and interesting path to follow in regards to adjuvant-mediated immune potentiation as it could also lead to the development of other adjuvants including some based on smaller and cheaper molecules. At the minimum, understanding the mechanistic action can shed light on optimizing rare short peptides for not only adjuvant use, but for alternative therapies and functions as well.

5.0 Summary and Future Directions

Taken together, these data show that rare peptides are able to stimulate higher IFNγ responses (Figure 5B) and increase survival rate (Figure 6A). Select rare peptides, such as 5mer4 show better efficacy compared to commercially available adjuvants (Figure 9B). Although peptide 97 did not enhance survival against influenza challenge (Figure 8B), it can stimulate high levels of IFNγ (Figure 7) which has potential to be applied to diseases where high IFNγ stimulation can be beneficial for immunity. The data suggests rare peptides are able to bind NK cells more effectively than common peptides (Figure 13 and 14). Once bound, rare peptides activate NK cells as observed by an increased expression of CD69 (Figure 11). Macrophages appear to be unaffected by the addition of rare or common peptides as surface expression levels of CD80, CD86 and I-A/I-E remained at similar levels of control macrophages (Figure 10). Mice vaccinated with 5mer4 + HA were able to survive influenza challenge but once depleted with NK cells, 5mer4 adjuvant activity could no longer be achieved as seen by decreased survival (Figure 12).

Further characterisation of peptide stimulated NK cells needs to be established, such as cytokine release and killing assays. Gene chip profiling would also be beneficial in determining pathways and receptors activated by rare peptides. Further characterisation is required to allow rare peptides to reach its full potential in multiple therapeutic areas.

Natural killer cells are the forefront of the immune response. Since NK cells do not require prior exposure to lyse viral infected cells, they are able to control the viral load while signalling for the adaptive immune response to develop. NK cells are important players in the immune response and there is great potential for therapeutic use. Rare short peptides are able to stimulate an increased immune response allowing an immense potential in multiple fields such as vaccine adjuvants. Their inclusion into currently licensed vaccines should be considered for rapid clinical development of improved immunisation strategies. Evidence of common peptides with immunosuppressing activity has been described (55) and this effect should be considered in areas such as allergy, transplantation and autoimmunity.

Although these are preliminary findings, the possibilities of rare peptides as an NK cell activator can be extrapolated to areas where NK activation desirable such as anti-tumour therapies. Overall, the evaluation of peptide frequency demonstrates a strong potential for the development of new adjuvants and immunomodulators.

6.0 References

(1) Egesten A, Schmidt A, Herwald H. Trends in Innate Immunity. : Karger; 2008.

(2) Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010 Mar 19;140(6):805-820.

(3) Call ME. Antigen presenting cells. UpToDate 2013.

(4) Bystry RS, Aluvihare V, Welch KA, Kallikourdis M, Betz AG. B cells and professional APCs recruit regulatory T cells via CCL4. Nat Immunol 2001 Dec;2(12):1126-1132.

(5) Bonilla FA. The adaptive cellular immune response. UpToDate 2012.

(6) Bonilla FA. The humoral immune response. UpToDate 2012.

(7) Ojo E, Wigzell H. Natural killer cells may be the only cells in normal mouse lymphoid cell populations endowed with cytolytic ability for antibody-coated tumour target cells. Scand J Immunol 1978 Apr;7(4):297-306.

(8) Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. Science 2011 Jan 7;331(6013):44-49.

(9) Brandstadter JD, Yang Y. Natural killer cell responses to viral infection. J Innate Immun 2011;3(3):274-279.

(10) Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med 1989 Jun 29;320(26):1731-1735.

(11) Yokoyama WM. Natural killer cell receptors. Curr Opin Immunol 1998 Jun;10(3):298-305.

(12) Caligiuri MA. Human natural killer cells. Blood 2008 Aug 1;112(3):461-469.

(13) Seaman WE. Natural killer cells and natural killer T cells. Arthritis Rheum 2000 Jun;43(6):1204-1217.

(14) Hall LJ, Clare S, Dougan G. NK cells influence both innate and adaptive immune responses after mucosal immunization with antigen and mucosal adjuvant. J Immunol 2010 Apr 15;184(8):4327-4337.

(15) Andoniou CE, Coudert JD, Degli-Esposti MA. Killers and beyond: NK-cell-mediated control of immune responses. Eur J Immunol 2008 Nov;38(11):2938-2942.

(16) Bots M, Medema JP. Granzymes at a glance. J Cell Sci 2006 Dec 15;119(Pt 24):5011-5014.

(17) Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002 Oct;2(10):735-747.

(18) Portales P, Reynes J, Pinet V, Rouzier-Panis R, Baillat V, Clot J, et al. Interferon-alpha restores HIV-induced alteration of natural killer cell perforin expression in vivo. AIDS 2003 Mar 7;17(4):495-504.

(19) Arase H, Arase N, Saito T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. J Exp Med 1995 Mar 1;181(3):1235-1238.

(20) Chen CC, Young JL, Monzon RI, Chen N, Todorovic V, Lau LF. Cytotoxicity of TNFalpha is regulated by integrin-mediated matrix signaling. EMBO J 2007 Mar 7;26(5):1257-1267.

(21) Reefman E, Kay JG, Wood SM, Offenhauser C, Brown DL, Roy S, et al. Cytokine secretion is distinct from secretion of cytotoxic granules in NK cells. J Immunol 2010 May 1;184(9):4852-4862.

(22) Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: "l'union fait la force". Blood 2005 Oct 1;106(7):2252-2258.

(23) Su HC, Nguyen KB, Salazar-Mather TP, Ruzek MC, Dalod MY, Biron CA. NK cell functions restrain T cell responses during viral infections. Eur J Immunol 2001 Oct;31(10):3048-3055.

(24) Watzl C, Long EO. Signal transduction during activation and inhibition of natural killer cells. Curr Protoc Immunol 2010 Aug;Chapter 11:Unit 11.9B.

(25) Nowbakht P, Ionescu MC, Rohner A, Kalberer CP, Rossy E, Mori L, et al. Ligands for natural killer cell-activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood 2005 May 1;105(9):3615-3622.

(26) Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008 May;9(5):495-502.

(27) Negishi I, Motoyama N, Nakayama K, Nakayama K, Senju S, Hatakeyama S, et al. Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature 1995 Aug 3;376(6539):435-438.

(28) Colucci F, Schweighoffer E, Tomasello E, Turner M, Ortaldo JR, Vivier E, et al. Natural cytotoxicity uncoupled from the Syk and ZAP-70 intracellular kinases. Nat Immunol 2002 Mar;3(3):288-294.

(29) Ljunggren HG, Karre K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. J Exp Med 1985 Dec 1;162(6):1745-1759.

(30) Garrido F, Ruiz-Cabello F, Cabrera T, Perez-Villar JJ, Lopez-Botet M, Duggan-Keen M, et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today 1997 Feb;18(2):89-95.

(31) Hewitt EW. The MHC class I antigen presentation pathway: strategies for viral immune evasion. Immunology 2003 Oct;110(2):163-169.

(32) Huard B, Fruh K. A role for MHC class I down-regulation in NK cell lysis of herpes virus-infected cells. Eur J Immunol 2000 Feb;30(2):509-515.

(33) Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, et al. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 1999 Apr;5(4):405-411.

(34) Gerosa F, Gobbi A, Zorzi P, Burg S, Briere F, Carra G, et al. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. J Immunol 2005 Jan 15;174(2):727-734.

(35) Rizzello V, Bonaccorsi I, Dongarra ML, Fink LN, Ferlazzo G. Role of natural killer and dendritic cell crosstalk in immunomodulation by commensal bacteria probiotics. J Biomed Biotechnol 2011;2011:473097.

(36) Cooper MA, Fehniger TA, Fuchs A, Colonna M, Caligiuri MA. NK cell and DC interactions. Trends Immunol 2004 Jan;25(1):47-52.

(37) Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med 2002 Feb 4;195(3):343-351.

(38) Vitale M, Della Chiesa M, Carlomagno S, Pende D, Arico M, Moretta L, et al. NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKp30 triggering receptor. Blood 2005 Jul 15;106(2):566-571.

(39) Wehner R, Dietze K, Bachmann M, Schmitz M. The bidirectional crosstalk between human dendritic cells and natural killer cells. J Innate Immun 2011;3(3):258-263.

(40) Wehner R, Lobel B, Bornhauser M, Schakel K, Cartellieri M, Bachmann M, et al. Reciprocal activating interaction between 6-sulfo LacNAc+ dendritic cells and NK cells. Int J Cancer 2009 Jan 15;124(2):358-366.

(41) Della Chiesa M, Vitale M, Carlomagno S, Ferlazzo G, Moretta L, Moretta A. The natural killer cell-mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors. Eur J Immunol 2003 Jun;33(6):1657-1666.

(42) Hamerman JA, Ogasawara K, Lanier LL. NK cells in innate immunity. Curr Opin Immunol 2005 Feb;17(1):29-35.

(43) Ornstein BW, Hill EB, Geurs TL, French AR. Natural killer cell functional defects in pediatric patients with severe and recurrent herpesvirus infections. J Infect Dis 2013 Feb 1;207(3):458-468.

(44) Orange JS. NK cell deficiency syndromes: Clinical manifestations and diagnosis. UpToDate 2012.

(45) Orange JS. Human natural killer cell deficiencies and susceptibility to infection. Microbes Infect 2002 Dec;4(15):1545-1558.

(46) Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999;17:189-220.

(47) Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. Curr Opin Immunol 2006 Aug;18(4):391-398.

(48) Cooper MA, Colonna M, Yokoyama WM. Hidden talents of natural killers: NK cells in innate and adaptive immunity. EMBO Rep 2009 Oct;10(10):1103-1110.

(49) O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat Immunol 2006 May;7(5):507-516.

(50) Paust S, Gill HS, Wang BZ, Flynn MP, Moseman EA, Senman B, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. Nat Immunol 2010 Dec;11(12):1127-1135.

(51) World Health Organization. Influenza. 2003; Available at: http://www.who.int/mediacentre/factsheets/2003/fs211/en/. Accessed August 7, 2013.

(52) Lamb RA, Krug RM. Chapter 46. In: Fields BN, Knipe DM, Howley PM, editors. Fields Virology. 4th ed.; 2001.

(53) Subbarao K, Joseph T. Scientific barriers to developing vaccines against avian influenza viruses. Nat Rev Immunol 2007 Apr;7(4):267-278.

(54) Matsuoka Y, Lamirande EW, Subbarao K. The mouse model for influenza. Curr Protoc Microbiol 2009 May;Chapter 15:Unit 15G.3.

(55) Patel A, Dong JC, Trost B, Richardson JS, Tohme S, Babiuk S, et al. Pentamers not found in the universal proteome can enhance antigen specific immune responses and adjuvant vaccines. PLoS One 2012;7(8):e43802.

(56) Patel A, Kobinger G. Evaluation of mismatched immunity against influenza viruses**.** Future Virology 2012;7(11):1065.

(57) Oyston P, Robinson K. The current challenges for vaccine development. J Med Microbiol 2012 Jul;61(Pt 7):889-894.

(58) Badgett MR, Auer A, Carmichael LE, Parrish CR, Bull JJ. Evolutionary dynamics of viral attenuation. J Virol 2002 Oct;76(20):10524-10529.

(59) Janeway CA, Travers P, Walport M, Shlomchik M. Immunobiology. Fifth ed.: Garland Science; 2001.

(60) Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK, Wannemuehler MJ, Narasimhan B. Vaccine adjuvants: current challenges and future approaches. J Pharm Sci 2009 Apr;98(4):1278-1316.

(61) Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol 2004 Oct;82(5):488-496.

(62) Osterholm MT, Kelley NS, Sommer A, Belongia EA. Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. Lancet Infect Dis 2012 Jan;12(1):36-44.

(63) Foged C. Subunit vaccines of the future: the need for safe, customized and optimized particulate delivery systems. Ther Deliv 2011 Aug;2(8):1057-1077.

(64) Abdulhaqq SA, Weiner DB. DNA vaccines: developing new strategies to enhance immune responses. Immunol Res 2008;42(1-3):219-232.

(65) Robertson JS, Griffiths E. Assuring the quality, safety, and efficacy of DNA vaccines. Methods Mol Med 2006;127:363-374.

(66) Saade F, Petrovsky N. Technologies for enhanced efficacy of DNA vaccines. Expert Rev Vaccines 2012 Feb;11(2):189-209.

(67) Schalk JA, Mooi FR, Berbers GA, van Aerts LA, Ovelgonne H, Kimman TG. Preclinical and clinical safety studies on DNA vaccines. Hum Vaccin 2006 Mar-Apr;2(2):45-53.

(68) Henke A. DNA immunization--a new chance in vaccine research? Med Microbiol Immunol 2002 Dec;191(3-4):187-190.

(69) Lu S. Immunogenicity of DNA vaccines in humans: it takes two to tango. Hum Vaccin 2008 Nov-Dec;4(6):449-452.

(70) MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, Bagarazzi ML, et al. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. J Infect Dis 1998 Jul;178(1):92-100.

(71) Liu MA. DNA vaccines: an historical perspective and view to the future. Immunol Rev 2011 Jan;239(1):62-84.

(72) Ferraro B, Morrow MP, Hutnick NA, Shin TH, Lucke CE, Weiner DB. Clinical applications of DNA vaccines: current progress. Clin Infect Dis 2011 Aug 1;53(3):296-302.

(73) Coban C, Kobiyama K, Aoshi T, Takeshita F, Horii T, Akira S, et al. Novel strategies to improve DNA vaccine immunogenicity. Curr Gene Ther 2011 Dec;11(6):479-484.

(74) Glenny AT, Barr M. The precipitation of diphtheria toxoid by potash alum. Journal of Pathology and Bacteriology 1931;34(2):131.

(75) Glenny AT, Pope CG, Waddington H, Wallace U. Immunological Notes: XVII–XXIV. Journal of Pathology and Bacteriology 1926;29(1):31-40.

(76) Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol 2009 Apr;9(4):287-293.

(77) Schijns VE, Lavelle EC. Trends in vaccine adjuvants. Expert Rev Vaccines 2011 Apr;10(4):539-550.

(78) Chen J, Ni G, Liu XS. Papillomavirus virus like particle-based therapeutic vaccine against human papillomavirus infection related diseases: immunological problems and future directions. Cell Immunol 2011;269(1):5-9.

(79) Nicholls EF, Madera L, Hancock RE. Immunomodulators as adjuvants for vaccines and antimicrobial therapy. Ann N Y Acad Sci 2010 Dec;1213:46-61.

(80) Kool M, Fierens K, Lambrecht BN. Alum adjuvant: some of the tricks of the oldest adjuvant. J Med Microbiol 2012 Jul;61(Pt 7):927-934.

(81) O'Hagan DT, Rappuoli R, De Gregorio E, Tsai T, Del Giudice G. MF59 adjuvant: the best insurance against influenza strain diversity. Expert Rev Vaccines 2011 Apr;10(4):447-462.

(82) Lambrecht BN, Kool M, Willart MA, Hammad H. Mechanism of action of clinically approved adjuvants. Curr Opin Immunol 2009 Feb;21(1):23-29.

(83) McKee AS, Munks MW, Marrack P. How do adjuvants work? Important considerations for new generation adjuvants. Immunity 2007 Nov;27(5):687-690.

(84) Tritto E, Mosca F, De Gregorio E. Mechanism of action of licensed vaccine adjuvants. Vaccine 2009 May 26;27(25-26):3331-3334.

(85) Dupuis M, Murphy TJ, Higgins D, Ugozzoli M, van Nest G, Ott G, et al. Dendritic cells internalize vaccine adjuvant after intramuscular injection. Cell Immunol 1998 May 25;186(1):18-27.

(86) Klinman DM. Adjuvant activity of CpG oligodeoxynucleotides. Int Rev Immunol 2006 May-Aug;25(3-4):135-154.

(87) Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK. Adjuvants--a balance between toxicity and adjuvanticity. Vaccine 1993;11(3):293-306.

(88) Hancock RE, Nijnik A, Philpott DJ. Modulating immunity as a therapy for bacterial infections. Nat Rev Microbiol 2012 Mar 16;10(4):243-254.

(89) U.S. Food and Drug Administration. **Common Ingredients in U.S. Licensed Vaccines**. 2007; Available at: http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/VaccineSafety/ucm187810.htm. Accessed 10/18, 2012.

(90) Garcon N, Van Mechelen M. Recent clinical experience with vaccines using MPL- and QS-21-containing adjuvant systems. Expert Rev Vaccines 2011 Apr;10(4):471-486.

(91) Rothenfusser S, Tuma E, Wagner M, Endres S, Hartmann G. Recent advances in immunostimulatory CpG oligonucleotides. Curr Opin Mol Ther 2003 Apr;5(2):98-106.

(92) Rappuoli R, Mandl CW, Black S, De Gregorio E. Vaccines for the twenty-first century society. Nat Rev Immunol 2011 Nov 4;11(12):865-872.

(93) Caskey M, Lefebvre F, Filali-Mouhim A, Cameron MJ, Goulet JP, Haddad EK, et al. Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. J Exp Med 2011 Nov 21;208(12):2357-2366.

(94) Sorensen OE, Borregaard N, Cole AM. Antimicrobial Peptides in Innate Immune Responses. Trends in Innate Immunity 2008;15:61-77.

(95) Sahl HG, Pag U, Bonness S, Wagner S, Antcheva N, Tossi A. Mammalian defensins: structures and mechanism of antibiotic activity. J Leukoc Biol 2005 Apr;77(4):466-475.

(96) Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, et al. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. J Biol Chem 1997 Jun 13;272(24):15258-15263.

(97) Bowdish DM, Davidson DJ, Lau YE, Lee K, Scott MG, Hancock RE. Impact of LL-37 on anti-infective immunity. J Leukoc Biol 2005 Apr;77(4):451-459.

(98) Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, et al. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood 2001 Jun 15;97(12):3951-3959.

(99) Nell MJ, Tjabringa GS, Vonk MJ, Hiemstra PS, Grote JJ. Bacterial products increase expression of the human cathelicidin hCAP-18/LL-37 in cultured human sinus epithelial cells. FEMS Immunol Med Microbiol 2004 Oct 1;42(2):225-231.

(100) Saenz R, Souza Cda S, Huang CT, Larsson M, Esener S, Messmer D. HMGB1-derived peptide acts as adjuvant inducing immune responses to peptide and protein antigen. Vaccine 2010 Nov 3;28(47):7556-7562.

(101) Schellack C, Prinz K, Egyed A, Fritz JH, Wittmann B, Ginzler M, et al. IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses. Vaccine 2006 Jun 29;24(26):5461-5472.

(102) Kanduc D. Epitopic peptides with low similarity to the host proteome: towards biological therapies without side effects. Expert Opin Biol Ther 2009 Jan;9(1):45-53.

(103) Kanduc D, Novello G, Mazzanti R. P-170 peptides with low similarity to the human proteome: tracing an effective and safe biological way towards effective and safe cancer chemotherapy. J Exp Ther Oncol 2009;8(2):151-155.

(104) Claverie JM, Kourilsky P, Langlade-Demoyen P, Chalufour-Prochnicka A, Dadaglio G, Tekaia F, et al. T-immunogenic peptides are constituted of rare sequence

patterns. Use in the identification of T epitopes in the human immunodeficiency virus gag protein. Eur J Immunol 1988 Oct;18(10):1547-1553.

(105) Stufano A, Capone G, Pesetti B, Polimeno L, Kanduc D. Clustering of rare peptide segments in the HCV immunome. Self Nonself 2010 Apr;1(2):154-162.

(106) Shanmugam A, Rajoria S, George AL, Mittelman A, Suriano R, Tiwari RK. Synthetic Toll like receptor-4 (TLR-4) agonist peptides as a novel class of adjuvants. PLoS One 2012;7(2):e30839.

(107) Seya T, Akazawa T, Tsujita T, Matsumoto M. Role of Toll-like receptors in adjuvant-augmented immune therapies. Evid Based Complement Alternat Med 2006 Mar;3(1):31-8; discussion 133-7.

(108) Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nat Med 1999 Nov;5(11):1249-1255.

(109) Patel A, Tran K, Gray M, Li Y, Ao Z, Yao X, et al. Evaluation of conserved and variable influenza antigens for immunization against different isolates of H5N1 viruses. Vaccine 2009 May 18;27(23):3083-3089.

(110) Weischenfeldt J, Porse B. Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. CSH Protoc 2008 Dec 1;2008:pdb.prot5080.

(111) Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. Curr Protoc Immunol 2008 Nov;Chapter 14:Unit 14.1.

(112) Kanduc D. Pentapeptides as minimal functional units in cell biology and immunology. Curr Protein Pept Sci 2013 Mar 1;14(2):111-120.

(113) Tuller T, Chor B, Nelson N. Forbidden penta-peptides. Protein Sci 2007 Oct;16(10):2251-2259.

(114) Ohmit SE, Petrie JG, Cross RT, Johnson E, Monto AS. Influenza hemagglutination-inhibition antibody titer as a correlate of vaccine-induced protection. J Infect Dis 2011 Dec 15;204(12):1879-1885.

(115) Fernandez-Botran R, Sanders VM, Mosmann TR, Vitetta ES. Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. J Exp Med 1988 Aug 1;168(2):543-558.

(116) Noble A, Staynov DZ, Kemeny DM. Generation of rat Th2-like cells in vitro is interleukin-4-dependent and inhibited by interferon-gamma. Immunology 1993 Aug;79(4):562-567.

(117) Atmar RL, Keitel WA. Adjuvants for pandemic influenza vaccines. Curr Top Microbiol Immunol 2009;333:323-344.

(118) McCall MB, Hopman J, Daou M, Maiga B, Dara V, Ploemen I, et al. Early interferon-gamma response against Plasmodium falciparum correlates with interethnic differences in susceptibility to parasitemia between sympatric Fulani and Dogon in Mali. J Infect Dis 2010 Jan 1;201(1):142-152.

(119) D'Ombrain MC, Robinson LJ, Stanisic DI, Taraika J, Bernard N, Michon P, et al. Association of early interferon-gamma production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children. Clin Infect Dis 2008 Dec 1;47(11):1380-1387.

(120) Gordon SB, Read RC. Macrophage defences against respiratory tract infections. Br Med Bull 2002;61:45-61.

(121) Segal BH. Role of macrophages in host defense against aspergillosis and strategies for immune augmentation. Oncologist 2007;12 Suppl 2:7-13.

(122) Yang Z, Grinchuk V, Urban JF,Jr, Bohl J, Sun R, Notari L, et al. Macrophages as IL-25/IL-33-responsive cells play an important role in the induction of type 2 immunity. PLoS One 2013;8(3):e59441.

(123) Wahlstrom J, Berlin M, Skold CM, Wigzell H, Eklund A, Grunewald J. Phenotypic analysis of lymphocytes and monocytes/macrophages in peripheral blood and bronchoalveolar lavage fluid from patients with pulmonary sarcoidosis. Thorax 1999 Apr;54(4):339-346.

(124) Girard M, Israel-Assayag E, Cormier Y. Mature CD11c(+) cells are enhanced in hypersensitivity pneumonitis. Eur Respir J 2009 Sep;34(3):749-756.

(125) Werfel T, Boeker M, Kapp A. Rapid expression of the CD69 antigen on T cells and natural killer cells upon antigenic stimulation of peripheral blood mononuclear cell suspensions. Allergy 1997 Apr;52(4):465-469.

(126) Nishikado H, Mukai K, Kawano Y, Minegishi Y, Karasuyama H. NK cell-depleting anti-asialo GM1 antibody exhibits a lethal off-target effect on basophils in vivo. J Immunol 2011 May 15;186(10):5766-5771.

(127) Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol 2004 Dec;5(12):1260-1265.

(128) Lin HT, Chuang CC, Wu HL, Chu DM, Wang YC. Characterization of cross protection of Swine-Origin Influenza Virus (S-OIV) H1N1 and reassortant H5N1

influenza vaccine in BALB/c mice given a single-dose vaccination. J Biomed Sci 2013 Mar 21;20:19-0127-20-19.

(129) Qiu C, Huang Y, Wang Q, Tian D, Zhang W, Hu Y, et al. Boosting heterosubtypic neutralization antibodies in recipients of 2009 pandemic H1N1 influenza vaccine. Clin Infect Dis 2012 Jan 1;54(1):17-24.

(130) Blanco JC, Boukhvalova MS, Shirey KA, Prince GA, Vogel SN. New insights for development of a safe and protective RSV vaccine. Hum Vaccin 2010 Jun;6(6):482-492.