# Evaluating the Immunogenic Potential of Synthetic Influenza T-B & B-T Peptides

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **AIDS** | Acquired immune deficiency syndrome |
| **ALP** | Alkaline phosphatase |
| **AM** | Alveolar macrophage |
| **APC** | Antigen presenting cell |
| **BLAST** | Basic local alignment search tool |
| **CCL5** | Chemokine (C-C motif) ligand 5 |
| **CPT** | Cell preparation tube |
| **CTL** | Cytotoxic T lymphocyte |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **ELISpot** | Enzyme-linked immunosorbent spot |
| **FBS** | Fetal bovine serum |
| **FMOC** | Fluorenylmethoxycarbonyl |
| **HA** | Hemagglutinin |
| **HIV** | Human immunodeficiency virus |
| **HLA** | Human leukocyte antigen |
| **HPLC** | High-performance liquid chromatography |
| **HRP** | Horseradish peroxidise |
| **IFN** | Interferon |
| **IgA** | Immunoglobulin A |
| **IgG** | Immunoglobulin G |
| **IgM** | Immunoglobulin M |
| **IL** | Interleukin |
| **IMDM** | Iscove's modified Dulbecco's medium |
| **LAIV** | Live attenuated influenza vaccine |
| **LANL ISD** | Los Alamos national laboratory influenza sequence database |
| **mAb** | Monoclonal antibody |
| **MAP** | Multiple antigen peptide |
| **MCP** | Monocyte chemoattractant protein |
| **MDCK** | Madin-Darby canine kidney |
| **MEM** | Minimum essential medium |
| **MHC** | Major histocompatibility complex |
| **MIP** | Macrophage inflammatory protein |
| **MPL** | Monophosphoryl lipid A |
| **M1** | Matrix protein 1 |
| **M2** | Matrix protein 2 |
| **NA** | Neuraminidase |
| **NK cell** | Natural killer cell |
| **NP** | Nucleoprotein |
| **NS1** | Non-structural protein 1 |
| **NS2** | Non-structural protein 2 |
| **OD** | Optical density |
| **PA** | Polymerase protein A |
| **PBMC** | Peripheral blood mononuclear cell |
| **PBS** | Phosphate buffered saline |
| **PB1** | Polymerase protein B1 |
| **PB2** | Polymerase protein B2 |
| **PFU** | Plaque-forming unit |
| **PMA** | Phorbol 12-myristate 13-acetate |
| **RCF** | Relative centrifugal force |
| **rHA** | Recombinant hemagglutinin |
| **RNP** | Ribonucleotide-NP |
| **RPMI** | Roswell park memorial institute |
| **SEM** | Standard error of the mean |
| **SFC** | Spot forming cell count |
| **SI** | Stimulation index |
| **TIV** | Trivalent inactivated influenza vaccine |
| **TLR** | Toll-like receptor |
| **TNF** | Tumor necrosis factor |
| **TRIF** | TIR-domain-containing adapter-inducing interferon-β |

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# 1. INTRODUCTION

## 1.1 Molecular biology of influenza

Influenza A virus is a negative-stranded segmented RNA virus belonging to the family *Orthomyxoviridae*. The viral particle is surrounded by a host-cell derived lipid bilayer envelope, which serves as an anchor to the interior matrix protein (M1), as well as to the external proteins hemagglutinin (HA), neuraminidase (NA) and ion-channel matrix protein 2 (M2) (162). The viral genome is made up of eight pieces of RNA. The genetic information codes for 10 distinct proteins: the nucleoprotein (NP), non-structural proteins 1 and 2 (NS1 and NS2), polymerase proteins A, B1 and B2 (PA, PB1 and PB2), as well as the four aforementioned envelope proteins M1, HA, NA and M2 (38). Each strand of RNA is encapsidated by a NP unit, thereby making up the ribonucleotide-NP (RNP) complex (53).

The two external proteins HA and NA are used to classify the influenza A virus into different subtypes (65). NA is a 240 kDa type 2 membrane protein attached to the viral envelope by its N-terminal end (53). Its function is to cleave the glycosidic linkages in sialic acid, thereby aiding in viral entry and release, and as of date there are 9 known types. On the other hand, there are 16 different HAs (so named because they may cause red blood cells to clump together or hemagglutinate) (67, 142). Structurally, the HA protein is made up of three identical subunits (156, 171), which altogether form a 220 kDa type 1 membrane protein that is attached to the viral envelope via its C-terminal end (53). Each HA monomer is made up of two domains; a globular portion and a stem-like anchor (43). Amino acid sequence analysis has shown that there is anywhere from 40-80% sequence overlap between subtypes (131). The HA subtypes have been further divided into five clades, depending on how the globular head of the protein is oriented in terms to its central trimeric coiled-coil (6, 78, 145). It should be noted that the HAs of

influenza viruses that circulate among human hosts tend to bind sialic acid in α2,6-linkages, in a cis conformation relative to the glycosidic oxygen (59, 77). On the other hand, the HAs of avian influenza viruses preferentially bind α2,3-linked sialic acid receptors, where the glycosidic oxygen of the α2,3 linkage is found directed towards the base of the site in a trans configuration (111, 141).

### 1.2 Influenza infection

Influenza virus is a leading cause of disease that affects people worldwide, with anywhere from 10 to 20% of the global population being infected each year (51). According to estimates by the World Health Organization, the virus kills up to 250,000-500,000 persons per season (3). In the Southern hemisphere influenza tends to circulate from May to September, while in the Northern Hemisphere epidemics usually occur in the fall and winter, but can extend as late as April and May (53). Most people are able to clear the virus without any major complications, and simply experience varied symptoms including chills, fever, aches and pains throughout the body, fatigue, sore throat, nasal congestion, as well as possible gastrointestinal symptoms such as vomiting, abdominal pain and diarrhea (38). Following an incubation period of up to 5 days (during which the first 3 days make up the period of highest potential for spread (29)), infection in healthy adults tends to resolve within two weeks without requiring hospitalization (53). Even so, influenza is responsible for increased outpatient medical visits and worker absenteeism in healthy 19–49 year-old patients (117), while an American study estimated that the virus caused approximately 226,000 hospitalizations per year during 1979–2001 and approximately 36,000 deaths per year during 1990–1999 (164, 165). Certain groups are even more at risk for influenza-related hospitalization and mortality; children under the age of 5,

persons over the age of 65 (19, 95, 116, 119, 125, 130, 134) and patients with chronic medical conditions, especially those of an immunocompromised nature (108). One group approximated that the mean age of persons who die from seasonal influenza is 76 years (170), while another investigation estimated 64–70 deaths per 100,000 seniors over the age of 65 (compared with 0.9–1.0 deaths per 100,000 persons aged 25–54 years) (106). Likewise, influenza has been found to cause prolonged and more severe infections in patients with human immunodeficiency virus (HIV) infection (52, 140, 146). Furthermore, Lin's study also found the risk for influenza-related death increased drastically to 94–146 deaths per 100,000 persons among patients with acquired immune deficiency syndrome (AIDS) (106).

The statistics presented above change drastically during pandemics, which occur when a new strain emerges to which the global population has no immunity (22, 41, 172). As previously stated, the HA proteins of avian influenza preferentially bind sialic acid linked to galactose via an α2,3-linkage. Since the human respiratory tract contains predominantly α2,6-linked sialic acid, these viruses were initially thought to be incapable of infecting human hosts (153). Indeed, influenza viruses that circulate among humans are generally restricted to three HA types: H1, H2, and H3 (100). However, key mutations in the receptor binding site have been found to allow viruses carrying HAs with α2,3-tropism to infect humans. These pandemic viruses can be human influenza strains that reassort with avian strains (as occurred during the 1957 A/H2N2 pandemic and the 1968 A/H3N2 pandemic), or completely avian in nature (as was seen during the 1918 A/H1N1 pandemic) (38). More recently, human infection with highly pathogenic H5N1 avian influenza viruses has sporadically been seen in Vietnam, Thailand, Indonesia and Cambodia. The latest pandemic influenza outbreak began in April of 2009, when a novel H1N1 strain caused respiratory illness throughout the world, and led to an estimated 18, 500 deaths by the time the

World Health Organization had declared the pandemic over in August of 2010 (99). The HA of this pandemic strain was found to be most closely related to the HA found in the influenza viruses that generally circulate among pigs, and hence the virus was termed "swine flu" (133). The gene encoding this particular HA protein had apparently evolved from the all-avian influenza H1N1 virus responsible for the 1918 pandemic (71), which caused an estimated 20–50 million deaths between 1918 and 1919, making it one of the most serious disease outbreaks in recorded history (82). As an interesting result of this, both hospitalization and mortality rates among seniors older than 65 years was below the rates usually observed in this group during seasonal epidemics; older persons had likely been exposed to similar influenza A viruses that circulated in the early to mid 20$^{th}$ century and still benefitted from partial or full immunity, lowering their risk for infection (44).

Highly pathogenic influenza viruses circulating during pandemics have been characterized as causing viral pneumonia, extensive pulmonary edema and acute respiratory distress syndrome, due to massive lung infiltration of mononuclear cells resulting in alveolar hemorrhage (85). In some patients, lymphopenia and multiple organ failure takes place (57). These complications are due to a phenomenon called "cytokine storm", whereby the same immunological factors that are critical for the efficient elimination of virus are produced in dysregulated and such exaggerated quantities that they instead cause extensive tissue damage (129, 163). The initial targets of influenza virus, whether a pandemic strain or not, are respiratory epithelial cells. The virus produced in these cells is released and goes on to infect alveolar macrophages (AMs) (25). Infected epithelial cells die by necrosis, while AMs predominantly undergo apoptotic death (63). Cell necrosis/apoptosis activates the production of a myriad of cytokines and chemokines: tumor necrosis factor (TNF)-α and interleukin (IL)-1, and monocyte

chemoattractant protein (MCP)-1, chemokine (C-C motif) ligand 5 (CCL5), macrophage inflammatory protein (MIP)-1α/β and IL-8 (89, 90, 158). This release of inflammatory factors by infected lung epithelium and AMs triggers the recruitment of macrophages, neutrophils and T-cells from the peripheral blood across the endo-epithelial barrier and into the lung tissue within the first 3 days post-infection (2). The movement of these cells is a distinguishing trait of the initial adaptive immune response (55, 84). Viral replication is still taking place in the epithelial cells and therefore newly-released virus can spread to the recruited cells (68, 84). However, these cells make minimal contribution to virus spread. Synthesis of viral proteins is interrupted before the first replication cycle because the infected cells die by apoptosis within 24–48 hours post-infection (63, 84). These apoptotic events cause further production of inflammatory and chemotactic cytokines, including TNF-α, IL-1β, IL-6 and interferon (IFN)-α/β (63, 74, 84, 94, 128). T-cells also produce IFN-γ, which inhibits viral replication, promotes T-cell proliferation, stimulates cytotoxic T lymphocyte (CTL) mediated killing, increases major histocompatibility complex (MHC) I expression, and activates macrophages and neutrophils, (107, 120, 166, 168, 177). With respect to the latter function, it should be pointed out that the HA protein has been shown to suppress transcription of the IL12p35 subunit, resulting in the down-regulation of neutrophil function (132). Meanwhile, the double-stranded RNA produced during the replicative process of the virus is recognized by Toll-like receptor (TLR)-3, and causes a signal through the PI3K/Akt apoptotic pathway and the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway, ultimately leading to the activation of INF-β and in turn the IFN-inducible genes coding for IL-6, IL-8 and CCL5 (101, 105, 115).

There is a lack of human data on cellular responses, but various studies have shown that CTLs make up a significant portion of the inflammatory cell infiltrate early after infection as

well as coinciding with clearance of illness (66), suggesting that the CTL response is important for clearance of virus and recovery from infection (23, 66, 83). Although its mechanism of action is not completely clear, it seems that CTLs, along with influenza-specific antibodies and complement, mediate the clearance of influenza-infected cells via cytolysis (112, 113, 139, 144). It is also known from work with mouse models that influenza infection induces a strong CD4 T-helper response. These cells produce cytokines that help regulate the immune response (121). In response to influenza infection, CD4 T-helper cells have been found to secrete IL-2, IL-10 and IFN-γ (42, 148), and this plays an important role in further augmenting the CTL response and stimulating antibody production against the virus (34, 173).

In terms of the humoral immune response to influenza, it is known that both mucosal and systemic immunity are important for resistance to infection and disease (48-50, 127). However, the main portal of entry of the virus is via the mucosal tissues of the respiratory tract, and thus the mucosal immune system serves as the first line of adaptive defence. Immunoglobulin A (IgA) and immunoglobulin M (IgM) play a major role in resistance to infection in the upper respiratory tract (48-50), while serum immunoglobulin G (IgG) is involved in protection of the lower respiratory tract (12). Levels of IgA and IgM peak 2 weeks after initial infection, while IgG levels peak at 4 to 6 weeks post-infection.

### 1.3 Available therapeutics

The M2 ion channel inhibitors rimantadine and amantadine, and the NA inhibitors oseltamivir and zanamivir are antiviral medications that are commonly used to alleviate the severity and duration of infection (53). However, these drugs are most effective if used within the first 48 hours following the onset of illness, and more importantly many virus strains have developed resistance to one or more of the antiviral agents (65). Influenza immunization is thus the preferred prophylactic agent against influenza virus, and it is recommended that all patients above the age of 6 months receive a yearly influenza vaccination to prevent infection (65). Depending on the closeness of the match between the circulating influenza strain and those included in the vaccine formulation, protection rates can be as high as 90% among healthy adults and 60% among senior patients. There are two available types of influenza vaccines: trivalent inactivated influenza vaccine (TIV) and live attenuated influenza vaccine (LAIV), with both types of preparations incorporating the three most important circulating strains (an influenza A H3N2 virus, an influenza A H1N1 virus, and an influenza B virus).

TIVs are licensed throughout the world (53), and are produced by growing virus in embryonated hens' eggs. Propagated virus is concentrated and highly purified from the allantoic fluid, and inactivated with either formaldehyde or β-propiolactone (3). Inactivated virus is then either chemically disrupted or its surface glycoproteins are isolated and purified to produce whole, split or subunit vaccine, respectively. It should be noted that whole influenza vaccine is more immunogenic, but is also associated with more frequent side reactions. Increases in serum antibody can be detected 2 to 6 days following administration of TIV (54, 179), and an estimated 90% of patients receiving the vaccine have protective antibody titres within 2 weeks (54). The

antibody response usually peaks between 2 to 3 weeks after immunization (32, 33), and gradually decreases reaching twofold lower concentrations at 6 months (138). The TIV-induced antibody response is dominated by IgG antibodies, but also includes IgM and IgA antibodies at lower levels (60, 61).

While TIVs contain inactivated viruses that cannot cause disease, LAIVs contain live yet attenuated virus that can potentially cause mild influenza-like symptoms. LAIVs (distributed in Canada under the name "FluMist") are not given to patients intramuscularly, but rather intranasally. The viral spray settles in the nasopharynx, more closely resembling a natural infection (1). As TIVs, LAIVs are trivalent and all three viral strains in the vaccine are updated yearly. Only the genes that encode the surface glycoproteins HA and NA are from the targeted wild-type strains, while the genes encoding the six remaining proteins (NP, NS1/NS2, PA, PB1, PB2, and M1/M2) are from an attenuated donor strain. These donor strains are cold-adapted and temperature sensitive, and as such the virus is only able to replicate efficiently at the lower temperatures found in the nasal mucosa and undergoes more limited replication at the warmer temperatures of the lower respiratory tract. In contrast to the TIV-induced antibody response, immunization by LAIV results in serum IgA and IgM levels that peak after 2 weeks and decline after 4 weeks, and IgG levels that peak after 4 to 12 weeks but remain constant for at least 1 year (53). Although no severe side effects have been attributed to LAIVs (24, 30, 58, 114, 160, 167), the vaccine is only approved for use in non-pregnant patients between the ages of 2 and 49 years (65) and its safety or effectiveness has not yet been assessed in patients with underlying medical conditions (65).

The vaccines currently on the market suffer from several weaknesses. Even though circulating neutralizing antibodies to HA and NA are sufficient to protect against influenza infection (37) and many countries have annual influenza immunization programs in place, seasonal influenza outbreaks are still a major cause of disease and death throughout the world (118). Viral replication of influenza involves an RNA-dependent polymerase complex that lacks any proofreading ability (53). As a result, the viral surface glycoproteins HA and NA regularly undergo amino acid changes that often lead to new variant strains that can bypass the host's acquired immunity (26). The effectiveness of influenza vaccination is dependent on the match between the strains included in the vaccine and those circulating in the community; vaccination with TIV is effective in 80% of healthy adults when there is a good match and only 50% when there is a poor strain match (88). The World Health Organization maintains a worldwide surveillance program that collects and sequences circulating influenza viruses. This data is compiled and analyzed biannually to recommend each year's vaccine strains (53). The seasonal influenza vaccine thus has to be reformulated and readministered on a yearly basis as based on these recommendations.

In any season, there are otherwise healthy vaccine recipients who do not generate protective neutralizing antibody responses post-immunization (70). Antibody production by B-cells is mediated by CD4 T-cells (10) which recognize antigens in association with human leukocyte antigen (HLA) class II molecules (40). The HLA gene complex is the most genetically variable region in the human genome. It is therefore likely that variable vaccine responses are linked to polymorphisms in these genes (72, 137). Indeed, a Utah-based study that examined the genetic relationships of patients who had died due to influenza infection over a span of 100 years found a relationship between death due to influenza infection and genetic distance (7, 123).

Moreover, HLA-DRB1*07 has been found to be over-represented in patients who do not mount a neutralizing antibody response to influenza (72).

In terms of the manufacturing process, production of vaccine is in itself laborious and costly; as previously mentioned each strain included in the formulation needs to be harvested, purified and inactivated or otherwise modified. Production can take up to 9 months, and vaccines need to be stored at temperatures between 2 and 8 degrees Celsius (45). Since the viruses used are grown in the allantoic cavity of chicken eggs, vaccines cannot be given to individuals with egg allergies (147). It is estimated that up to 2.6% of the general pediatric population suffers from egg allergies. Although the condition usually resolves by school age, up to 56% of cases are persistent (91). This is an important consideration, given that children tend to have the highest influenza infection rates during epidemics (as high as 40%) (73). Reactions following exposure range from vomiting, hives, and general swelling to anaphylaxis (81).

### 1.4 Novel alternatives

An interesting alternative to “traditional” vaccines would be the use of synthetic viral peptides as immunogens. These compounds can be rapidly and relatively affordably mass-produced, are sterile and do not elicit allergic responses. Additionally, the need for refrigeration is eliminated as peptides can be stably kept as dry powder for long periods of time. From an immunological point of view, vaccination with short and well-defined peptides may be preferential to immunizing with whole viral proteins. Viral proteins are internalized, denatured, and proteolytically degraded by antigen presenting cells (APC) into short linear segments called epitopes (8, 152, 159, 169, 178). The HLA genes mentioned in the previous section encode

major histocompatibility complex (MHC) molecules, which bind and present processed epitopes to T-cells (56, 150). Interestingly, efficient T-cell stimulation does not depend on presentation of every possible epitope but is instead limited to specific epitopes (4, 20, 28, 64, 69, 102, 103, 143, 149, 157). Analysis of hundreds of overlapping synthetic peptides has proven that some epitopes preferentially induce humoral and cellular immune responses – these epitopes are called immunodominant (4, 21, 31, 69, 80, 102, 109, 143, 161). Since an effective T-cell response is needed for both cellular and humoral immunity, it is fair to say that T-cell epitopes play a vital role in vaccines and strong immune responses could theoretically be generated based on a single immunodominant T-cell epitope. However, immune responses are HLA-linked and therefore single epitope vaccines are not feasible for universal use. Still, restricting vaccine components to immulogically important epitopes could result in more focused and thus stronger cellular and humoral responses (16, 26). Additionally, *in vitro* work suggests that short peptides may not need to be as extensively processed as whole proteins thereby resulting in decreased response times to vaccination (36).

On the other hand, peptide vaccines suffer from inherent weaknesses and to date none are licensed for human or animal use or have entered advanced phases of clinical trials. Proteases present on the surface of dendritic cells may degrade the peptides before they reach their intended targets, and there is also the risk of formation of dimers and other types of aggregates (via reactive terminal cysteine residues) (175). This would result in decreased presentation of normal epitopes and/or presentation of irregularly truncated or cryptic epitopes. Of greatest significance is the fact that without the aid of adjuvants, short peptides elicit only moderate immune responses at best (3, 87). Yet most experimental adjuvants are highly toxic and therefore inadequate for human administration (122).

However, there are several approaches that might increase the stability and immunogenicity of peptide constructs. Coupling peptides to lipid moieties increases the biological half-life of synthetic peptides (36). More importantly, the addition of simple lipid moieties has an adjuvant–like effect; lipidated influenza peptides were found to enhance specific CD8+ immune responses (87). Furthermore, addition of lipid chains to peptides results in more efficient cytosolic uptake and prolonged presentation events (104). As opposed to chemical adjuvants such as alum, lipid moieties have been tested in human trials with few or no side effects (47). The precise mechanisms whereby lipid side chains achieve their adjuvanticity have to date not been elucidated. Lipidation may facilitate vaccine anchoring to the cell membrane via interactions with lipid receptors (122), with various studies implicating TLR-2 (39, 135). This is of particular importance to a potential influenza vaccine candidate, as this receptor is expressed on the epithelia of air passages (87). Lipid-TLR-2 interaction has been found to lead to activation of dendritic cells (as evidenced by the up-regulation of MHC class II molecules) and to induce nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (47, 176), as well as trigger inflammatory signalling pathways in macrophages resulting in the production of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) (86).

Incorporating glycine spacer residues between epitopes has also been found to lead to increased humoral immune responses (122). Linking several epitopes in a dendrimer-like arrangement may represent yet another method of enhancing the stability of peptides; that is, constructing poly-peptide structures or Multiple Antigen Peptides (MAPs). Increased molecular size and branched structures have been correlated to decreased proteolysis and thus a longer serum half-life (87, 136). Both computer modeling and electrophoretic analysis also suggest that

linking influenza T-helper and B-cell epitopes orients the peptides in a more compact and globular shape than when these are not linked (36). Moreover, linked epitopes in a branched MAP construct should attain a more natural conformation, which may in turn lead to enhanced recognition and increased binding. Furthermore, having multiple antigens per molecule means that more epitopes are available to interact with APCs, and may thus activate stronger immune responses (5). Lastly, incorporation of multiple distinct epitopes could be a simple solution to the issues posed by seasonal antigenic diversity and HLA polymorphism (46, 87).

### 1.5 Rationale behind project

As outlined in the previous section, current influenza vaccines have several shortcomings. Synthetic peptide-based vaccines are customizable, safe, and relatively easy to mass-produce. However, their development has been stalled due to low immunogenicity in the absence of adjuvants. In a previous study, mice were immunized with a short synthetic peptide consisting of a T-helper epitope linked to an immunodominant B-cell epitope, both based on the influenza H1N1 strain A/Puerto Rico/8/34 (36). Modest humoral and cellular immune responses were detected in vaccinated animals. On the other hand, no significant responses were detected in the group that had been administered equimolar amounts of non-linked T and B peptides, suggesting the linked T-B construct as a fair starting design for a peptide vaccine against influenza. The addition of lipid moieties and the construction of branched peptides are both known ways to increase the immunogenicity of peptides. It is therefore worthwhile to combine the aforementioned T-B peptide design with these modifications. *In vitro* humoral and cellular assays can then be used to screen individual constructs for differences in reactivity to elucidate their immunogenic potential.

## 1.6 Aims

1 – To design and synthesize modifications of a previously tested simple linked T-B peptide.

2 – To characterize and compare how different modifications affect the original T-B peptide's immunogenic potential.

## 1.7 Hypothesis

By modifying a simple synthetic influenza T-B diepitope, we will be able to also modify its immunogenic potential and thereby construct a more effective vaccine candidate.

## 2. MATERIALS AND METHODS

### 2.1 Peptide synthesis and purification

Peptides based on the influenza A/Puerto Rico/8/34 H1N1 strain HA were synthesized by the solid phase method on a Symphony Peptide Synthesizer (Protein Technologies, Tucson AZ). Briefly, amino acids were coupled in sequential format from the COOH terminus using standard N-(9 fluorenyl)methoxycarbonyl (FMOC) chemistry. Peptide stock solutions were then prepared by dissolving lyophilized preparations in double distilled water at a concentration of 5 mg/ml and stored at -80°C until use.

Altogether 16 peptides were constructed; a simple construct consisting of a T-helper epitope (110HA120, sequence SFERFEIFPKE) linked by two glycine spacers (GG) to a B-cell epitope (150HA159, sequence WLTEKEGSYP), and 15 modifications thereof. Peptides that were lipidated had palmitic acid ($CH_3(CH_2)_{14}COOH$) added at the N-terminus; multiple antigen peptides (MAPs) were constructed by creating branching points with lysine residues; mutated peptides were designed by aligning all post-1934 H1N1 sequences available from the Los Alamos National Laboratory Influenza Sequence Database (LANL ISD) (110), and comparing them using a proprietary alignment program. Protein sequence analysis was done using the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) (9).

A non-influenza peptide (a T-cell epitope from the HIV gag protein, with sequence HKGRPGNFLQNRPEPTAP) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, Maryland) and was included in all assays

at the same concentration as the influenza peptides as a negative control. Likewise, recombinant influenza hemagglutinin protein from the A/New Caledonia/20/99 H1N1 strain (Protein Sciences, Meriden, Connecticut) was included in each assay as a positive control.

**2.2 Human samples**

Approximately 100 ml of peripheral blood was obtained via venipuncture with the informed consent from 16 healthy donors. For confidentiality purposes, donors were assigned a number between 1144 and 1161[1]. The group of volunteers consisted of 8 females and 8 males ranging in age from 23 to 55 years old, who had been immunized with the 2007/2008 FluViral influenza vaccine (GlaxoSmithKline, London, UK). Blood was collected in 8 ml Cell Preparation Tubes (CPTs) with sodium citrate as an anticoagulant (BD, Franklin Lakes, New Jersey). The CPTs were allowed to sit at room temperature for 30 minutes and were gently mixed by inversion prior to density gradient centrifugation at 1,700 Relative Centrifugal Force (RCF) for 25 minutes without brake. The top layer of plasma was removed from the CPTs by pipetting and stored until use at -80°C. The lower layer of peripheral blood mononuclear cells (PBMCs) was then isolated by washing twice with 10 ml Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific, Waltham, Massachusetts). PBMCs were counted in Türk stain (0.01% gentian violet, 1.0% acetic acid) and aliquoted in freezing media consisting of 90% heat-inactivated fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri) at cell concentrations ranging between 3 x $10^6$ to 10 x $10^6$ PBMCs/ml prior to storing at -80°C.

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[1] Numbers 1152 and 1153 were assigned, but donors failed to show for the blood draw hence these numbers were skipped.

### 2.3 Viruses and cells

Influenza A/New Caledonia/20/99 (H1N1) and A/Puerto Rico/8/34 (H1N1) viruses were acquired from the Children's Hospital of Eastern Ontario (CHEO, Ottawa, Ontario) and the American Type Culture Collection (ATTC, Manassas, Virginia), respectively. Viruses were propagated via inoculation into the allantoic cavity of 10-day-old embryonated chicken eggs (Canadian Food Inspection Agency, Ottawa, Canada). Virus stocks were stored at -80°C until use.

Madin-Darby Canine Kidney (MDCK) cells (ATTC, Manassas, Virginia) were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin (Cellgro, Manassas, Virginia) in a humidified 5% $CO_2$ atmosphere at 37°C.

### 2.4 ELISA

Screening by enzyme-linked immunosorbent (ELISA) assay was performed as previously described (13, 15). Briefly, EIA/RIA Stripwell 96-well plates (Corning Incorporated, Corning, New York) were coated with 100 µl per well of recombinant A/New Caledonia/20/99 hemagglutinin protein (Protein Sciences, Meriden, Connecticut) diluted to 1 µg/ml in PBS or individual influenza or HIV gag peptides diluted to 10 µg/ml in PBS, sealed with adhesive film and incubated overnight at 4°C.

Plates were washed 6 times with 300 µl/well of PBS/0.05% Tween20 (Sigma-Aldrich, St. Louis, Missouri) and incubated for 1 hour at 37°C after the addition of 300 µl per well of blocking buffer (either PBS/5% FBS for plates used for influenza strain-specific sheep sera, or

PBS/10% FBS/2% skim milk for plates used for human plasma). The plates were washed as before and incubated 1.5 hours at 37°C after the addition of 100 µl per well of influenza strain-specific antiserum (sheep serum positive for neutralizing antibodies against A/New Caledonia/20/99, obtained from the National Institute for Biological Standards and Controls, Herts, UK) diluted to 1/100 in PBS/5% FBS, or heat-inactivated human plasma diluted to 1/100 in PBS/10% FBS/2% skim milk. After this incubation, the plates were washed again as previously and incubated 1 hour at 37°C with 100 µl per well of 1/10,000 rabbit anti-sheep IgG horseradish peroxidise (HRP)-conjugate (Abcam, Cambridge, Massachusetts) or 1/5000 goat anti-human IgG HRP-conjugate (Abcam, Cambridge, Massachusetts). Plates were washed again and incubated at room temperature with 100 µl per well TMB One Component HRP Microwell Substrate (SurModics, Eden Prairie, Minnesota). The colorimetric reaction was stopped after 8 minutes by adding 100 µl per well of 450 nm Stop Reagent for TMB Microwell Substrates (SurModics, Eden Prairie, Minnesota). The optical density (OD) of each well was then read at 450 nm on a 3550-UV microplate reader (Bio-Rad Laboratories, Hercules, California).

**2.5 Competitive microneutralization assay**

The competitive microneutralization assay was a modified version of a protocol previously described by our group (155). Briefly, heat-inactivated human plasma samples were diluted to a concentration of 1/80 in 2% FBS/IMDM. 50 µl of plasma were combined with 50 µl of individual influenza or HIV gag peptide at 50 µg/ml or 50 µl of recombinant A/New Caledonia/20/99 hemagglutinin protein at 3 µg/ml, and incubated 1 hour at 37°C. 50 µl of plasma/peptide mixture were then added to 50 µl of influenza virus (1/15 dilution of $TCID_{50}$ of A/Puerto Rico/8/34 virus in 2% FBS/IMDM) in a flat-bottom 96-well plate (Corning

Incorporated, Corning, New York). The following controls were in place on every plate: virus control (50 µl 2% FBS/IMDM plus 50 µl virus); cell control (100 µl 2% FBS/IMDM); no peptide control (25 µl diluted plasma plus 25 µl 2% FBS/IMDM plus 50 µl virus). After these 96-well plates were incubated for 1.5 hours at 37°C, 1 x $10^5$ freshly trypsinized MDCK cells were added to each well. Plates were incubated overnight for 18–22 hours in a humidified 5% $CO_2$ atmosphere at 37°C.

The following day, media was removed by inversion and plates were washed once with 200 µl of PBS. Cells were then fixed by the addition of 100 µl of cold 80% acetone to each well for 10 minutes. After removal of acetone by inversion, the plates were air-dried for 20 minutes. Plates were then washed 5 times with 300 µl PBS/0.05% Tween20 and incubated for 1 hour at room temperature with 100 µl per well of biotinylated influenza A anti-NP antibody (Chemicon International, Temecula, California) at a dilution of 1/2000 in 5% FBS/PBS. Plates were washed as before and incubated for 1 hour at room temperature after the addition of 100 µl per well HRP-conjugated streptavidin (Upstate, Temecula, California) at a dilution of 1/10,000 in 5% FBS/PBS. Plates were once again washed and incubated at room temperature with 100 µl per well TMB One Component HRP Microwell Substrate. The colorimetric reaction was stopped after 12 minutes by adding 100 µl per well of 450 nm Stop Reagent for TMB Microwell Substrates. The optical density of each well was read at 450 nm using an Emax microplate reader (Molecular Devices, Sunnyvale, California).

**2.6 Competitive plaque reduction assay**

A previously described and tested protocol was modified by our group (75). Briefly, freshly trypsinized MDCK cells were washed and resuspended in supplemented IMDM at a concentration of $1.125 \times 10^5$ cells/ml. Three millilitres of cell suspension were then seeded into each well of 6-well tissue-culture treated polystyrene, flat-bottom plates (BD, Franklin Lakes, New Jersey). After 48 hours incubation in a humidified 5% $CO_2$ atmosphere at 37°C, cells were verified to have reached 90-95% confluency.

Heat-inactivated A/New Caledonia/20/99 H1N1 strain-specific sheep antiserum or human plasma samples were diluted to a concentration of 1/10 or 1/80, respectively in supplemented IMDM. Individual influenza or HIV peptides were diluted to 50 µg/ml while recombinant A/New Caledonia/20/99 hemagglutinin protein was diluted to 10 µg/ml. 50 µl diluted peptide or diluted protein were added to 50 µl diluted sheep serum or diluted human plasma and incubated for 1 hour at 37°C. 100 µl of influenza virus diluted to 120 plaque-forming units (PFUs) were then added to the mixture from the previous step, and once again incubated for 1 hour at 37°C.

Pre-seeded 6-well plates were washed twice with warm PBS, and 100 µl of mixture were added to the appropriate well. Upon addition to the sample/peptide mixture the influenza virus was diluted by a factor of 2, therefore 60 PFUs were added to each well. To verify that this was indeed the amount of virus added, a virus control (100 µl of virus diluted to 60 PFUs) was run for each sample. A cell control (100 µl PBS alone), and a no peptide control (wherein 50 µl PBS were added to 50 µl serum/plasma prior to the first incubation) were also included for each serum/plasma tested. Plates were incubated for 1 hour at 37°C in a humidified 5% $CO_2$

atmosphere, and were gently rocked back and forth every 15 minutes throughout the incubation period to ensure even distribution of virus.

At the end of the incubation, 3 ml of warm overlay were gently added to each well. Overlay consisted of equal parts supplemented 2x Minimum Essential Medium (MEM) (VWR, West Chester, Pennsylvania) containing 2% penicillin/streptomycin, 2% L-Glutamine (Invitrogen, Carlsbad, California) and 0.25% sodium bicarbonate (Sigma-Aldrich, St. Louis, Missouri) and 1.3% agarose (Invitrogen, Carlsbad, California) dissolved and melted in 100 ml of distilled water. Once the MEM/agarose solution had cooled to 37°C, 0.9 μg/ml of TPCK-trypsin was added.

After 20 minutes at room temperature, the overlay had solidified and plates were returned to the incubator for 3 days. Carnoy's fixative (three parts methanol to one part acetic acid) was then added to each well for 30 minutes, and discarded by inversion. The agarose discs were removed by carefully rinsing plates under a gentle stream of tap water, and a 0.1% crystal violet solution was used to stain the wells. After 30 minutes, stain was rinsed off, plates were allowed to air dry, and plaques were counted.

A small-scale competitive plaque reduction assay utilizing the plasma of donor 1158 was carried out to investigate whether peptide concentration affected the rate of neutralizing antibody binding. Two modifications were made to the protocol listed above: peptides 124, 131, and 136 were diluted to 100, 250, 500, 1000, 2500, and 5000 μg/ml rather than 50 μg/ml, and 100 PFUs of virus were added to each condition instead of 60 PFUs.

### 2.7 Cellular proliferation assay

A protocol previously used in our laboratory was optimized for use with human cells (13-15, 17). Briefly, frozen PBMCs were thawed in a 37°C water bath and washed twice with 10 ml warm Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. Viable cells were counted by Trypan blue dye exclusion, and cell suspensions were prepared to a concentration of 2.0x $10^6$ cells/ml. 100 µl of these cell suspensions were added to 100 µl of each stimulant (individual influenza or HIV gag peptide at 10 µg/ml, or recombinant A/New Caledonia/20/99 hemagglutinin protein at 1 µg/ml). All conditions were tested in triplicate in a round-bottom 96-well plate (BD, Franklin Lakes, New Jersey). Unstimulated cells and cells stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin were also included for each donor as negative and positive controls, respectively.

Plates were incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 48 or 120 hours (PMA/ionomycin-stimulated cells and peptide/recombinant protein-stimulated cells, respectively). At these time points, 50 µl of [$^3$H] thymidine (Amersham, Amersham, UK) diluted 1/100 (to give 0.01 mCi/ml) in supplemented RPMI were added to each well. Plates were then returned to the incubator for 16-18 hours.

Cells were then harvested onto glass-fibre filter mats (PerkinElmer, Waltham, Massachusetts) using an automated cell harvester (Tomtec, Hamden, Connecticut). The filter mats were allowed to air dry overnight, and were then placed in a plastic sample bag and saturated with Betaplate scint scintillation fluid (PerkinElmer, Waltham, Massachusetts).

Radioactivity was counted on a Wallac 1450 Microbeta Plus Liquid Scintillation and Luminescence Counter (PerkinElmer, Waltham, Massachusetts).

**2.8 ELISpot assay**

Enzyme-linked immunosorbent spot (ELISpot) assay was performed as previously described (14, 15). Briefly, sterile 96-well MultiScreenHTS filter plates (Millipore, Billerica, Massachusetts) were activated by the addition of 15 µl of sterile-filtered 35% ethanol per well. After 1 minute at room temperature the plates were washed 5 times with 200 µl PBS, and 100 µl of anti-human IFN-γ monoclonal antibody (mAb) (Mabtech, Nacka Strand, Sweden) at a concentration of 10 µg/ml in PBS were added to each well. Coated plates were incubated overnight at 4°C.

The following day, coating antibody was removed by washing the plates 5 times with 200 µl per well using sterile PBS. Plates were blocked for 1 hour at room temperature with 200 µl per well supplemented RPMI. After the incubation, blocking medium was removed by inversion and the cell suspensions containing the appropriate stimulatory agents were added to each well as follows.

Frozen PBMCs were thawed and counted using the same method as in Section 2.7, and cell suspensions were prepared at a concentration of 2.0x $10^6$cells/ml. 100 µl (2.0 x $10^5$) cells were then added to each well and stimulated with individual influenza peptides, HIV gag peptide, or recombinant A/New Caledonia/20/99 hemagglutinin protein (diluted to a concentration of 10 µg/ml and 4 µg/ml respectively in supplemented RPMI). Unstimulated cells were used as a negative control and PMA/ionomycin stimulated cells were used as a positive

control. All conditions were tested in duplicate for each donor. The plates were placed in a plastic container lined with $ddH_2O$-moistened paper towel. This container in turn was placed in a humidified incubator with 5% $CO_2$ at 37°C and incubated for 48 hours.

Following this incubation, the cells were decanted and the plates were washed 5 times with 200 µl per well of sterile PBS. Biotinylated anti-human IFN-γ (Mabtech, Nacka Strand, Sweden) was diluted to a concentration of 1 µg/ml in PBS/0.5% FBS, and added at 100 µl per well prior to an incubation of 2 hours at room temperature. The plates were washed as before and 100 µl of streptavidin-alkaline phosphatase *(*ALP) (Mabtech, Nacka Strand, Sweden) diluted 1/1000 in PBS/0.5% FCS were added to each well. Plates were incubated for 1 hour at room temperature and washed one more time as before. After 100 µl of BCIP/NBT ALP substrate (Mabtech, Nacka Strand, Sweden) were added to each well, the plates were incubated in the dark at room temperature for 20 minutes. Colour development was stopped by thoroughly washing the plates under running tap water. The plates were allowed to dry, and spots were counted using a dissection microscope at a magnification of 40x.

**2.9 Statistical analysis**

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance of the human donor data was evaluated using a two-tailed Mann–Whitney t-test, where a $p$ value equal to or less than 0.05 was considered significant when compared with the HIV gag peptide control.

# 3. RESULTS

## 3.1 Characterization of peptide constructs

Sixteen synthetic peptides representing the hemagglutinin protein of A/Puerto Rico/8/34 H1N1 strain of influenza were designed. The parent design of these short constructs consisted of a T-helper epitope linked by two glycine spacers to a B-cell epitope (T-B peptide). Fifteen variations of this basic construct were synthesized; 6 different MAPs, a T-B peptide with an added N-terminal cysteine residue, a lipidated T-B peptide, and 7 mutated versions of the T-B peptide (Table 1).

Branching peptides, or MAPs, were generated by incorporating lysine residues into the constructs between the T and B epitopes. Altogether, three different types of MAPs were designed: a construct with one lysine branching point and two chains coming off the backbone, a similar construct that was lipidated with palmitic acid at the N-terminus of each of the two branches, and a larger construct with two lysine branching points and thus four chains coming off the backbone (Figure 1). Each of the three types of MAPs was synthesized using the B-cell epitope as a backbone and the T-helper epitope as branches, and vice versa, for a total of 6 unique designs.

In order to design the mutated peptides, the HA sequences of all available H1N1 genomes from after 1934 were downloaded from the LANL ISD (110). When these sequences were aligned and compared, it was found that the T-helper epitope had been conserved. Conversely, the B-cell epitope had three variable sites: WLT[E→G]K[E→N]G[S→L]YP (where variable sites are shown as [original A/Puerto Rico/8/34 residue→divergent residue]). All 7

possible mutations of the original epitope were synthesized and these were reverse-analysed using the NCBI BLAST program to determine the number of influenza genomes that each unique epitope could be found in (Table 2). Comparing the generated epitopes to the protein sequences available in the NCBI database revealed that not all epitope mutations were generated at the same frequency. In fact, three of the mutated epitopes were very favourable: the B-cell epitopes of constructs 138 (mutated at all three variable positions), 134 (mutated at the second and third variable positions), and 133 (mutated at the second variable position) had over 500, 199, and 86 exact genome matches, respectively. The unmutated epitope from the A/Puerto Rico/8/34 strain (construct 122) had 4 exact matches in the database, including the original strain, while construct 137 (mutated at the first and the second variable positions) had two exact matches. On the other hand, three mutated epitopes were not naturally occurring: the B-cell epitopes of constructs 132, 135 and 136 (mutated at the third variable position, mutated at the first variable position, and mutated at both the first and third variable position, respectively) had no exact matches in the database.

To determine if the constructs to be evaluated shared any similarity with human proteins, the peptides were also screened against the human sequences available in the NCBI BLAST program. There was no significant similarity found for the peptides, thereby eliminating the possibility of any autoimmune reactions should our constructs be administered to human subjects.

All of the influenza peptides were synthesized in-house by the solid phase method using an automated peptide synthesizer, and high-performance liquid chromatography (HPLC) was

used to analyze the sixteen lyophylates. Analysis of the HPLC peaks showed that the purity of the peptide preparations ranged between 90–95% (data not shown).

**Table 1. Summary of synthetic influenza peptides designed**

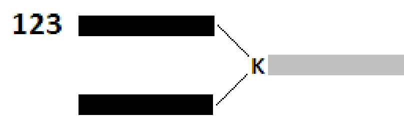
Sixteen different peptide constructs consisting of a T-helper epitope (SFERFEIFPKE) linked to an immunodominant B-cell epitope (WLTEKEGSYP) based on the HA of influenza virus A/Puerto Rico/8/34 H1N1 were synthesized. Peptide 122 was a simple, unmodified T-B diepitope construct. Peptides 123-128 are MAPs (as depicted schematically in Figure 1), where ›K designates a lysine branching point; peptides 124, 128 and 131 are lipidated with palmitic acid, as indicated by $CH_3(CH_2)_{14}COOH$; peptides 132-138 incorporate mutated versions of the original A/Puerto Rico/8/34 B-cell epitope, with each mutation shown in bold type (as summarized in Table 2).

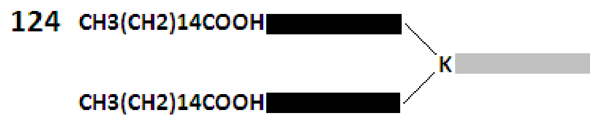
| ID | Description | MW | Sequence |
|---|---|---|---|
| **122** | T-B peptide | 2733 | SFERFEIFPKE-GG-WLTEKEGSYP |
| **123** | T-B peptide, 2-branched MAP | 4332 | SFERFEIFPKE<br>›K-WLTEKEGSYP<br>SFERFEIFPKE |
| **124** | T-B peptide, lipidated 2-branched MAP | 4809 | *$CH_3(CH_2)_{14}COOH$*-SFERFEIFPKE<br>›K-WLTEKEGSYP<br>*$CH_3(CH_2)_{14}COOH$*-SFERFEIFPKE |
| **125** | T-B peptide, 4-branched MAP | 7410 | SFERFEIFPKE<br>›K<br>SFERFEIFPKE<br>›K-WLTEKEGSYP<br>SFERFEIFPKE<br>›K<br>SFERFEIFPKE |
| **126** | B-T peptide, 2-branched MAP | 4113 | WLTEKEGSYP<br>›K-SFERFEIFPKE<br>WLTEKEGSYP |
| **127** | B-T peptide, 4-branched MAP | 6752 | WLTEKEGSYP<br>›K<br>WLTEKEGSYP<br>›K-SFERFEIFPKE<br>WLTEKEGSYP<br>›K<br>WLTEKEGSYP |
| **128** | B-T peptide, lipidated 2-branched MAP | 4590 | *$CH_3(CH_2)_{14}COOH$*- WLTEKEGSYP<br>›K- SFERFEIFPKE<br>*$CH_3(CH_2)_{14}COOH$*- WLTEKEGSYP |
| **129** | T-B peptide, N-terminal cysteine | 2837 | SFERFEIFPKE-GG-WLTEKEGSYP-Cys |
| **131** | T-B peptide, lipidated | 3338 | *$CH_3(CH_2)_{14}COOH$*- SFERFEIFPKE-GG-WLTEKEGSYP |
| **132** | Mutated T-B peptide 1 | 2760 | SFERFEIFPKE-GG-WLTEKEG**L**YP |
| **133** | Mutated T-B peptide 2 | 2718 | SFERFEIFPKE-GG-WLTEK**N**GSYP |
| **134** | Mutated T-B peptide 3 | 2745 | SFERFEIFPKE-GG-WLTEK**N**G**L**YP |
| **135** | Mutated T-B peptide 4 | 2661 | SFERFEIFPKE-GG-WLT**G**KEGSYP |
| **136** | Mutated T-B peptide 5 | 2688 | SFERFEIFPKE-GG-WLT**G**KEG**L**YP |
| **137** | Mutated T-B peptide 6 | 2646 | SFERFEIFPKE-GG-WLT**G**K**N**GSYP |
| **138** | Mutated T-B peptide 7 | 2672 | SFERFEIFPKE-GG-WLT**G**K**N**G**L**YP |

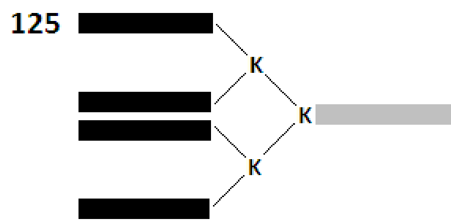
**Figure 1. Schematic depiction of MAPs**

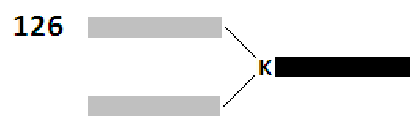
Eight unique MAPs were designed and synthesized. T-helper and B-cell represent the T-helper epitope SFERFEIFPKE and B-cell epitope WLTEKEGSYP, respectively. Both sequences are from the HA of influenza virus A/Puerto Rico/8/34 H1N1 strain. $CH_3(CH_2)_{14}COOH$ depicts the addition of a palmitic acid lipid moiety, while K represents a lysine residue used as a branching point.

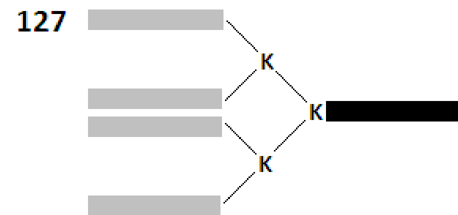
T-helper B-cell



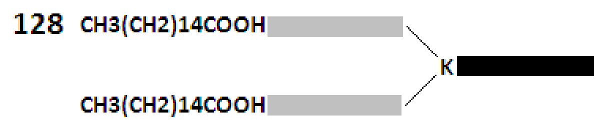



**Table 2. B-cell epitope mutations and protein BLAST analysis**

Three variable positions were found in the B-cell epitope of post-1934 H1N1 genomes (larger font as compared to non-variable positions). All possible combinations of mutations were designed and synthesized, and these mutations are bolded in red for emphasis. The relevance of the constructs was evaluated by using the NCBI protein BLAST analysis to determine if the mutations were naturally occurring, and if so how many influenza genomes encompassed each combination.

| ID | B-cell epitope | Relevance |
|---|---|---|
| **122** | WLTEKEGSYP | 4 exact matches, including "original" A/Puerto Rico/8/34 epitope |
| **132** | WLTEKEGLYP | No exact matches |
| **133** | WLTEKNGSYP | 86 exact matches |
| **134** | WLTEKNGLYP | 199 exact matches |
| **135** | WLTGKEGSYP | No exact matches |
| **136** | WLTGKEGLYP | No exact matches |
| **137** | WLTGKNGSYP | 2 exact matches |
| **138** | WLTGKNGLYP | >500 exact matches, including A/New Caledonia/20/99 |

### 3.2 Antibody binding profile by ELISA screening

In order to determine the antibody binding and recognition patterns of our various influenza peptide construct, the individual peptides were tested against commercial influenza strain-specific sheep serum and immune plasma from vaccinated human donors. ELISA plates were coated with the sixteen different influenza peptides, an unrelated HIV gag peptide as negative control, and recombinant hemagglutinin protein from A/New Caledonia/20/99 influenza strain. Each condition was coated in triplicate; serum and plasma samples were tested in duplicate under each coating condition, while the third well was left as a blank and subtracted from the average OD value.

A/New Caledonia/20/99 strain-specific sheep serum (H1N1 subtype) exhibited preferential binding of four peptides: 124, 125, 127 and 131, which are lipidated 2-branched MAP T-B peptide; 4-branched MAP T-B peptide; 4-branched MAP B-T peptide; and lipidated T-B peptide, respectively (Figure 2).

The peptides were screened using immune human samples, and peptide 124 (lipidated 2-branched MAP T-B peptide) showed the most reactivity in terms of antibody binding activity (Table 3 and Figure 3). Three donors bound 124 at exceptionally high rates: the plasma of donors 1147, 1155 and 1158 bound the peptide very strongly (at an OD value above 2.00, which is on par with the OD values of the positive control, the recombinant hemagglutinin protein). An additional two donors, 1146 and 1148, were able to bind peptide 124 at strong rates (OD values between 1.00 and 1.99). Of the remaining donors, all except four donors bound peptide 124 at a higher rate than all other peptides. Of these, donor 1149 bound peptide 125 at the highest rate, while donors 1150, 1157 and 1159 showed the highest OD value with peptide 131 (Table 3).

Interestingly, donor 1145 bound both peptides 124 and 131 at the highest rate. Examining mean antibody binding levels, peptide 124 and peptide 131 were clearly preferentially bound even when averaged across all 16 donors (Figure 3).

**Figure 2. Antibody binding by influenza strain-specific serum**

Ability of sheep serum positive for antibodies against A/New Caledonia/20/99 to bind 16 different influenza T-B and B-T peptides, HIV gag peptide (HIV) and recombinant A/New Caledonia/20/99 HA protein (rHA NC) as measured by ELISA. Absorbance values (OD 450nm) are given as a mean of duplicates at a 1/100 dilution of sera ± SEM with the subtraction of non-specific binding levels (absorbance value of serum alone control wells). ELISA was run on three separate days and data shown is representative of all results.

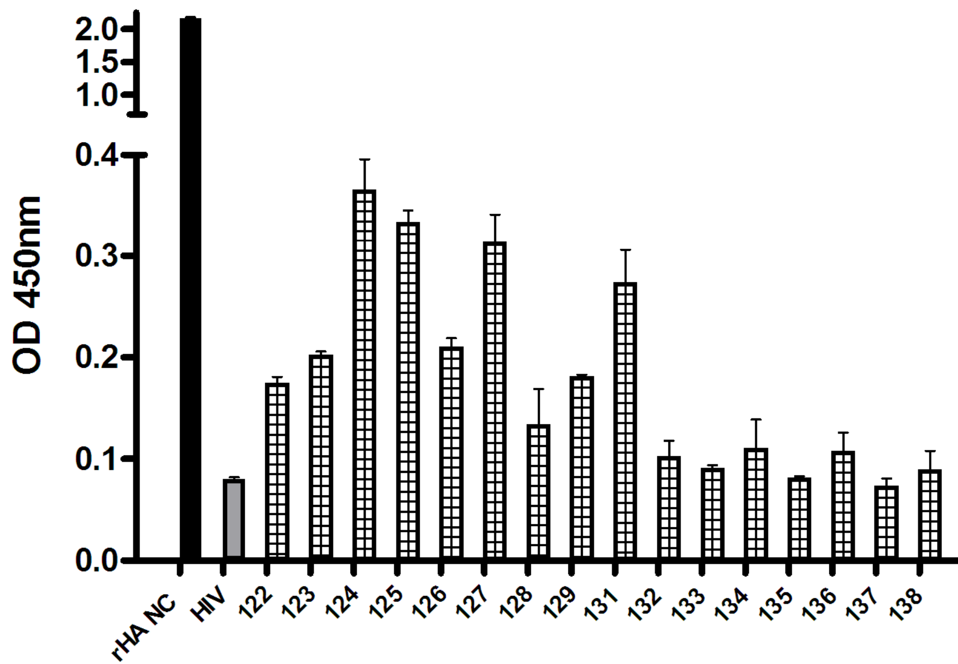

Commercial strain-specific sera:
Anti-A/New Caledonia/20/99
OD 450nm
2.0
1.5
1.0
0.4
0.3
0.2
0.1
0.0
rHA NC
HIV
122
123
124
125
126
127
128
129
131
132
133
134
135
136
137
138

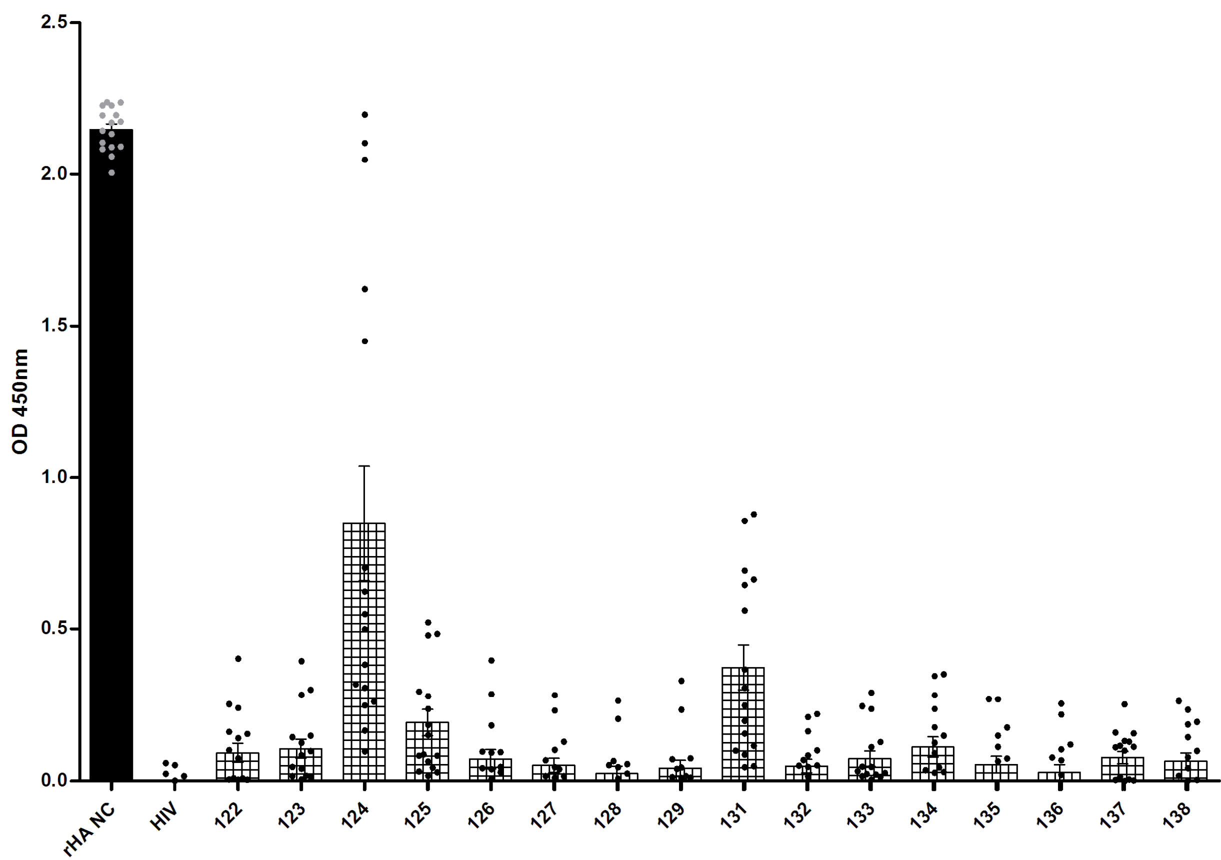
**Table 3. Antibody binding levels by plasma from human donors**

Average OD values of each of the sixteen donors against the sixteen synthetic influenza peptides and the included controls as determined by ELISA. The peptide that resulted in the highest OD value is bolded for each donor, and higher OD values are highlighted (yellow if between 0.250 and 0.99, orange if between 1.00 and 1.99 and red if above 2.00).

| | Donor | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1144** | **1145** | **1146** | **1147** | **1148** | **1149** | **1150** | **1151** | **1154** | **1155** | **1156** | **1157** | **1158** | **1159** | **1160** | **1161** |
| **rHA** | 2.060 | 2.145 | 2.084 | 2.238 | 2.091 | 2.106 | 2.172 | 2.196 | 2.175 | 2.196 | 2.092 | 2.006 | 2.228 | 2.134 | 2.228 | 2.239 |
| **HIV** | -0.011 | -0.038 | 0.001 | -0.017 | -0.049 | 0.017 | 0.059 | -0.036 | -0.014 | 0.052 | -0.048 | -0.032 | -0.022 | -0.010 | -0.005 | 0.024 |
| **122** | 0.102 | -0.025 | 0.240 | 0.009 | 0.006 | 0.141 | 0.402 | 0.005 | 0.005 | 0.253 | 0.155 | 0.162 | 0.010 | 0.074 | -0.037 | -0.023 |
| **123** | 0.085 | 0.006 | 0.299 | 0.098 | -0.006 | 0.149 | 0.394 | -0.023 | 0.017 | 0.282 | 0.126 | 0.144 | 0.014 | 0.047 | 0.017 | 0.041 |
| **124** | **0.305** | **0.248** | **1.622** | **2.104** | **1.450** | 0.098 | 0.549 | **0.499** | **0.317** | **2.049** | **0.383** | 0.261 | **2.198** | 0.166 | **0.623** | **0.701** |
| **125** | 0.184 | 0.029 | 0.484 | 0.237 | 0.044 | **0.293** | 0.479 | 0.017 | 0.083 | 0.521 | 0.151 | 0.278 | 0.064 | 0.083 | 0.031 | 0.088 |
| **126** | -0.025 | -0.004 | -0.051 | 0.093 | 0.039 | 0.182 | 0.396 | 0.047 | 0.096 | 0.285 | 0.095 | 0.042 | 0.029 | 0.008 | -0.060 | -0.025 |
| **127** | 0.017 | -0.052 | 0.039 | 0.068 | -0.004 | 0.129 | 0.231 | 0.017 | 0.046 | 0.282 | 0.103 | 0.011 | -0.008 | -0.006 | -0.045 | -0.004 |
| **128** | -0.084 | -0.087 | 0.008 | 0.056 | -0.044 | 0.025 | 0.263 | -0.027 | 0.052 | 0.204 | 0.046 | -0.036 | -0.031 | 0.066 | -0.018 | -0.004 |
| **129** | -0.059 | -0.061 | 0.011 | 0.075 | -0.010 | 0.045 | 0.329 | 0.071 | 0.017 | 0.234 | 0.040 | 0.013 | -0.004 | 0.011 | -0.023 | -0.012 |
| **131** | 0.049 | **0.248** | 0.663 | 0.367 | 0.645 | 0.086 | **0.879** | 0.115 | 0.100 | 0.856 | 0.197 | **0.306** | 0.560 | **0.692** | 0.155 | 0.045 |
| **132** | 0.051 | -0.079 | 0.210 | 0.069 | -0.022 | 0.101 | 0.220 | -0.038 | -0.038 | 0.163 | 0.015 | -0.029 | -0.018 | 0.051 | 0.047 | 0.084 |
| **133** | 0.128 | -0.066 | 0.047 | 0.111 | 0.016 | 0.236 | 0.290 | 0.005 | -0.005 | 0.245 | 0.021 | 0.023 | 0.032 | 0.027 | 0.014 | 0.047 |
| **134** | 0.092 | -0.014 | 0.282 | 0.149 | 0.045 | 0.236 | 0.345 | 0.036 | 0.028 | 0.351 | 0.126 | 0.177 | 0.030 | -0.015 | -0.055 | -0.035 |
| **135** | -0.020 | -0.074 | 0.149 | 0.065 | -0.013 | 0.176 | 0.269 | -0.002 | -0.040 | 0.268 | 0.074 | 0.112 | -0.013 | -0.031 | -0.039 | -0.020 |
| **136** | -0.050 | -0.108 | 0.104 | 0.068 | -0.063 | 0.120 | 0.254 | -0.038 | -0.063 | 0.218 | 0.021 | 0.077 | -0.037 | -0.006 | -0.040 | -0.012 |
| **137** | 0.014 | 0.129 | 0.131 | 0.100 | 0.004 | 0.157 | 0.252 | 0.002 | 0.159 | 0.112 | 0.115 | 0.111 | 0.000 | 0.006 | -0.034 | -0.025 |
| **138** | 0.004 | -0.082 | 0.185 | 0.099 | 0.017 | 0.194 | 0.262 | -0.003 | 0.144 | 0.234 | 0.078 | 0.042 | -0.009 | 0.002 | -0.085 | -0.051 |

**Figure 3. Overall antibody binding levels by plasma from human donors**

Antibody binding levels of sixteen different influenza T-B and B-T peptides, HIV gag peptide (HIV) and recombinant A/New Caledonia/20/99 HA (rHA NC) as measured by ELISA. Absorbance values (OD 450nm) are given as a mean of duplicates at a 1/100 dilution of plasma with the subtraction of non-specific binding levels (absorbance value of plasma alone control wells) ± SEM. Individual absorbance values (as presented in Table 3) are shown by •, while mean OD values (average of all donors) are represented by a bar. The assay was run on three separate days and data shown is representative of all results. All peptides except 128, 129, 132, 135, and 136 had a $p$ value lower than 0.05 in a Mann Whitney test.


2.5
2.0
1.5
1.0
0.5
0.0
OD 450nm
rHA NC
HIV
122
123
124
125
126
127
128
129
131
132
133
134
135
136
137
138

### 3.3 Neutralizing antibody binding profile by functional assay screening

To determine if the synthetic influenza peptides were able to bind neutralizing antibodies, we developed two modified functional assays – a competitive microneutralization assay and a competitive plaque reduction assay. Both assays assessed the peptides' ability to inhibit virus neutralization by binding the neutralizing antibodies found in immune samples.

In the competitive microneutralization assay, individual peptides were incubated with human plasma samples, and after one hour of incubation influenza virus was added to the samples. Following an additional incubation time of 1.5 hours, this mix was added to freshly trypsinized MDCK cells, In theory, any virus that had not been neutralized by the immune samples would be free to infect the cells, as evidenced by the detection of newly-formed virus. As such, cells were incubated overnight, and then fixed and lysed. Virus growth was measured by using an antibody directed at the influenza virus NP. Note that for each sample a peptide-free control was included, and this represented the baseline level of virus neutralization, which was different for each donor. This baseline level was subtracted from each raw value to normalize results across donors. Lower levels of virus neutralization would result in higher levels of virus growth (as shown by higher OD values), and it was thus possible to determine which peptides were able to bind neutralizing antibodies.

The synthetic influenza peptides were screened by competitive microneutralization assay using immune human plasma, and donors 1145, 1147, and 1158 showed increased reactivity (Table 4). Examining the average neutralizing antibody binding levels of all 16 donors to each peptide, 5 different designs showed increased reactivity. Peptides 123, 125, 127, 128, and 129 (2-branched MAP T-B peptide, 4-branched MAP T-B peptide, 4-branched MAP B-T peptide,

lipidated 2-branched MAP B-T peptide, and T-B peptide with an N-terminal cysteine residue, respectively) were able to bind neutralizing antibodies at a higher rate, as demonstrated by a decrease in virus neutralization (Figure 4).

For the competitive plaque reduction assay, peptides were incubated with influenza strain-specific sheep serum or human plasma samples for one hour. Influenza virus was added to the samples, and after an additional hour of incubation the mixture was added to MDCK cells pre-seeded onto 6-well plates. As in the competitive microneutralization, any virus that had not been neutralized would infect the cells. Infected cells would be lysed, and therefore virus growth could be quantified by counting how many plaques were formed. The plates were thus incubated for 3 days, after which cells were fixed and stained to determine plaque formation under each condition. As before, a no peptide control was included for each sample to determine the baseline level of virus neutralization for each donor and this value was subtracted from each plaque count. Like in the competitive microneutralization assay, higher plaque counts indicated stronger influences of specific peptides on virus neutralization and in turn a more effective binding of neutralizing antibodies.

All constructs (although peptide 136, which is mutated T-B peptide 5, only to a low extent) were able to inhibit virus neutralization by commercial A/New Caledonia/20/99-strain specific sera (Figure 5). In particular, peptides 123 and 125 (2-branched MAP T-B peptide and 4-branched MAP T-B peptide, respectively) showed slightly higher binding of neutralizing antibodies as compared to other constructs.

The assay was conducted with immune human plasma, and donors 1145, 1147 and 1154 were associated with higher general inhibition of virus neutralization upon addition of peptides.

It should be noted that the first two of these donors also showed stronger responses in the competitive microneutralization assay. On the other hand, addition of influenza peptides to the plasma of donors 1149, 1151, 1160 and 1161 resulted in very weak changes in levels of virus neutralization (Table 5). Comparing differences in reactivity between peptides, preferential binding was not limited to one or two designs; peptides 126, 133, 135, and 138 (2-branched MAP B-T peptide, mutated T-B peptide 2, mutated T-B peptide 4, and mutated T-B peptide 6, respectively) all showed increased activity when neutralizing antibody binding was averaged across all donors (Figure 6).

To further characterize the neutralizing antibody binding ability of peptides of interest, a modified competitive plaque reduction assay was designed and carried out. Peptide 124 (lipidated 2-branched MAP T-B peptide), peptide 131 (lipidated T-B peptide), and peptide 136 (mutated T-B peptide 5) were added in increasing amounts to the plasma of donor 1158. As higher concentrations of peptide 124 were added, a positive dose-effect was observed; increasing amounts of peptide were able to bind increasing amounts of neutralizing antibodies, as evidenced by decreased levels of virus neutralization (Figure 7). Peptide 131 showed a similar trend, although to a lesser extent. Interestingly, the rate of neutralizing antibody binding rate of this construct decreased sharply at the highest concentration tested (5000 μg/ml). On the other hand, peptide 136 did not seem to be able to bind neutralizing antibodies at higher rates as higher concentrations of peptide were used.

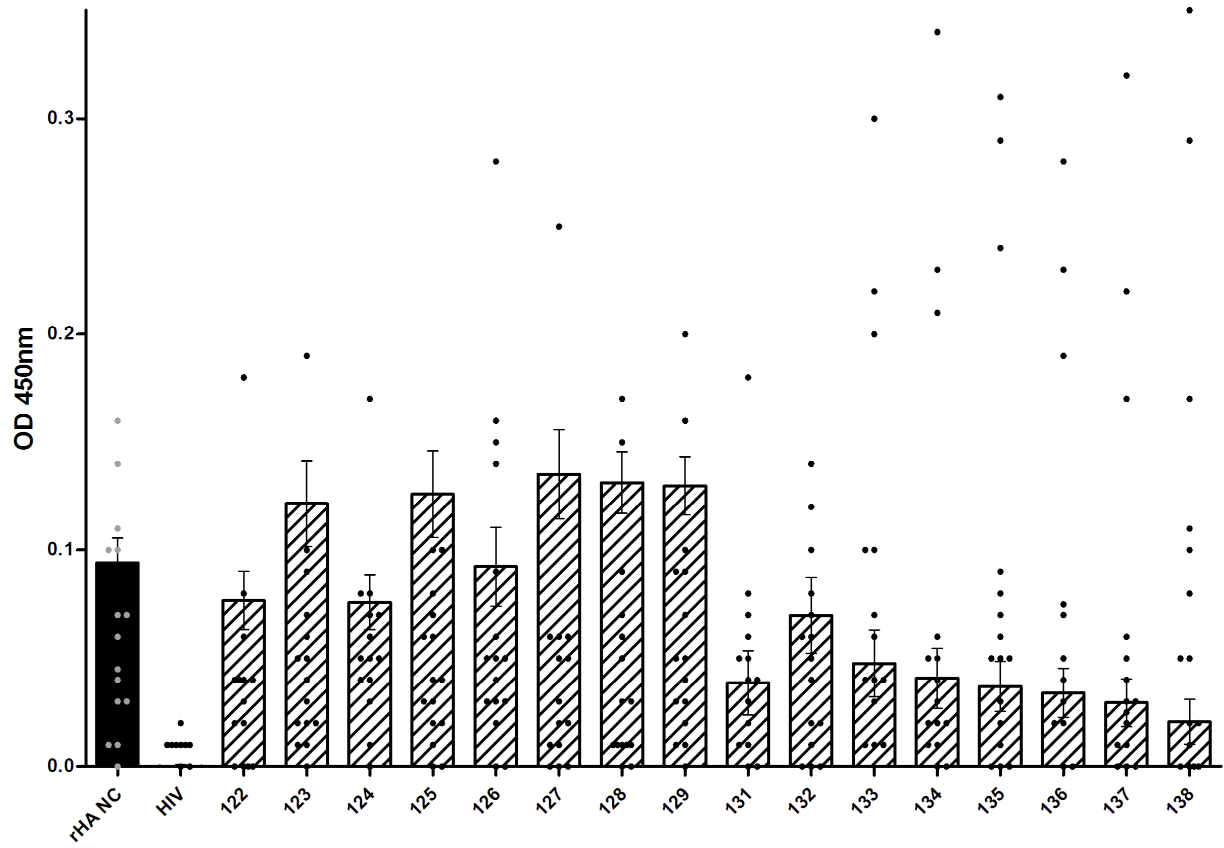
**Table 4. Neutralizing antibody binding levels by plasma from human donors in competitive microneutralization assay**

Average OD values (after total background subtraction; adjusted for cells alone control and peptide-free control wells, except the virus alone control which is shown with only cells alone control wells subtracted) of each of the sixteen donors against the sixteen synthetic influenza peptides and the included controls as measured by competitive microneutralization assay. The peptide that resulted in the largest change in absorbance value as compared to the peptide-free control is bolded for each donor, and higher differences are highlighted (yellow if between 0.200 and 0.349 and red if above 0.350).

| | **Donor** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1144** | **1145** | **1146** | **1147** | **1148** | **1149** | **1150** | **1151** | **1154** | **1155** | **1156** | **1157** | **1158** | **1159** | **1160** | **1161** |
| **Virus alone** | 1.064 | 1.011 | 1.026 | 1.075 | 1.112 | 1.104 | 1.097 | 1.123 | 0.991 | 1.013 | 1.021 | 0.974 | 1.110 | 1.105 | 1.094 | 1.018 |
| **rHA** | 0.061 | 0.215 | 0.064 | 0.134 | 0.127 | 0.117 | 0.111 | 0.094 | 0.130 | 0.059 | 0.041 | 0.032 | 0.089 | 0.055 | 0.076 | 0.100 |
| **HIV** | 0.001 | -0.009 | -0.002 | 0.004 | -0.011 | 0.010 | 0.001 | -0.002 | -0.004 | -0.002 | -0.005 | 0.004 | 0.009 | 0.004 | -0.001 | -0.004 |
| **122** | 0.110 | 0.145 | 0.055 | 0.176 | 0.047 | 0.036 | 0.091 | 0.045 | 0.035 | 0.048 | 0.038 | **0.073** | 0.162 | 0.119 | -0.025 | 0.072 |
| **123** | 0.104 | **0.244** | 0.064 | 0.204 | 0.125 | 0.121 | 0.131 | 0.047 | 0.074 | 0.047 | 0.072 | 0.053 | **0.324** | 0.165 | 0.041 | **0.129** |
| **124** | 0.014 | 0.109 | 0.048 | 0.198 | 0.095 | 0.059 | 0.089 | 0.030 | 0.089 | 0.026 | 0.107 | 0.023 | 0.124 | 0.115 | 0.007 | 0.081 |
| **125** | 0.185 | 0.214 | 0.062 | 0.271 | 0.142 | 0.052 | **0.193** | **0.084** | 0.096 | 0.052 | 0.097 | 0.020 | 0.258 | 0.162 | 0.021 | 0.106 |
| **126** | 0.006 | 0.235 | 0.025 | 0.176 | 0.151 | 0.048 | 0.124 | 0.025 | 0.084 | 0.048 | 0.079 | 0.001 | 0.203 | 0.149 | 0.035 | 0.087 |
| **127** | **0.279** | 0.227 | 0.033 | **0.278** | 0.200 | 0.093 | 0.092 | 0.037 | 0.118 | 0.075 | **0.175** | 0.016 | 0.166 | 0.174 | 0.098 | 0.100 |
| **128** | 0.207 | 0.140 | 0.007 | 0.179 | 0.166 | **0.141** | 0.155 | 0.053 | **0.161** | **0.102** | 0.163 | 0.059 | 0.142 | **0.221** | 0.094 | 0.111 |
| **129** | 0.162 | 0.205 | **0.076** | 0.124 | 0.247 | 0.114 | 0.120 | 0.065 | 0.158 | 0.038 | 0.169 | 0.064 | 0.157 | 0.137 | **0.126** | 0.113 |
| **131** | -0.001 | -0.003 | 0.046 | 0.154 | 0.069 | -0.056 | 0.166 | 0.032 | 0.035 | -0.013 | -0.012 | 0.025 | 0.054 | 0.052 | -0.013 | 0.086 |
| **132** | 0.018 | 0.120 | 0.038 | 0.225 | 0.049 | 0.069 | 0.094 | 0.017 | 0.074 | 0.046 | 0.126 | 0.003 | 0.208 | 0.028 | -0.017 | 0.020 |
| **133** | 0.075 | 0.083 | 0.019 | 0.232 | 0.025 | 0.035 | 0.090 | 0.020 | 0.063 | -0.010 | 0.060 | -0.028 | 0.062 | 0.059 | -0.027 | 0.005 |
| **134** | 0.054 | 0.080 | -0.043 | 0.165 | -0.017 | 0.039 | 0.035 | 0.011 | 0.116 | 0.014 | 0.070 | -0.031 | 0.106 | 0.036 | 0.005 | 0.011 |
| **135** | 0.035 | 0.096 | 0.012 | 0.113 | 0.086 | 0.034 | -0.048 | 0.025 | 0.074 | -0.026 | 0.011 | -0.030 | 0.049 | 0.078 | 0.022 | 0.063 |
| **136** | 0.119 | 0.025 | 0.009 | 0.047 | 0.068 | 0.025 | -0.033 | 0.003 | 0.086 | -0.003 | -0.050 | 0.004 | 0.062 | 0.058 | 0.035 | 0.089 |
| **137** | 0.029 | 0.077 | 0.016 | -0.066 | 0.131 | -0.006 | 0.023 | 0.009 | 0.039 | 0.055 | 0.002 | 0.000 | -0.001 | 0.038 | 0.062 | 0.063 |
| **138** | -0.021 | 0.106 | 0.020 | 0.060 | 0.028 | -0.033 | 0.035 | -0.016 | 0.055 | 0.002 | -0.024 | 0.048 | 0.075 | -0.007 | -0.030 | 0.032 |

**Figure 4. Overall neutralizing antibody binding levels by plasma from human donors in competitive microneutralization assay**

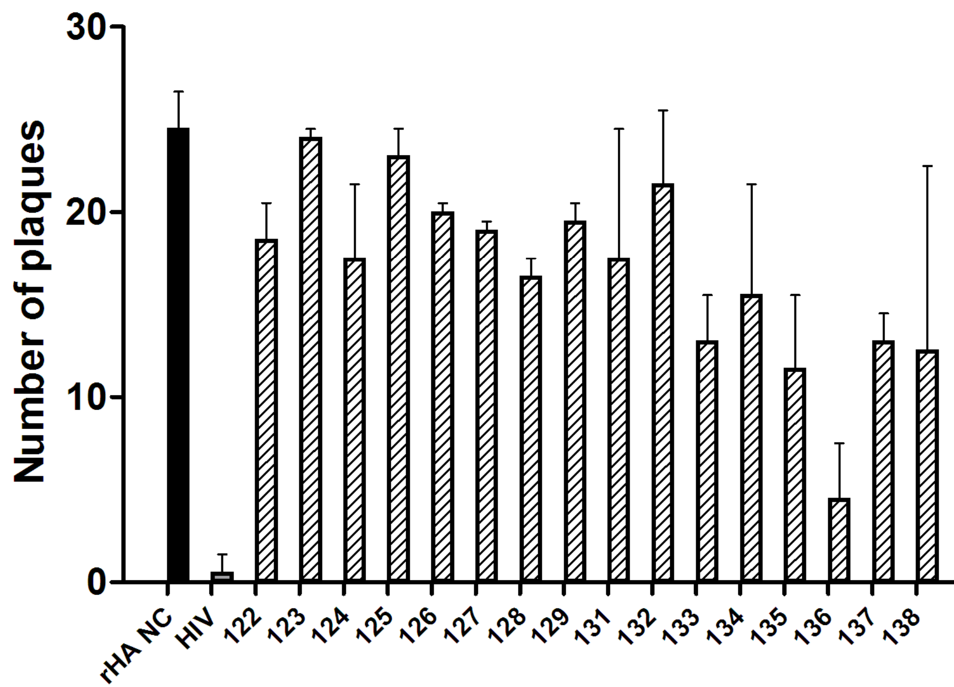
Comparison of neutralizing antibody binding levels of human plasma from 16 different donors against 16 different influenza T-B and B-T peptides, HIV gag peptide (HIV) and recombinant A/New Caledonia/20/99 HA protein (rHA NC), as measured by the inhibition of neutralization of A/Puerto Rico/8/34 H1N1 influenza virus in a competitive microneutralization assay. Absorbance values are given as mean of duplicates at a 1/80 dilution of plasma ± SEM. Total background (absorbance of cells alone control and peptide-free control wells) was subtracted from each raw absorbance value. Individual OD values (as presented in Table 4) are shown by •, while mean OD values across all donors (average of all donors) are represented by a bar. All peptides except 131 and 138 had a *p* value lower than 0.05 in a Mann Whitney test.



OD 450nm
0.3
0.2
0.1
0.0
rHA NC
HIV
122
123
124
125
126
127
128
129
131
132
133
134
135
136
137
138

**Figure 5. Neutralizing antibody binding by influenza strain-specific serum**

Ability of 16 different T-B and B-T influenza peptides, HIV gag peptide (HIV) and recombinant A/New Caledonia/20/99 HA protein (rHA NC) to bind neutralizing antibodies in sheep serum positive against A/New Caledonia/20/99 strain, and thereby inhibit neutralization of A/New Caledonia/20/99 influenza virus. Number of viral plaques given as mean of duplicates at a 1/80 dilution of plasma ± SEM. Background (number of plaques in peptide-free serum wells) was subtracted from each plaque count.



Commercial strain-specific sera:
Anti-A/New Caledonia/20/99
Number of plaques
30
20
10
0
rHA NC
HIV
122
123
124
125
126
127
128
129
131
132
133
134
135
136
137
138

**Table 5. Neutralizing antibody binding levels by plasma from human donors in competitive plaque reduction assay**

Average plaque count (after background subtraction, except the virus alone control which is shown directly as counted) of each of the sixteen donors against the sixteen synthetic influenza peptides and the included controls. The peptide that resulted in the largest change in plaque count as compared to the peptide-free control is bolded for each donor, and higher differences are highlighted (yellow if between 6 and 15 and red if above 16).

| | Donor | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1144** | **1145** | **1146** | **1147** | **1148** | **1149** | **1150** | **1151** | **1154** | **1155** | **1156** | **1157** | **1158** | **1159** | **1160** | **1161** |
| **Virus alone** | 57 | 58 | 60 | 61 | 57 | 60 | 60 | 60 | 60 | 59 | 59 | 60 | 57.5 | 60 | 57 | 62 |
| **rHA** | 14 | -1 | 0 | 6 | 10 | 1 | 7 | 11 | 16 | 4 | 10 | 7 | 4.5 | 3 | 1 | 3 |
| **HIV** | -1 | 1 | 1 | 1 | 1 | 0 | -2 | -1 | 1 | 0 | -1 | 1 | -1 | -1 | -1 | 2 |
| **122** | -2 | 4 | 4 | 4 | 6 | 0 | 8 | -1 | 18 | 3 | 0 | 2 | 0 | 4 | 0 | 2 |
| **123** | -1 | 2 | 1 | 3 | 9 | 2 | **10** | 1 | 19 | 4 | 6 | 7 | 5 | 5 | 0 | 2 |
| **124** | 4 | 7 | 5 | 8 | 6 | -1 | 5 | 1 | 17 | 5 | 3 | 8 | 0 | 7 | -1 | **4** |
| **125** | -3 | 4 | 7 | 10 | 4 | 0 | **10** | 2 | 6 | 3 | 2 | 6 | 0 | 8 | 1 | 3 |
| **126** | 3 | 16 | 3 | 14 | 5 | 0 | 9 | 0 | 28 | 5 | 6 | 15 | 5 | 3 | **2** | **4** |
| **127** | 2 | 2 | -1 | 5 | 6 | 0 | 6 | -1 | 25 | 1 | 0 | 5 | 0 | 6 | 1 | 3 |
| **128** | 0 | 17 | 1 | 7 | 1 | 1 | 5 | 1 | 9 | 3 | 1 | 6 | **15** | -1 | 0 | 3 |
| **129** | 5 | 16 | 4 | 20 | **10** | 2 | 9 | 1 | 9 | 3 | 0 | 7 | 5 | 1 | -1 | 3 |
| **131** | 0 | 4 | 7 | 3 | 6 | 0 | 4 | 1 | 18 | **8** | -1 | -1 | 5 | 5 | 1 | 2 |
| **132** | 0 | 14 | -1 | 8 | 0 | 2 | **10** | 0 | 12 | 4 | **7** | 6 | 5 | 6 | 1 | 2 |
| **133** | 6 | 22 | -1 | 3 | 4 | 1 | **10** | 1 | 30 | 7 | 4 | **20** | -1 | **10** | 1 | **4** |
| **134** | -2 | 21 | 2 | 23 | 5 | 0 | 2 | **3** | **34** | 1 | 2 | 6 | 1 | 5 | 0 | **4** |
| **135** | 5 | 29 | 5 | 24 | 9 | 0 | 1 | 2 | 31 | 7 | 5 | 8 | 0 | 6 | 0 | 3 |
| **136** | **7** | 19 | 4 | **28** | 5 | -1 | 2 | -1 | 23 | 0 | 3 | -2 | 7.5 | 0 | -2 | 2 |
| **137** | 4 | 22 | 5 | 17 | 2 | 0 | 1 | 0 | 32 | 6 | 3 | 0 | 2.5 | 1 | -1 | 3 |
| **138** | 0 | **35** | **8** | 17 | **10** | 0 | 0 | 0 | 29 | 2 | 1 | 11 | 5 | 5 | -1 | 2 |

**Figure 6. Overall neutralizing antibody binding levels by plasma from human donors in competitive plaque reduction assay**

Comparison of neutralizing antibody binding levels of human plasma from 16 different donors against 16 different influenza T-B and B-T peptides, HIV gag peptide (HIV) and recombinant A/New Caledonia/20/99 HA protein (rHA NC), as measured by the inhibition of neutralization of A/New Caledonia/20/99 influenza virus. Number of viral plaques are given as mean of duplicates at a 1/80 dilution of plasma ± SEM. Background (number of plaques in peptide-free serum wells) was subtracted from each plaque count. Individual plaque counts (as presented in Table 5) are shown by •, while mean plaque counts across all donors (average of all donors) are represented by a bar. All peptides had a *p* value lower than 0.05 in a Mann Whitney test.

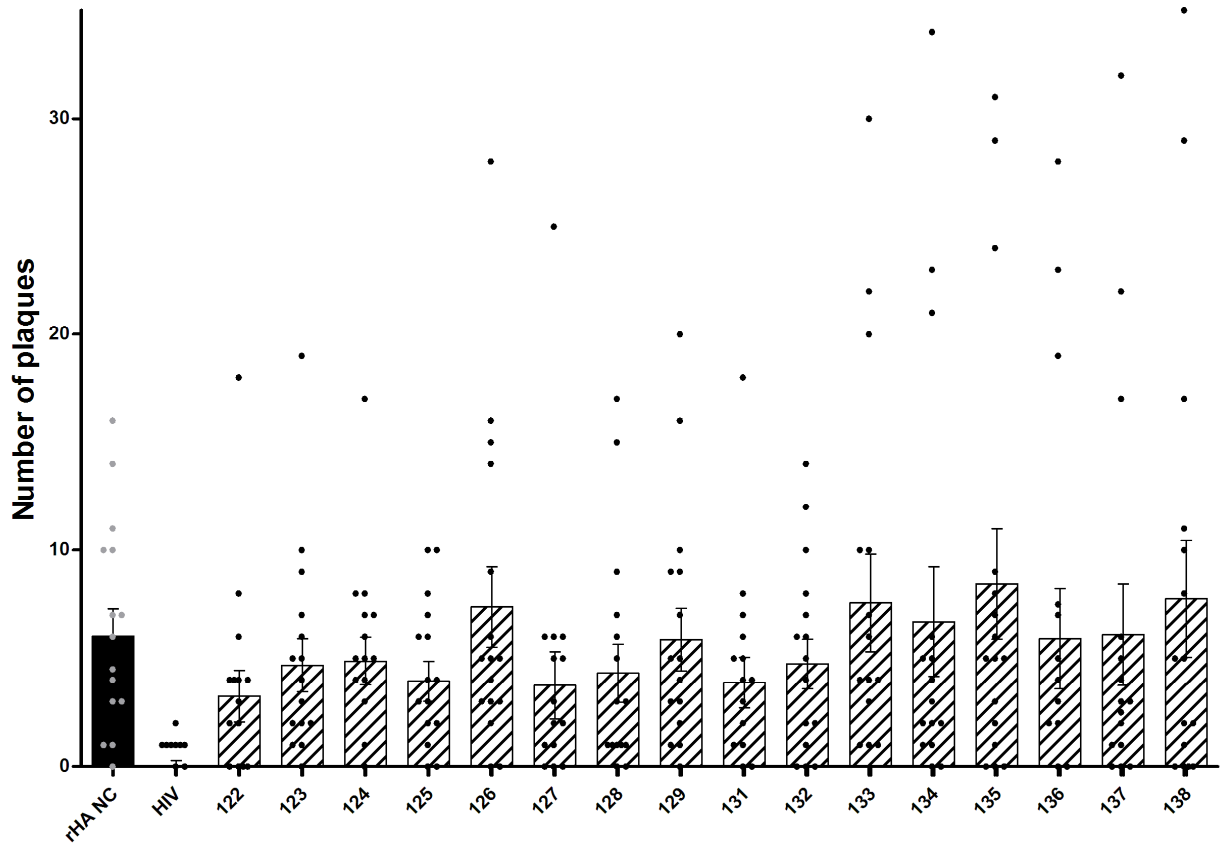
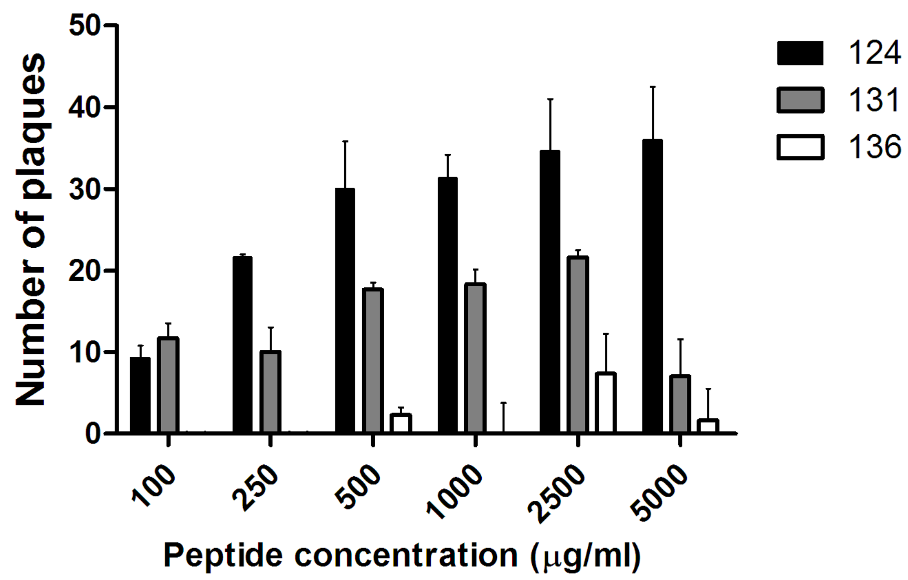

Number of plaques
0
10
20
30
rHA NC
HIV
122
123
124
125
126
127
128
129
131
132
133
134
135
136
137
138

**Figure 7. Dose-response effect of increasing amounts of peptide in competitive plaque reduction assay**

Average plaque count of donor 1158 against three synthetic influenza peptides. Peptides 124, 131, and 136 were added in graded amounts at 100, 250, 500, 1000, 2500, and 5000 μg/ml. Number of viral plaques are given as mean of triplicates (after background subtraction) at a 1/80 dilution of plasma ± SEM.


Number of plaques
50
40
30
20
10
0
100
250
500
1000
2500
5000
Peptide concentration (μg/ml)
124
131
136

### 3.4 Induction of cellular immune responses

The degree to which our synthetic influenza peptides activate cellular immune responses was determined with both cellular proliferation and IFN-γ ELISpot assays.

First, human PBMCs isolated from donor samples were stimulated with individual influenza peptides, an HIV gag peptide (as a negative control), recombinant A/New Caledonia/20/99-strain hemagglutinin protein (rHA), and PMA/ionomycin (as a positive control). Levels of cell division in response to the different stimulants were assessed by [$^{3}$H] thymidine incorporation; dividing cells become radiolabelled and could thus be quantified with the use of scintillation fluid and a luminescence counter. The stimulation index (SI) was then calculated by dividing the average number of cells in each stimulated condition by the average number of cells in unstimulated control wells.

Among the sixteen human samples tested, several donors had intermediate SIs across all peptides that were assessed; PBMCs from donors 1145, 1156 and 1161 were not stimulated above an index of 5 by most of the peptides. However, these values were still significantly higher than those associated with the HIV gag peptide-stimulated PBMCs. On the other hand, donors 1148, 1151, 1159 and 1160 showed a five-fold or greater increase in proliferation in response to most peptides. Donor 1159 in particular was a very high responder, with most peptides inducing SI values above 10 (Table 6).

In terms of peptide-specific differences, there was not a clear bias to a particular peptide. As summarized in Table 6, lipidated MAP construct 124 was able to induce greater than 10-fold increases in levels of proliferation in four donors (donor 1147, 1148, 1154 and 1160 with SI

values of 12.835, 11.375, 10.280 and 18.650, respectively), and increases between 5-fold and 9.999-fold in another five donors (donor 1149, 1150, 1151, 1159, and 1159 with SI values of 6.625, 9.150, 6.590, 6.990, and 5.695, respectively). Although peptides 126 and 131 (2-branched MAP B-T peptide and lipidated T-B peptide, respectively) stimulated somewhat higher levels of cellular proliferation (mean SI values across all donors), these were only slight increases compared to the other peptides (Figure 8).

Furthermore, the frequency of IFN-γ secretion in the human PBMCs in response to peptide stimulation was evaluated. As in the proliferation assay, responses to the sixteen individual influenza peptides, an HIV gag peptide (as a negative control), recombinant A/New Caledonia/20/99-strain hemagglutinin protein (rHA), and PMA/ionomycin (as a positive control) were evaluated. Use of a human IFN-γ-specific antibody allowed quantification of cytokine production, as each IFN-γ producing cell was detected as a distinct coloured spot. Results are presented as average spot forming cell counts (SFC), where average values have been normalized to account for one million cells and spontaneous IFN-γ production (background levels in unstimulated control cells had been subtracted).

Like in the lymphocyte proliferation assay, donors 1145 and 1161 were low responders, with most peptides resulting in SFC counts of less than 20 cells per million (although donor 1156 who had low cellular proliferative responses showed moderate cytokine production). In applying the above criteria that SFC counts of less than 20 cells per million designate low general responders, PBMCs from donors 1144, 1146, 1148, 1149, 1150 and 1155 can also be classified as such, since these donors only produced low levels of IFN-γ in response to most peptides (Table 7). In stark contrast, donors 1151, 1154, 1156, 1157, 1159 and 1160 were high general

responders; stimulation with most peptides resulted in SFC counts higher than 20. It should be noted that donors 1151, 1159 and 1160 also had high general stimulation indexes in the proliferation assay. Peptide 124 was able to induce very high levels of IFN-γ production (more than 100 SFC per million cells) among six donors: donors 1148, 1150, 1151, 1154, 1156 and 1159 had counts of 195, 122.5, 120, 182.5, 127.5 and 132.5 SFC per million cells, respectively. It was interesting that peptide 124 elicited high responses even in donors 1148 and 1150, who in general were low responders. Furthermore, in all but two donors stimulation with peptide 124 resulted in IFN-γ production higher than with any other peptide. Only donors 1144 and 1147 did not have the highest peptide-specific response associated with peptide 124. Rather, these two donors showed the best response to peptide 134 (mutated T-B peptide 3) and 131 (lipidated T-B peptide), respectively. Examining peptide-induced IFN-γ responses, it is clear that peptide 124 is the best design as it elicited the highest SFC count when averaged across all the donors (Figure 9).

**Table 6. Cellular proliferation levels in PBMCs from human donors**

Average Stimulation Index (ratio of cellular proliferation between stimulated and unstimulated cells) of each of the sixteen human donors against the sixteen synthetic influenza peptides and the included controls as determined by proliferation assay. The peptide that resulted in the highest SI value is bolded for each donor, and higher SI values are highlighted (yellow if between 5.000 and 9.999 and red if above 10.000).

| | Donor | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1144** | **1145** | **1146** | **1147** | **1148** | **1149** | **1150** | **1151** | **1154** | **1155** | **1156** | **1157** | **1158** | **1159** | **1160** | **1161** |
| **PMA/iono** | 195.750 | 297.430 | 194.255 | 401.480 | 125.410 | 91.335 | 168.070 | 179.650 | 60.110 | 412.330 | 405.900 | 315.485 | 164.550 | 486.100 | 189.140 | 239.190 |
| **rHA** | 5.280 | 6.485 | 5.735 | 1.490 | 12.680 | 12.110 | 6.080 | 10.590 | 1.995 | 4.625 | 3.965 | 4.720 | 13.565 | 5.960 | 11.710 | 4.300 |
| **HIV** | 0.950 | 1.110 | 0.860 | 1.200 | 1.240 | 0.960 | 1.030 | 0.880 | 0.870 | 1.100 | 1.095 | 1.150 | 1.070 | 0.970 | 0.790 | 1.030 |
| **122** | 6.030 | 1.500 | 4.890 | 20.185 | 8.035 | 4.655 | 4.620 | 8.510 | 3.275 | 5.240 | 3.540 | 4.160 | 6.605 | 6.400 | 11.365 | 4.595 |
| **123** | **9.335** | 3.080 | 5.900 | 6.885 | 10.485 | 6.870 | 2.860 | 9.000 | 2.065 | 3.210 | 1.730 | 9.065 | 4.870 | 11.660 | 3.655 | **8.470** |
| **124** | 3.185 | 1.425 | 4.175 | 12.835 | 11.375 | 6.625 | **9.150** | 6.590 | **10.280** | 2.795 | 2.520 | 4.600 | 4.960 | 6.990 | **18.650** | 5.695 |
| **125** | 5.135 | 2.755 | 4.720 | 8.160 | 9.680 | 3.120 | 7.645 | 7.440 | 2.820 | 3.050 | 2.710 | 10.775 | 4.490 | 6.385 | 5.780 | 5.405 |
| **126** | 6.305 | 2.795 | 3.835 | **27.680** | 9.060 | 4.355 | 4.470 | 5.385 | 4.630 | 2.290 | 6.155 | 7.185 | 6.660 | 25.130 | 18.285 | 4.235 |
| **127** | 4.585 | 2.315 | 5.005 | 4.560 | 9.865 | 6.425 | 6.230 | 8.745 | 2.370 | 2.915 | 1.960 | 6.820 | 3.195 | 20.510 | 10.125 | 2.780 |
| **128** | 2.465 | 2.490 | 3.815 | 8.075 | 7.785 | 2.750 | 3.345 | 5.025 | 2.190 | **15.880** | 4.045 | 3.895 | 6.065 | 24.885 | 17.935 | 2.300 |
| **129** | 6.255 | 1.790 | **6.905** | 3.765 | 7.075 | 4.725 | 4.060 | 7.505 | 4.035 | 9.325 | 3.015 | **15.325** | 6.510 | **30.400** | 6.440 | 2.345 |
| **131** | 7.815 | 3.960 | 6.825 | 2.835 | 6.860 | 7.410 | 7.075 | **9.085** | 4.690 | 11.080 | 2.945 | 8.090 | 4.685 | 29.310 | 11.320 | 5.795 |
| **132** | 5.040 | **5.120** | 5.000 | 3.085 | 11.790 | **9.250** | 5.945 | 5.860 | 8.170 | 6.840 | **6.435** | 6.185 | 3.580 | 14.555 | 5.930 | 5.095 |
| **133** | 6.115 | 3.850 | 4.515 | 2.880 | 9.700 | 3.685 | 3.155 | 6.685 | 4.765 | 3.195 | 5.130 | 5.465 | 2.820 | 16.205 | 7.035 | 3.660 |
| **134** | 7.585 | 2.610 | 3.300 | 2.395 | 10.055 | 4.780 | 3.255 | 8.075 | 7.735 | 2.435 | 3.520 | 10.770 | **11.965** | 18.870 | 7.900 | 5.085 |
| **135** | 4.115 | 1.810 | 5.650 | 4.175 | 9.205 | 3.640 | 4.000 | 8.415 | 9.940 | 3.795 | 1.750 | 8.345 | 8.875 | 14.295 | 6.225 | 4.500 |
| **136** | 7.530 | 2.825 | 4.350 | 2.320 | 9.000 | 6.770 | 4.105 | 8.585 | 3.290 | 13.055 | 2.860 | 7.320 | 10.600 | 15.655 | 6.710 | 4.665 |
| **137** | 4.025 | 2.350 | 6.220 | 2.285 | 8.880 | 2.185 | 6.245 | 6.310 | 5.705 | 3.880 | 5.325 | 9.520 | 6.280 | 21.755 | 6.865 | 3.465 |
| **138** | 8.955 | 3.430 | 4.540 | 3.635 | **12.055** | 8.395 | 5.540 | 5.295 | 5.835 | 2.910 | 2.845 | 7.005 | 9.665 | 6.205 | 8.835 | 3.600 |

**Figure 8. Overall cellular proliferation levels in PBMCs from human donors**

Comparison of ability of 16 different influenza T-B and B-T peptides, HIV gag peptide (HIV), recombinant A/New Caledonia/20/99 hemagglutinin protein (rHA NC) and PMA/ionomycin (PMA/iono) to induce proliferation of human PBMCs, as tracked by [$^3$H] thymidine incorporation. Stimulants were tested in triplicate and the most outlying value was discarded for each count. Stimulation Index is given as the ratio between the number of cells dividing in response to antigen-stimulation and unstimulated cells ± SEM. Individual SI values (as presented in Table 6) are shown by •, while mean SI values across all donors is represented by a bar. All peptides had a *p* value lower than 0.0001 in a Mann Whitney test.

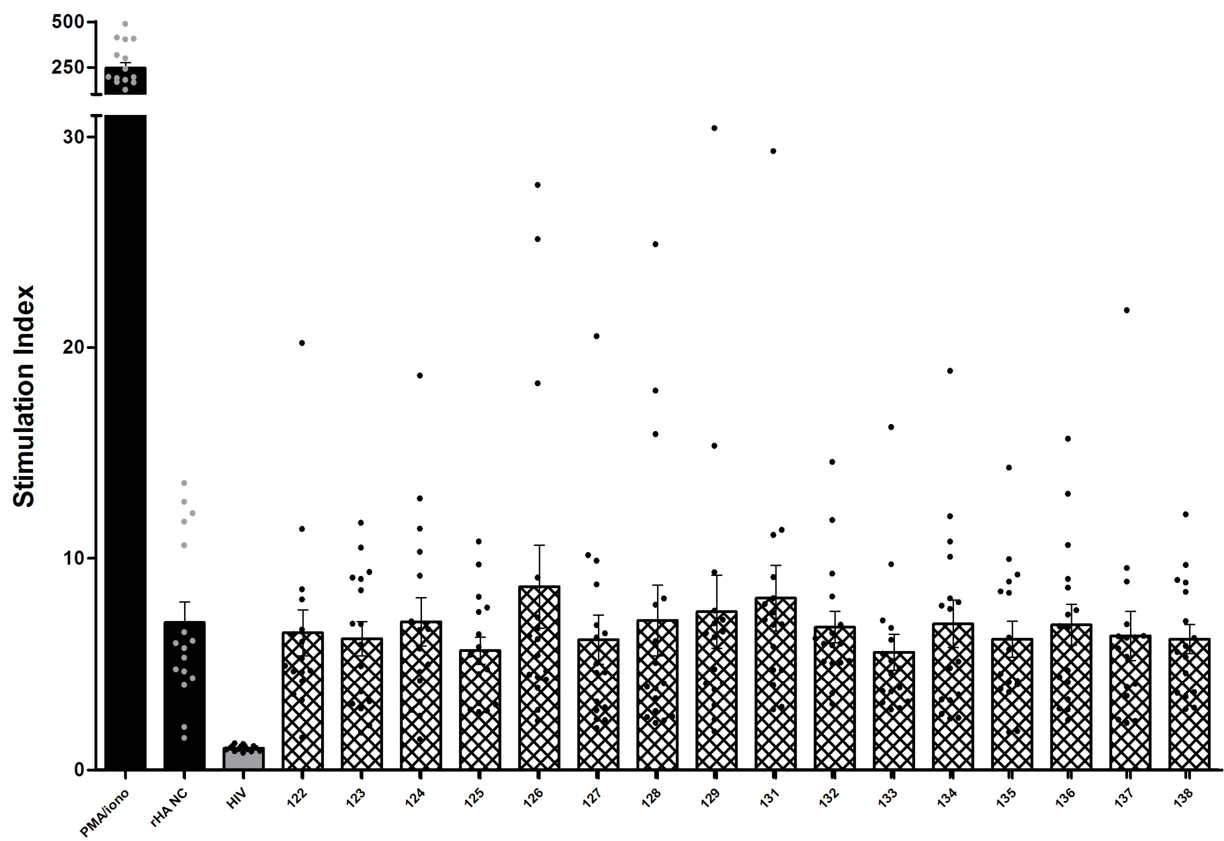

500
250
30
20
10
0
Stimulation Index
PMA/iono
rHA NC
HIV
122
123
124
125
126
127
128
129
131
132
133
134
135
136
137
138

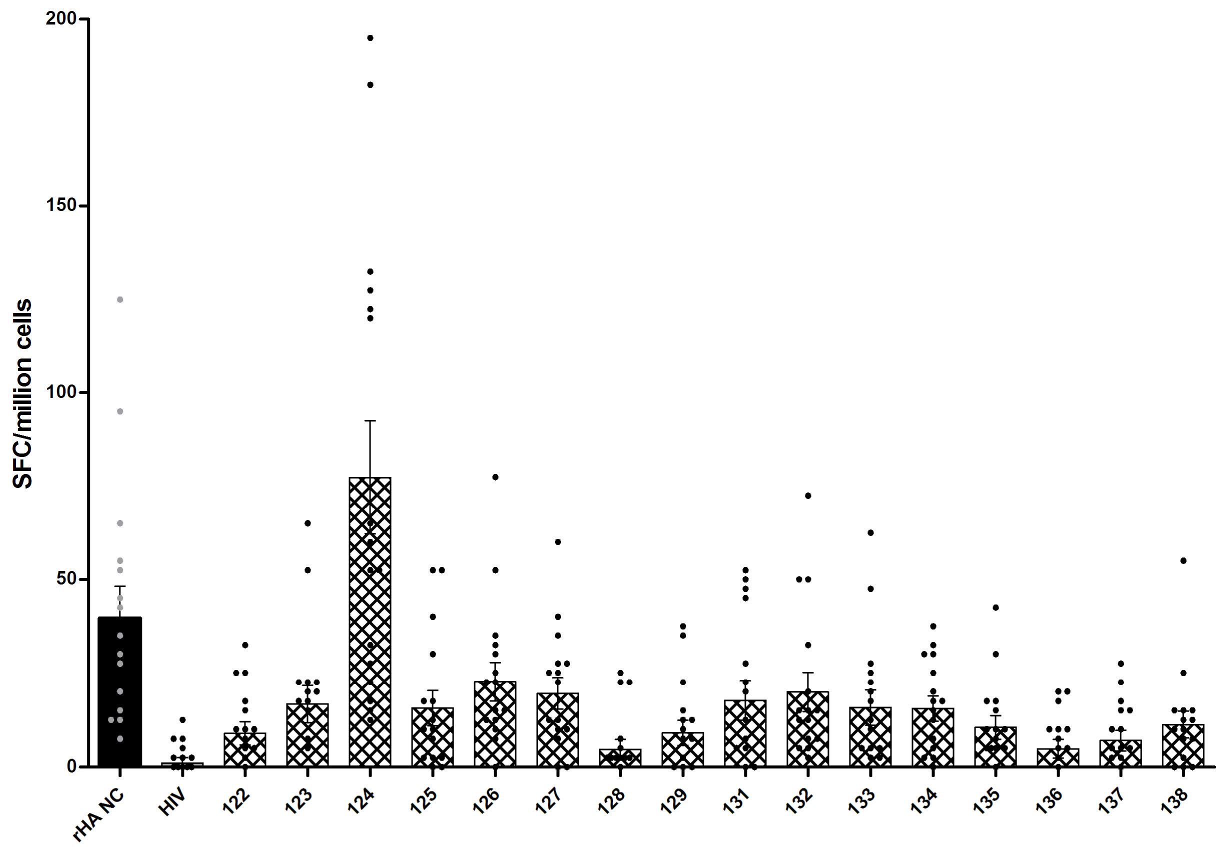
**Table 7. IFN-γ secretion levels in PBMCs from human donors**

Average spot-forming cell counts (as determined by ELISpot assay specific for human IFN-γ, per million cells with baseline IFN-γ production in unstimulated cells subtracted) of each of the sixteen donors against the sixteen different synthetic influenza peptides and the included controls. The peptide that resulted in the highest SFC value is bolded for each donor, and higher SFC values are highlighted (yellow if between 20 and 99 and red if above 100).

| | Donor | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1144** | **1145** | **1146** | **1147** | **1148** | **1149** | **1150** | **1151** | **1154** | **1155** | **1156** | **1157** | **1158** | **1159** | **1160** | **1161** |
| **rHA** | 12.5 | -5 | 42.5 | 45 | 20 | 35 | 12.5 | 125 | 30 | 7.5 | 52.5 | 55 | 95 | 27.5 | 65 | 15 |
| **HIV** | 5 | -10 | -2.5 | 2.5 | -7.5 | 0 | -2.5 | 2.5 | 12.5 | 0 | 7.5 | 0 | 0 | 0 | 7.5 | 2.5 |
| **122** | 25 | -7.5 | 2.5 | -2.5 | 10 | 17.5 | 7.5 | -12.5 | 15 | 0 | 32.5 | 10 | 5 | 25 | 10 | 5 |
| **123** | 17.5 | -7.5 | 20 | 22.5 | -7.5 | 5 | -2.5 | 20 | 52.5 | -2.5 | 65 | 22.5 | 7.5 | 22.5 | 15 | 17.5 |
| **124** | 17.5 | **12.5** | **22.5** | 27.5 | **195** | **32.5** | **122.5** | **120** | **182.5** | **15** | **127.5** | **52.5** | **65** | **132.5** | **60** | **52.5** |
| **125** | 10 | -7.5 | 2.5 | 12.5 | 0 | 0 | 2.5 | 10 | 40 | 7.5 | 52.5 | 17.5 | 2.5 | 30 | 52.5 | 17.5 |
| **126** | 15 | -7.5 | **22.5** | 0 | 7.5 | 12.5 | 12.5 | 52.5 | 77.5 | 15 | 35 | 25 | 32.5 | 22.5 | 30 | 10 |
| **127** | 22.5 | -2.5 | 12.5 | 27.5 | 0 | 12.5 | 7.5 | 25 | 60 | 0 | 10 | 35 | 25 | 40 | 27.5 | 10 |
| **128** | 0 | -5 | -2.5 | 2.5 | -12.5 | 2.5 | 5 | 22.5 | 7.5 | 2.5 | 2.5 | 25 | 2.5 | -2.5 | 22.5 | 2.5 |
| **129** | 0 | -5 | 2.5 | 12.5 | -7.5 | 7.5 | 0 | 0 | 37.5 | 10 | 22.5 | 15 | -5 | 12.5 | 35 | 7.5 |
| **131** | -5 | 5 | 0 | **50** | -5 | -2.5 | 20 | 27.5 | 52.5 | 7.5 | 22.5 | 5 | 0 | 47.5 | 45 | 12.5 |
| **132** | 12.5 | -5 | 15 | 5 | 7.5 | 2.5 | 12.5 | 15 | 50 | 5 | 72.5 | 15 | 20 | 32.5 | 50 | 7.5 |
| **133** | 5 | -7.5 | 2.5 | 17.5 | -5 | 5 | 2.5 | 25 | 62.5 | 5 | 12.5 | 20 | 22.5 | 27.5 | 47.5 | 10 |
| **134** | **25** | -7.5 | 17.5 | 15 | 2.5 | 12.5 | 2.5 | 20 | 30 | 5 | 17.5 | 30 | 7.5 | 37.5 | 32.5 | 0 |
| **135** | 15 | -10 | 5 | 10 | 7.5 | 10 | 5 | 42.5 | 5 | 0 | -2.5 | 30 | 10 | 17.5 | 17.5 | 5 |
| **136** | 10 | -10 | -2.5 | 2.5 | -12.5 | 7.5 | -2.5 | 20 | 20 | -2.5 | 10 | 17.5 | 5 | 10 | 5 | 0 |
| **137** | 5 | 10 | -2.5 | 0 | -10 | 5 | 10 | 2.5 | 17.5 | 2.5 | 22.5 | 15 | -12.5 | 15 | 27.5 | 5 |
| **138** | 7.5 | 0 | 7.5 | 15 | -7.5 | 2.5 | 0 | 10 | 15 | 10 | 12.5 | 25 | 0 | 15 | 55 | 12.5 |

**Figure 9. Overall IFN-γ secretion levels in PBMCs from human donors**

Comparison of ability of 16 different influenza T-B and B-T peptides, HIV gag peptide (HIV) and recombinant A/New Caledonia/20/99 HA (rHA NC) to induce IFN-γ production in human PBMCs. Stimulants were tested in duplicate, and an adjusted average number of spot-forming cells (SFC) per one million cells are shown ± SEM (baseline level of IFN-γ production in unstimulated cells was assessed in each donor and subtracted from individual counts). Individual SFC values (as presented in Table 7) are shown by •, while mean SFC values across all donors (average of all donors) are represented by a bar. All peptides except 128, 129, 136, and 137 had a $p$ value lower than 0.05 in a Mann Whitney test.



SFC/million cells
200
150
100
50
0
rHA NC
HIV
122
123
124
125
126
127
128
129
131
132
133
134
135
136
137
138

# 4. DISCUSSION

In the field of vaccine development, novel alternatives to traditional immunization approaches are presently being explored; in the case of influenza virus currently available vaccines have several inherent drawbacks, as outlined above. Utilizing short synthetic peptides presents an exciting possibility to replace whole protein or inactivated virus vaccines. Such constructs are attractive options, as synthetic vaccine components can be mass-produced and purified with relative ease, and are fully customizable (3, 27). However, without the addition of powerful adjuvants, synthetic epitope-based vaccines are not highly immunogenic (87, 154). This presents a concern, as only alum and monophosphoryl lipid A (MPL) have been tested for human use, and are still under evaluation (16). We therefore thought it worthwhile to examine whether the immunogenicity of these potential vaccine alternatives could be improved.

In the present study, we have investigated the immunogenic potential of totally synthetic influenza peptide constructs, consisting of a T-helper epitope and a B-cell epitope of the HA of the A/Puerto Rico/8/34 H1N1 influenza virus strain. It has previously been shown that such a linked T-B peptide construct successfully elicits both humoral and cellular immune responses when administered to mice (36). Although the responses induced by the linked peptide were only moderate, no significant responses were detected in mice immunized with equimolar amounts of non-linked T and B peptides, suggesting that such a conjugated epitope design is a fair starting design to develop synthetic peptides of improved immunogenicity. In an effort to improve the effectiveness of this simple T-B peptide, we used various bioinformatic tools (an approach that made use of sequence databases, software to align and compare sequences, and BLAST analysis) (154) to design and analyze a set of influenza peptides based on Brumeanu *et al*'s original

design. In the studies described herein, several factors that could improve the efficacy of short synthetic peptide immunogens were investigated. Namely, we evaluated lipidation, construction of MAPs, inclusion of a cysteine residue and various mutations for differences in activity in both humoral and cellular assays.

In line with previous findings that T-cell epitopes are less variable than B-cell epitopes (38), the sequence used for our T-helper epitope was found to be conserved in all H1N1 strains recorded since 1934. Such non-variable regions are postulated to evade mutations during replication because they have vital viral functions (82), and therefore inclusion of conserved epitopes in a synthetic vaccine may result in broad-spectrum protection against multiple strains and potentially even future variants (38). Indeed, various studies, especially those focusing on the hypervariable HIV-1 virus, have suggested an advantage in using conserved sequences when designing prophylactic agents (82).

On the other hand, upon aligning post-1934 H1N1 genomes against the B-cell epitope, this region was found to be variable at three positions. All possible mutants were synthesized and evaluated, but the epitope from the A/Puerto Rico/8/34 strain was chosen as the "parent epitope" used for the MAP and/or lipidated constructs, as this sequence has been previously characterized as being immunodominant (35, 43).

The NCBI BLAST software employed in the above analysis was also used to compare the epitopes contained in our constructs against the proteins encoded by the human genome. This is an important step when designing peptides for use as immunogens, as similar sequences could result in autoimmune reactions upon vaccination of human patients (16, 162). Upon analysing the sixteen influenza peptides and the individual epitope components no similarity whatsoever to

the human genome was found, suggesting that the peptide constructs would not induce adverse self-immune reactions.

In order to assess the potential efficacy of the different B-cell epitope designs in eliciting humoral responses, we screened the various influenza peptides by ELISA assay that measured antibody binding using sera from immunized sheep and plasma from humans. When the peptides were tested against sera from animals immune to A/New Caledonia/20/99 H1N1 strain, peptides 124, 125, 127 and 131 bound the antibodies at high rates. Constructs 124, 125, and 131 incorporate two lipid chains, four T-helper epitopes, or one lipid chain respectively, suggesting that increased molecular complexity enhances binding, possibly by orienting the B-cell epitope in a more natural configuration. It was not surprising that peptide 127 was among the most reactive in the ELISA assay, as this design consists of four B-cell epitopes on a T-helper epitope backbone and therefore there were more epitopes present to bind available antibodies. However, this peptide was not associated with the highest binding, perhaps because the added B-cell epitopes were obscuring one another. Upon screening the peptides with samples from immunized human patients, a similar trend arose; constructs 124, 125, and 131 once again bound the antibodies at higher rates (at statistically significant levels, with $p < 0.0001$). As in the assays performed with sheep sera, peptide 124 was the most reactive. Interestingly, the constructs with multiple B-cell epitopes did not perform well in the assays utilizing human plasma.

Neutralizing antibodies, specifically those directed at the HA protein, have been characterized as being of major importance in protection against influenza infection (11, 26, 38, 62). The ELISA assays above measured direct antibody binding, but not all these antibodies may be neutralizing; this assay assesses binding of all virus-specific antibodies while only those that

actually inhibit viral functions might offer protection (62). We therefore modified two developed assays (75, 155) that quantify functional binding to incorporate a competitive aspect. Binding of neutralizing antibodies was characterized indirectly via inhibition of virus neutralization, whereby a peptide was deemed an effective construct if it was able to preferentially bind neutralizing antibodies resulting in decreased virus neutralization by immune samples.

It should be noted that we chose to not screen the different peptides by hemagglutination assay, even though this assay is very commonly used in influenza vaccine functional analysis. We had previously found that the microneutralization assay is a slightly more sensitive test. (155) Of more importance to this particular investigation, however, is the fact that neutralizing antibodies are not always hemagglutination inhibiting and vice versa (18), and therefore an absence of hemagglutination inhibition activity is not necessarily accompanied by an absence of virus neutralization activity (96-98, 174). Thus, the results of a hemagglutination assay may or may not be of any relevance in terms of the peptides assessed herein.

Pre-incubating immune human samples with any of the synthetic influenza peptides prior to testing by competitive microneutralization assay resulted in decreased virus neutralization as compared to the HIV gag peptide control, indicating that neutralizing antibodies were indeed being bound. Specifically, constructs 123, 125, 127, 128, and 129 were associated with larger changes in virus neutralization ($p < 0.0001$). Peptides 125 (MAP with a B-cell epitope backbone and four T-helper epitope arms) and 127 (MAP with a T-helper epitope backbone and four B-cell epitope arms) were both associated with increased binding activity in at least one of the two types of ELISA screening. Meanwhile, constructs 123 and 128 (branched peptides with a B-cell epitope backbone and two T-helper epitope arms or a T-helper epitope backbone with two

lipidated B-cell epitope arms, respectively) are also MAP constructs, in which the B-cell epitopes were presumably found in an orientation similar to that in the native HA protein, a factor that leads to enhanced recognition and binding by neutralizing antibodies. The other lipidated MAP design (peptide 124), which was the most reactive in the ELISA screenings, was not included in the above group of peptides that showed high reactivity. However, this construct was still able to bind neutralizing antibodies, and performed on par with the unmodified peptide (construct 122). Construct 129, with an N-terminal cysteine residue at the T-helper epitope end, was included in this study to address the concern that oxidation of this residue could cause peptides to crosslink via disulfide bonding, resulting in impaired activity. Indeed, a previous study had found that an air-oxidized T-cell epitope from hen egg-white lysozyme inefficiently stimulated T-cells (92). However, this peptide contained two cysteine residues within the same epitope which presumably caused intra-peptide bonds (and incorrect folding). The construct in our study, containing a single cysteine residue, seems to be undergoing inter-peptide disulfide bonds. Oxidation thus actually enhanced the activity of peptide 129 in functional humoral assays, possibly because cross-linking resulted in heavier molecules.[2] Also of note, all mutant peptides (132 – 138) were associated with decreased virus neutralization relative to the original construct (122). The non-mutated B-cell epitope has previously been characterized as being immunodominant, and therefore these results suggest that the mutations that were incorporated were sufficient to decrease activity.

It should be noted that it was necessary to use two different virus strains for the competitive microneutralization assay and the competitive plaque reduction assay. The latter

[2] In terms of immunoreactivity in cellular assays, construct 129 proved as efficient or a better immunogen than its non-cysteinated equivalent, peptide 122 (refer to Figure 8 and 9).

assay was incubated for 3 days, during which the A/New Caledonia/20/99 strain used grew to high enough viral titers to detect infectivity. On the other hand, the competitive microneutralization assay had previously been optimized for overnight incubation. The influenza virus strain used in the competitive plaque reduction assay was tested, but did not grow to detectable levels in this shorter amount of time (data not shown). Thus another influenza H1N1 virus strain (A/Puerto Rico/8/34) was used. However, the results of the competitive plaque reduction assay were still somewhat in agreement with those of the competitive microneutralization assay. When screening the constructs for neutralizing antibody binding activity by competitive plaque reduction assay using sera from sheep immune to the H1N1 strain A/New Caledonia/20/99, again all constructs were able to inhibit virus neutralization relative to the negative control (HIV gag peptide). Furthermore, peptides 123 and 125 gave better results, as determined by a higher plaque count. These two peptides were also among the high binders in the competitive microneutralization assay. When the various peptides were screened using immune human plasma samples, the results were quite a bit different. Once more, all influenza peptides were able to bind neutralizing antibodies to some degree when compared to the HIV gag peptide control. Peptides 123 and 125 (MAP constructs with two or four T-helper epitope arms on a B-cell epitope trunk, respectively) were, as in the previous functional binding assays, more efficient than peptide 122, the "original" backbone construct. However, these two peptides were not associated with the highest reactivity. Rather, peptides 126, 133, 135, and 138 showed the largest change in plaque count (statistically significant, with $p < 0.0001$, $p = 0.0004$, $p = 0.0002$, and $p = 0.0027$, respectively). Peptide 126 is a MAP construct with two B-cell epitopes, and should in theory be able to bind more neutralizing antibodies simply because there are more available binding sites. However, this peptide had not been among the better constructs in the

other binding assays, whether functional or not. It was quite interesting that three mutated B-cell epitopes were among the higher binding peptides. Although the B-cell sequences of mutated constructs 133 and 138 had been found to have 86 and over 500 matches in the NCBI database, respectively, mutated peptide 135 did not have any exact matches. In other words, a sequence that does not actually occur under natural circumstances was able to effectively bind neutralizing antibodies. It should be stressed that these results are those of the average plaque counts of all 16 human donors. Individual responses were fairly varied, both in terms of which peptides had the largest effect on plaque count and the degree of this effect.

In order to study cellular responses as stimulated by our peptide constructs, the ability of the various synthetic influenza peptides to induce human PBMCs to mount a proliferative response and to produce IFN-γ was investigated. Examining peptide-induced proliferation across all 16 donors, all synthetic influenza T-B dipeptide constructs were effective stimulants (comparable to the recombinant influenza hemagglutinin protein). Stimulation with peptide 126 or 131 (two B-cell epitope arms on a T-helper epitope trunk or lipidated T-B peptide, respectively) led to slightly higher average stimulation (with $p < 0.0001$), however overall cell-mediated immune responses as evidenced by cell division were not substantially high. It should be noted that the T-helper epitope included in this study is known to be recognized by the murine MHC class II molecule $IE^d$ (35, 79, 151), which based on its amino acid sequence is structurally equivalent to the human HLA-DR molecule (76, 93). As previously stated, T-cell responses depend on proper antigen presentation by HLA molecules, which are highly polymorphic (56, 162). It is reasonable to expect that the T-helper epitope investigated herein can elicit T-cell epitope responses in humans and in the context of several HLA haplotypes. It is also fair to expect that different haplotypes will bind the epitope at varying specificities. Since we were

working with cells from a varied human donor group, it was not surprising that the general stimulation was low. It is thus necessary to examine donor-specific responses, and it becomes important to note that PBMCs from >50% of patients proliferated at levels higher than 5-fold relative to unstimulated control cells in response to peptide 124. We expected this construct to induce a robust proliferative response, as it has two T-helper epitopes that are flanked by lipid moieties. Thus it has multiple and structurally stable epitopes available for presentation, and may therefore be captured by more APCs thereby initiating a stronger cellular immune response.

Examining the IFN-γ secretion profile it becomes even more clear that peptide 124 is a suitable construct. This construct, even when looking at average IFN-γ production in all 16 donors, induces far higher levels of this cytokine than any of the other constructs ($p < 0.0001$).

In summary, although peptide 125 (MAP with four T-helper epitopes on a B-cell backbone) was best across all antibody binding assays, peptide 124 was best overall – this construct performed well in binding assays and resulted in the highest overall cellular immune responses. Thus it appears that the design of construct 124 (lipidated MAP with two T-helper epitopes on a B-cell backbone) is optimal as evaluated herein. The branched and lipidated nature of this molecule may be positioning the B-cell epitope in an optimal conformation, while there are several T-helper epitopes that are lipid-stabilized, leading to proper presentation to multiple T-cells.

## 5. CONCLUSION AND FUTURE DIRECTION

In modifying a simple synthetic influenza T-B epitope, we were able to improve its activity in both antibody binding and cellular assays. Screening the different designs revealed the best overall construct to be a lipidated influenza MAP with two T-helper epitopes on a B-cell backbone. These results suggest that the most critical factor in creating an immunogenic peptide is mimicking a native epitope conformation.

High activity in *in vitro* assays is a fair starting point with which to evaluate vaccine candidates but does not necessarily guarantee *in vivo* protective effects. These constructs will therefore have to be evaluated in animal experiments. It would also be worthwhile to apply similar modifications to other known epitopes of the influenza virus, and perhaps even to other hypervariable viruses such as HIV or hepatitis C virus.

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