Investigation of a Trimeric Hemagglutinin Stem Domain from Influenza B for a Universal Vaccine

Amparo Duran

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Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

ABSTRACT

Influenza infection occurs in as much as 5–15% of the world population, resulting in 3–5 million cases of severe illness and up to 500,000 deaths annually. According to the CDC, on average 24% of all influenza positive respiratory samples during 2001 to 2011 tested positive for Influenza B. Influenza has two main surface glycoproteins, neuraminidase (NA) and hemagglutinin (HA), HA being responsible for the binding of the virus to the host cell. Currently, seasonal influenza vaccines are produced using two strains of Influenza A and one or two strains of Influenza B viruses recommended by the World Health Organization (WHO). These vaccines are mainly targeting the head domain of the HA protein, which mutates constantly, hence the need for annual vaccine updates. The goal of this research is to develop an experimental universal vaccine against influenza B and increase our knowledge to help pave the way for finding a one-time vaccination alternative, reducing the need for a yearly flu shot. To achieve the above, protection and toxicity studies were conducted in DBA/2 mice immunized with a designed HA2 adenoviral-vectored vaccine targeting the HA stem region of influenza B. Results showed that this designed vaccine was able to confer 100% survival protection, this was supported by lower viral titer in trachea and lung tissues. Additionally, we studied the influence of CD40L as a targeting adjuvant, by analyzing its effect on the humoral and cellular immune response, where results showed that it has a significant effect by inducing a higher T_H1-bias response. This research is the first report that leads us to a better understanding of the potential use of a conserved consensus HA2 sequence to induce protection against influenza B virus.

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DEDICATION

A mi mamá y papá.

Por impulsarme a seguir mis metas por mas locas que estas parecian.

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LIST OF ABREVIATIONS

Abs: Antibodies

Ads: Adenovirus

ADCC: Antibody-dependent cell. Mediated cytotoxicity

ANOVA: Analysis of variance

APC: Antigen Presenting Cells

ATCC: American Type Culture Collection

BALF: Bronchoalveolar lavage fluid

BGTD: Biologics and Genetic Therapies directorate

bp: Base pairs

BSA: Bovine serum albumin

B/Vic: Influenza B virus strain Victoria 2/87

B/Flo: Influenza B virus strain Florida 04/06

CD40L: CD40 ligand

CMV: Cytomegalovirus

CTL: Cytotoxic T-lymphocytes

DC: Dendritic cells

DMEM: Dulbecco's modified Eagle's medium

dsRNA: double strand RNA

E. Coli: *Escherichia coli*

ELISA: Enzyme linked immunosorbent assay

F: Trimerization motif

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

G: grams

GC: Germinal Centers

H&E: Hematoxylin and Eosin

HA: Hemagglutinin

HA1: Hemagglutinin subunit 1

HA2: Hemagglutinin subunit 2

HCl: Hydrogen chloride

HeLa: Henrietta Lacks, uterine cell variety

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP: Horseradish peroxidase

IFN: Interferon

Ig: Immunoglobulin

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgG1: Immunoglobulin G1

IgG2a: Immunoglobulin G2a

IgG2b: Immunoglobulin G2b

IIV: Inactivated influenza vaccines

IL: Interleukin

kDa: Kilodalton

L: liters

LNs: Lymph nodes

M1: Matrix protein 1

M2: Matric protein 2

MDCK: Madin-Darby canine kidney

MEM: Minimum Essential Medium

µg: Microgram

mg: Milligram

MHC: major histocompatibility complex

µl: Microlitre

ml: Millilitre

mM: Millimolar

MOI: Multiplicity of infection

NA: Neuraminidase

NAI: NA inhibitor

NCBI: National Center for Biotechnology Information

NK : Natural killer cells

NP: Nucleoprotein

NEP: Nuclear export protein

NS1: non-structural protein 1

PA: Polymerase acidic protein

PB1: Polymerase basic protein 1

PB2: Polymerase basic protein 2

PB1-F2: Polymerase basic protein 1-F2

PBS: Phosphate buffered saline

PBST: PBS with 0.05% Tween 20

PBS-T: PBS with 0.1% Tween 20

Pe-Cy5: Phycoerythrin-Cyanine 5

PCR: Polymerase chain reaction

PKR: RNA-dependent protein kinase

PFU: Plaque forming units

PVDF: polyvinylidene difluoride

QIV: Quadrivalent influenza vaccine

rAd- HBHA2F: rAd expressing secreted influenza B HA2-fusion protein

rAd-Empty: rAd empty control vector

rAd-HAFCD40L: rAd expressing secreted influenza A HA2-CD40L fusion protein

rAd-HBHA2FCD40L: rAd expressing secreted influenza B HA2-CD40L fusion protein

rAd: Recombinant adenovirus

RDE: Receptor-Destroying Enzymes

RNP: Ribonucleoprotein

PKR: RNA-dependent protein kinase

RPM: Revolutions per minute

S: Secretion signal

SDS: Sodium dodecyl sulfate

x g: Centrifugal force x gravity

TBS-T: 1x Tris buffer saline with 0.01% Tween 20

TBST: 1x Tris buffer saline with 0.1% Tween 20

$TCID_{50}$: Median tissue culture infectious dose

TIV: Trivalent influenza vaccine

T_H1: Type I helper $CD4^+$ T cells

T_H2: Type II helper $CD4^+$ T cells

TMB: Tetramethylbenzidine

TPCK: L-(tosylamido-2-phenyl) ethyl chloromethyl ketone

WHO: World Health Organization

vRNP: Viral Ribonucleoprotein

Chapter 1 General Introduction

1.1 Influenza Viruses

Influenza is a viral infection, commonly known as flu; usually presented as fever, aching muscles, chills, sweat, headache, fatigue, nasal congestion and sore throat, with a chance of resulting in complications such as pneumonia (CDC; Mayo Clinic, 2017); it can cause highly contagious respiratory tract infections with symptoms associated with high morbidity and mortality.

The influenza virus was first isolated in 1931 from pigs (Shope, 1931); and since then the number of isolated strains has increased considerably. The virus infects persons of all ages, being the most severe cases among the young children and elderly (Fiore et al., 2008); up to a quarter of the world population are infected annually, resulting in over 500,000 deaths (Nicholson, K.G. and Lamb, R.A.; WHO, 2003). Additionally, influenza virus evolves in an unpredictable fashion, with new strains generated as a result of antigenic drift and/or antigenic shift (van de Sandt et al., 2012 and CDC-NCIRD, 2017), allowing the virus to escape host immune responses.

1.1.1 Classification and nomenclature

Influenza is part of the *Orthomyxoviridae* family; a particular characteristic is their external layer of approximately 500 spike-like projections on the envelope, which are glycoproteins known as hemagglutinin (HA) and neuraminidase (NA) (Nicholson, K.G. et al., 1998; Lamb, R.A, 2001).

Influenza viruses are classified into three types based on its genetic makeup: A, B and C. The first two are known to cause seasonal epidemics, while influenza C causes a mild respiratory illness and is not known to cause epidemics. The influenza A viruses are divided into subtypes based on their two surface proteins, hemagglutinin and neuraminidase. Up to date, there are 18

different HA subtypes (1-18) and 11 different NA subtypes (1-11) whereas influenza B viruses are not divided into subtypes but can be classified based on its genetic lineages, i.e., B/Victoria and B/Yamagata (CDC, 2017; Zambon, 1999).

The current internationally accepted nomenclature for influenza viruses is based on the antigenic type, the host of origin, the geographical origin, strain number and the year of isolation (CDC, 2017).

1.1.2 Influenza virus structure

Influenza is a single-stranded, negative-sense RNA virus that possesses a segmented genome: influenza A and B contain eight RNA segments that encode 17 proteins and 11 proteins respectively. Of all identified viral components, these viral proteins were found in both subtypes: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NS1), nuclear export protein (NEP), polymerase acidic protein (PA), polymerase basic protein (PB1) and prolymerase basic protein 2 (PB2) (James, S. 2016; van de Sandt, et al. 2015). The virion has an irregular spherical shape with a lipid envelope.

The surface is covered by the glycoproteins as mentioned above, HA and NA, both of which are found on the surface of the virus, with HA playing a role in viral entry and HA viral release (Samji, T. 2009). One matrix protein, M2, can also be found in the viral envelope, functioning as a transmembrane ion channel component participating in the viral uncoating step during replication. The other matrix protein, M1, is within the lipid bilayer, surrounding the virus core, which is a ribonucleoprotein (RNP) complex, consisting of the RNA segments, the NP and RNA polymerase complex comprised of three polymerase proteins (PB1, PB2 and PA) (James, S. 2016 and Samji, T. 2009).

1.1.3 Influenza's life cycle

The influenza virus life cycle can be divided into 5 stages: entry into the host cell; entry of viral RNPs into the nucleus; transcription and replication of the viral genome; export of the vRNPs from the nucleus and assembly and budding at the host cell plasma membrane (Samji, T. 2009).

The first stage occurs when the HA glycoproteins bind to sialic acid on the host cell surface, facilitating the viral entry by receptor-mediated endocytosis. In the host cell cytoplasm, the vesicle containing the virus undergoes an acidification process, triggering a conformational change in the HA, exposing the fusion peptide and permitting the exit from the endosome; at the same time, hydrogen ions are pumped from the endosome through the M2 ion channel into the virus particle, allowing the viral RNPs to be released, followed by the second stage involving active transportation of the viral components into the nucleus through the nuclear pore complex (James, S. 2016; Samji, T. 2009). The third stage starts with the transcription of the viral genomic RNA into messenger RNA (mRNA), followed by the mRNA being exported to the cytoplasm where replication takes place. Next, the newly synthesized viral RNAs are exported to the host cell surface where RNP-M1-NEP complex assembles at the cytoplasmic membrane (fourth stage). The fifth and final stage of the cycle is for the viral particles to be formed and leave the cell. As an enveloped virus, the virus takes advantage of the host cell's plasma membrane to form its own viral particles. New virus particles bud on the apical side of polarized cells where the viral genome is packaged into virions before NA removes the sialic acid from the virus, allowing the viral particle to be released (James,S. 2016; Nayak, D.P. et al, 2009; Samji, T. 2009).

1.1.4 Influenza's glycoproteins

The two main proteins on the viral surface are HA and NA, both of which are involved in the life cycle of the virus. Specifically, HA mediates the binding and fusion of the virus to the host cell while NA releases the new viral particles from the infected cell. (see *1.1.4 Influenza's Life cycle*). Given their importance in the virus life cycle, antibodies against HA block viral infection. However, constant immune pressure from the hosts also plays important roles in generating escaping mutants (Gamblin, S.J. et al. 2010; Samji, T., 2009).

1.1.4.1 Influenza neuraminidase (NA)

Neuraminidase (NA) is a mushroom shaped tetramer, with approximately 470 amino acids. The protein can be defined into three regions including the cytoplasmic portion, transmembrane and stem. NA is the second most abundant glycoprotein, constituting approximately 17% of the viral envelope (Samji, T. 2009). Up to date there are 11 NA subtypes for influenza A with about 50% homology between them, whereas only one NA subtype for influenza B was found. There are about 30% homology between type A and type B NA. It is known that the amino acid residues involved in its catalytic function and the dimensional structure of the active site are among the homologies between influenza A and B NA (Shtyrya, Y.A. et at. 2009).

As mentioned earlier, NA is very important in that it is responsible for removing the sialic acid from the budding virus particle on the cell surface, thereby releasing the newly formed viral particles from the surface of the infected cell. It is a critical step in aiding virus transmission and preventing virus aggregation. Currently, anti-NA drugs have been used in clinics for the treatment of influenza, particularly in the elderly or immune-compromised individuals. In

addition, there is evidence suggesting that the NA protein may also facilitate the entry of the virus into the respiratory tract, in addition to its role in the release of the virus as discussed earlier. It appears that NA can bind the sialic acid present in the mucosal surface that coats the epithelial cells of the respiratory tract, thereby functioning in coordination with HA proteins in the viral entry process (Racaniello, V. 2013; Yang, J. et al. 2016).

1.1.4.2 Influenza hemagglutinin (HA)

Hemagglutinin (HA) is the major envelope glycoprotein responsible for mediating the entry of the virus into the host cell, and it is one of the primary targets of host neutralizing antibodies. Constituting approximately 80% of the viral envelope, it is the most abundant glycoprotein; HA is initially synthesized in the infected cell as a single polypeptide known as HA precursor (HA0), which is subsequently cleaved into two subunits HA1 and HA2 linked by a disulphide bond (Samji, T. 2009).

HA1 is the globular domain at the distal end of the spike, containing the receptor-binding site (Fan et al., 2015; Gerhard, W., et al., 1981; Hashem, A.M., et al., 2010; Wiley, DC., et al, 1987), while HA2 forms the fibrous stem of the viral spike which anchors the globular domain to the viral membrane. In its N-terminus HA has a conserved region of 20 amino acid residues; this sequence is generally referred to as the fusion peptide which is essential in mediating the membrane fusion between the viral envelope and the host cell membrane (Ambrose C.S., et al., 2004; Boyce WM, et al., 2009; Fan et al., 2015; Skehel J.J., et al., 2000; Vijaykrishna D. et al., 2015; Wilson I.A, et al., 1981).

1.2 Influenza B

Previous studies indicate that even though influenza A and B are known to be adapted to humans, they diverged at some point during the evolution process. First isolated in 1940, influenza B cases may generally constitute ¼ of all influenza annual cases, but it is not uncommon for influenza B to dominate in a given season. Specifically, according to the last reports by the World Health Organization (WHO), among all confirmed influenza cases, the cases of influenza B went from 22.7% to 50.9% in the last year, while 94.4% of cases belong to B-Yamagata lineage and 5.6% to the B/Victoria lineage (WHO, 2017).

Contrary to influenza A, there is no clear evidence supporting continuously circulating influenza B viruses among animals, although there have been sporadic reports on isolation of influenza B strains in animals including dogs and two seal species. It was also reported that infections of influenza B were found in animals living in close proximity to humans, such as guinea pigs, pigs, horses and ruminants (Romvary J, et al., 1980; Osterhaus A.D. et al., 2000; van de Sandt, 2015). However, animals susceptible to influenza B infection are not as reliable as that for influenza A infections in terms of clinical manifestations similar to that in humans. Although a variety of animals have been explored to study influenza B virus, such as ferrets, guinea pigs, pigs and macaques, the observed clinical signs and severity were found to vary depending on the virus strain and route of administration (Huang, S.S. et al. 2011; Huang, S.S., et al., 2014; Pica, N., et al. 2011; van de Sandt, 2015).

Similar to influenza A, influenza B virus can infect people of all age groups, with a higher incidence in young children and seniors, with similar symptoms including fever, cough, abdominal pain, headache, vomiting and in some cases even conjunctivitis. Pathological examination reveals evidence of virally infected epithelial cells in the upper respiratory tract, ie,

the presence of virus and inflammatory reactions in trachea and bronchi (Kumlin, U., et al., 2008; Nicholls, J.M., et al., 2008; Paddock, C.D., et al., 2000; Rogers,G.N., et al., 1983; van de Sandt, 2015).

There are some interesting studies on influenza B virus-induced pathogenesis. One of the studies was focused on the role of glycosylation of the HA protein with respect to the infection of epithelial cells in the upper respiratory tract; it appears the extent of glycosylation might be related to the efficiency of viral infection. Some studies have been conducted to determine if the relatively less effective infection by influenza B virus compared with influenza A virus is due to the lower receptor affinity, particularly in the infection of the lower respiratory tract (Hillaire M.L, et al., 2015; Huang, S.S. et al., 2014; Mccullers, J.A., et al., 2012; van de Sandt, 2015; Velkov. T., 2013; Wang, Y.F., et al., 2012). These previous studies indicate that the lower receptor-binding affinity of influenza B virus could be related to a difference in the receptor binding sites in the HA protein. Specifically, influenza B virus has a Phe-95 instead of a Tyr-98 in influenza A HA. The difference between this amino acid position could result in the loss of hydrogen bonds that are required to stabilize the base of the receptor binding sites in the HA side chain (Matrosovich, M.N., et al., 1993; van de Sandt, 2015; Wang, Q., et al., 2007).

1.2.1 Influenza B genomic organization

Although influenza B viruses are not as diversified as influenza A viruses, there are two lineages known to be genetically distinct. Even though both lineages diverged from a single ancestor virus, it is believed B/Victoria lineage started slowly drifting apart around 1970s in China (Chen, J.M. et al., 2007; Lindstrom, S.E., et al., 1999; van de Sandt, 2015). Moreover, although both influenza B lineages co-circulate every season along with influenza A, cases of

influenza B/ Yamagata are generally more common, with the exception that in 1980s influenza B/Victoria cases were found to be more predominant.

It is also noted that while both influenza B lineages originated from a single ancestor, they follow different patterns, with strong evidence indicating that similar to influenza A viruses, influenza B viruses are drifting in antigenicity. It is estimated that influenza B has a mutation rate of $2.0x10^{-3}$ substitutions per site per year; these mutations have been predominantly observed in B/Victoria lineage. Studies also show that these mutations are frequently found in the HA glycoprotein, particularly surrounding the receptor binding site in HA1 subunit (Bedford T, et al., 2014; Koel BF, et al., 2013; Krystal M, et al., 1983; Rota PA, et al., 1992; Smith, DJ. et al., 2004; van de Sandt, 2015; Vijaykrihna D., et al., 2015).

1.2.2 Influenza B virus HAs

Antigenic differences between the two influenza B lineages are due to the HA glycoproteins. Approximately 93% to 95% of the HA sequence of influenza B is conserved between both lineages of influenza B, whereas only 35% of homology is found between influenza A and B. Clearly, this indicates that an influenza A vaccine would be incapable of inducing protection against influenza B viral infection.

Sequence analyses have revealed four major epitopes on the influenza B HA1 protein, responsible for the differences between influenza B strains. The 120-loop, 150- loop, 160-loop and 190-helix and their respective surrounding regions have a high structural flexibility, making them prone to drifting mutations without compromising the structural integrity but these mutations would be enough to allow the virus to evade attack by neutralizing antibodies. In contrast, sequence differences, ie., amino acid substitutions are very few in the two genetic

lineages in influenza B HA2. Specifically, substitutions at positions 132 and 158 were found not to cause marked structural disruption and were fairly conserved (Berton et al., 1984; Berton and Webster, 1985; Hovanec and Air, 1984; Ni, F. et al., 2013; Kordyukova, et al., 2011; Rivera et al., 1995; Wang et al., 2008; Webster and Berton, 1981).

1.2.3 Innate and adaptive immune response to influenza B

The first line of protection after infection is the innate antiviral immune response. Studies indicate that interferon (IFN) response starts when endosomal membrane fusion occurs and the vRNPs are released into the cytosol. It is believed that IFN response is faster in influenza B infection than influenza A. The virus can employ a variety of strategies to counteract IFN response. Specifically, influenza viruses are encapsidated by NP, thereby avoiding the formation of dsRNA (van de Sandt, et al., 2012; van de Sandt, 2015). Additionally, several viral proteins of influenza virus can also interfere with the innate immune response. In the case of influenza B virus, NS1 protein can partially antagonize the response of IFN by binding to dsRNA produced during viral replication, thus inhibiting RNA-dependent protein kinase (PKR) activation (Dauber, B. et al., 2006; Dauber, B. et al., 2009; van de Sandr, 2015; Yin, C., et al., 2007).

The second line of defense is the adaptive immune system, which is mediated by virus-specific antibodies (humoral response) and virus-specific $CD4^+$ and $CD8^+$ T lymphocytes (cellular response). While cellular response will not prevent viral infection, it aids in the reduction on the severity of infection and the viral clearance; it is also possibly contributing to cross protections. As discussed above, both influenza B as influenza A viruses have developed their capabilities to evade immune responses through antigenic drift or antigenic shift (Mckinstry, K.K., et al, 2011; Sridhar, S., et al., 2013).

1.3 Current influenza vaccines, surveillance, selection and efficacy

Up to now, annual vaccination is the most effective way of preventing and reducing the number of influenza cases and their severity. Vaccination against influenza is highly recommended for elderly, young children and pregnant women. These current vaccines mainly induce strain-specific neutralizing antibodies (Abs) against the highly variable head domain of the viral HA (Beyer, W.E., et al., 2002; Cox, R.J., et al., 2004; van de Sandt, 2015).

Seasonal influenza vaccines are produced using the strains recommended by the WHO based on global epidemiological surveillance about 6-8 months ahead of the targeted flu season, and they currently have a trivalent or quadrivalent formulation, containing two strains of influenza A (H1N1 and H3N2) and one strain of one or both influenza B lineages (Carrat, F. et al., 2007; Cate, T.R., et al., 2010; WHO, 2004). This quadrivalent influenza vaccine (QIV) was developed because two influenza B lineages have been co-circulating since the 2001-2002 influenza season and the mismatch between the vaccine and the circulating B viruses during 2001/ 2011 flu season reduced the effectiveness of vaccine-induced protection against the B virus (Tisa, V., et al., 2016). However, the quadrivalent formulation is not available in every country, mainly due to its cost-effectiveness related to the virus epidemiology, prevalence and unit cost (Boer de, P.T., 2018).

Given the constant evolution of the viruses, both vaccine formulations have inherent disadvantages including the need for annual strain specific selection and the uncertainty of the actual circulating strains, potentially resulting in vaccine/virus mismatch which can significantly reduce the efficacy of the annual immunization. Moreover, there is also a need to update the reagents for quality analyses of strain-specific vaccines. All these shortcomings would be

dramatically exacerbated in the event of a pandemic influenza outbreak, given a much-shortened timeframe available for the production of a vaccine for global needs.

To circumvent the need for annual vaccination, great efforts have been put forward by the research community to develop universal influenza vaccines. While several studies have been reported for the development of universal vaccines against type A viruses, very little has been done on the development of universal type B influenza vaccines, even though influenza B viruses continuously co-circulates with the type A viruses and constitute at least a quarter of the cases of influenzas during seasonal epidemics.

1.3.1 Inactivated influenza vaccines (IIV)

The inactivated influenza vaccines (IIV) have been approved for all populations, including pregnant women and persons with chronic medical conditions. Initially developed as a whole inactivated virus, this type of vaccine encountered various problems in early years of the development due to the presence of impurities derived from egg production systems.

Today, IIV consist of either split virus or subunit antigens. Split virus is made by disrupting virus particles by either diethyl ether or detergent. The split vaccines may contain HA and other viral components, yet the amounts of other viral antigens have been monitored. It remains unclear as to if the loss of other viral components including NA and nucleoproteins in the vaccine preparations could have contributed to the loss of some immunogenicity (Geeraedts, F., et al. 2008; Soema, P.C., et al., 2015). Some studies indicate that subunit antigens devoid of other antigenic components would require the addition of adjuvants in order to enhance immune responses, particularly for vaccines targeting the elderly (Soema, P.C., et al., 2015; Squearcione, et al., 2003).

Virosomal influenza vaccines have been used mainly in the EU countries, for the production of IIV formulations. Virosomes are reconstituted influenza virus envelopes composed of external main glycoproteins HA and NA in addition to viral phospholipids (Herzog, et al., 2009; Soema, P.C., et al., 2015).

Most comparative studies were conducted amongst the different IIVs, with the results mostly suggesting similar immunogenicity and safety. However, such comparative studies in immunogenicity evaluated immune responses against the HA protein, it remains unclear as to how humans would respond to other viral antigenic components and what the true immunogenicity of these vaccines would be causing a lack in the immune response against the other viral components (Beyer, W.E.P, et al., 1998; Beyer, W.E.P, et al., 2011; Soema, P.C., et al., 2015).

1.3.2 Live attenuated influenza vaccines (LAIV)

LAIVs are produced by genetic assortment involving two strategies. Specifically, they could be made via cold adaptation or reverse genetics. The resulting virus contains the HA and NA coding genes of the circulating influenza virus strain, with remaining genes derived from an attenuated or weakened strain which is known to be less pathogenic to humans.

Approved for use in children and adults between 2 and 49 years old, LAIVs are administrated by intranasal route, mimicking the natural route of infection of the virus, resulting in a higher localized mucosal immune response. Compared to IIV, LAIVs elicit stronger IgA and cell-mediated immune responses. Indeed, studies performed on children and adults immunized with LAIVs where analysis of IFN-gamma-producing NK and T cells in blood were studied, have shown that LAIVs may induce antigen-specific CD4+T and CD8+T lymphocyte response

and have been reported to induce cross-reactive immunity. (Hai, R. et al., 2008; He, X.S, et al., 2006; Hoft, D.F., et al., 2011; van de Sandt, 2015).

Although LAIV has some advantages over IIV, there are also some concerns surrounding LAIV. Specifically, there are some questions raised with respect to likelihood that LAIV virus may undergo genetic reversion into a pathogenic influenza strain and observations that when administrated to infants under 2 years old LAIV may be associated with adverse reactions such as wheezing (Barria, M.I., et al., 2013, Carter, N.J., et al., 2011; Soema, P.C., et al., 2015; Tosh, P.K., 2008).

1.3.3 Experimental vaccines under development

Current influenza vaccines have been proven to be an effective strategy in helping decrease the occurrence, severity and mortality of influenza infections. However, there are obvious drawbacks associated with the current flu vaccines as discussed earlier, particularly the need for annual reformulation of vaccines because of the constant evolution of the circulating viruses as well as the mismatch between the vaccines and the actual circulating virus strains.

Some new and novel types of influenza vaccines have been vigorously developed including different forms of the vaccine such as recombinant proteins, viral vectors, peptides and DNA vaccines (Soema, P.C., et al., 2015). Importantly, efforts in developing universal vaccines that are capable of inducing a broadly cross-protective immune response have been strengthening in recent years. Specifically, investigators are trying to find antibodies that recognize a highly conserved sequence of the influenza virus, particularly the HA and NA proteins. Our laboratory has identified the first universal antibodies capable of binding to all HA and NA proteins (Chun, S., et al., 2008; Doyle, T.M., et al., 2013; Gravel, C., et al., 2015; Li, C., et al. 2010). Numerous

attempts have also been made to develop highly conserved stalk region of the HA proteins as prototype universal vaccines. Initial results have been promising as these prototype vaccines can induce immune protections in animals challenged with diverse strains of viruses (Berlanda, F., et al., 2016: Bianchi, E. et al., 2005; van de Sandt, 2015).

1.3.3.1 Influenza vaccines delivered by viral vector

Vaccines delivered by viral vectors are aimed at generating a recombinant replication-deficient virus that is able to produce influenza antigens. These vectored vaccines have been shown to be more effective in eliciting immune responses in many cases. One of the viral vectors extensively explored is modified adenovirus, which is linear non-enveloped DNA virus that can infect a wide range of mammalian cell; this virus can grow in very high titers in tissue culture and activate the innate immune response *in vivo*. The generation of recombinant adenovirus is rather straightforward, using two standard methods. One of them involves an *in vitro* ligation of a DNA fragment with the Ads genome (Stow ND, 1981; Vemula, Sai V., et al., 2010), while the second method involves homologous recombination in permissive cell lines (Bett, A.J., et al., 1994; Vemula, Sai V., et al., 2010).

Recombinant adenovirus can induce highly effective immune responses; they activate innate immune responses by expressing pathogen-associated molecular patterns (PAMPs), which bind to pathogen recognition receptors on host cells, activating the production of proinflammatory cytokines and differentiation of DC into APCs (Medzhitov, R. 2000; Tatsis, N., 2004). Additionally, recombinant adenovirus can induce neutralizing antibodies mainly directed to the surface of the virus (Hong, S.S., et al. 2003; Tatsis, N., 2004).

Despite the advantages associated with recombinant adenovirus for its high efficiency in vaccine or gene delivery, efforts have been made to circumvent the problems of anti-vector immunity which remains the main risk for this type of vaccines (Soema, P.C., et al., 2015; Vemula, Sai V., et al., 2010).

1.3.3.2 CD40L as a vaccine adjuvant

CD40L is a type II transmembrane multimeric protein mainly expressed by activated CD4+ T cells, but it can also been found in activated B cells, dendritic cells (DC), platelets and smooth muscle cells. During the interaction of dendritic cells and $CD4^+$ T cells, CD40L promotes their maturation into competent APCs by enhancing the survival of DC, the secretion of cytokines and the upregulation of costimulatory receptors and MHC class I and II molecules. (Bishop, G.A et al., 2003; Fan et al., 2015; Hashem, A.M., et al., 2014; Quezada, S.A. et al., 2004; van Kooten et al., 2000).

CD40 is a member of the TNF receptor family, expressed by B cells of all stages of development and differentiation, along with APCs, activated $CD4^+$ T cells, $CD8^+$ T cells and other cell types. Data from previous studies have shown CD40 is involved in amplification and regulation of inflammatory immune responses. CD40 signaling on B cells facilitates Ig production, Ig isotype switch, germinal center (GC) formation, and memory B cell maturation (Bennett, S.R., et al., 1998; Fan et al., 2015; Oxenius et al., 1996; Schoenber, S.O. et al., 1998; Schultze J.L. et al., 1997; van Kooten et al., 2000).

CD40/CD40L interaction is known to play key roles in the regulation of humoral and cellular immune response. The importance of this interaction was first observed when studying hyper IgM syndrome, where in some cases, it was found that in some immunodeficiency patients

there is a genetic defect in CD40L, while in other patients there is a deficiency in CD40 signaling. Such defect or deficiency was found to result in an impairment of T cell-dependent antibodies, inducing a reduction in the circulating antibody isotypes and defective production of IgG and IgA (Durandy, A., et al., 1997; Notarangelo, L.D., et al., 1996; van Kooten et al., 2000). Moreover, it was found that CD40/CD40L interaction is necessary for the cross-priming of cytotoxic T lymphocytes (CTL) response by DC and increased levels of cytokines such as IL-12. It was also reported that CD40L could skew immune responses towards TH1 (van Kooten et al., 2000). These findings collectively suggest that enhanced CD40/CD40L interaction could be considered as a viable approach in the treatment of diseases which might directly be related to the imbalance of the immune system like lupus and cancer (van Kooal., 2000).

There have also been reports on the use of CD40L as a molecular adjuvant to improve vaccine-induced immune responses in mice against a lethal dose of influenza A virus. On this subject, our laboratory has succeeded in proving that the addition of CD40L as a targeting ligand and molecular adjuvant induces early and persistent B cell GC formation and accelerated Ig isotype switching, with a TH1-skewed response. (Bar, T. et al., 2003; Cao, J. et al., 2010; Fan et al., 2015; Hashem et al., 2014; Huang, D. et al., 2004; Lin, F.C. et al., 2009; Liu, J et al., 2008; Tripp, R.A., et al., 2000).

CHAPTER 2 A consensus influenza B HA2 provides complete protection against a high dose of Influenza B

2.1 ABSTRACT

Influenza B has two main surface glycoproteins, neuraminidase (NA) and hemagglutinin (HA). HA consists of a head and stem domain and is responsible for the binding of the virus to the host cell. Current vaccines target the head domain of the HA protein, resulting in the need for an annual dose because of high mutation rates in the head of the viral HA. Sequence analysis shows that the stem domain of HA has a lower mutation rate and is highly conserved between the different influenza B strains from its two lineages, making this an attractive target for future vaccine development. Using a bioinformatics approach, a synthetic consensus influenza B HA2 sequence was designed and evaluated for protection in mice. Results demonstrated that mice immunized with adenovirus vectors containing this HA2 consensus sequence were fully protected against a high infectious dose of influenza and showed significantly decreased viral replication in lung and trachea tissues.

2.2 INTRODUCTION

The HA protein is an attractive target for preventive and therapeutic intervention, due to its roles in the early stages of virus infection, including its binding to the host cell and mediating viral fusion to the cellular membrane (Skehel and Wiley, 2000). HA is a transmembrane, homotrimeric protein, and is composed of two subunits, HA1 and HA2. The HA2 subunit anchors the HA1 domain to the viral membrane, it contains the viral fusion protein and has proven to be a highly conserved structure (Chen, J. et al., 1995; Chun, S. et al., 2008). Even though current vaccines protect the hosts by mainly inducing antibodies against the HA, they afford a limited protection due to the ability of the virus to drift and shift in antigenicity in addition to strain specifity (Gerhard et al., 2006 & Epstein, 2006).

Through bioinformatics analysis of the available HA2 sequences of influenza B viruses, a 223 amino acid synthetic, highly conserved consensus sequence was synthetically designed. This sequence was codon optimized for expression in mouse and used to generate an experimental adenovirus-vectored vaccine. I postulated that this vaccine could protect mice against a lethal dose of influenza B virus. To test this hypothesis, the ability of the experimental vaccine to reduce or eliminate severe symptoms of illness in immunized mice lethally challenged with an influenza B virus was studied.

2.3 OBJECTIVES

1. To design a synthetic conserved consensus HA2 domain for influenza B.
2. To use the consensus HA2 sequence to engineer an adenovirus-vectored vaccine and evaluate it's potential to protect mice from influenza B.

2.4 MATERIALS AND METHODS

2.4.1 Cell lines, viruses and recombinant proteins.

293A cells, Madin-Darby canine kidney (MDCK) cells and HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). 293A and MDCK cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies Inc., Burlington, ON, Canada), while HeLa cells were cultured in complete Minimum Essential Medium (MEM) with 10% FBS. Media were supplemented with 2mM L-glutamine, 25 mM HEPES, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 110 mg/L of sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin.

Influenza B strain B/Victoria/2/87 (B/Vic) was obtained from Dr. Kramer's laboratory at the Icahn School of Medicine at Mount Sinai (New York, NY, USA) and was propagated at 33ºC in the allantoic cavities of 10-day old embryonated hen eggs (Canadian Food Inspection Agency, Ottawa, ON, Canada) for 72 hours. Allantoic fluid was clarified by ultracentrifugation, aliquoted and stored at -80ºC. All viruses were titered by plaque assay in MDCK cells.

2.4.2 Design of universal HA2 sequence

To identify a universally conserved sequence in the HA2 subunit of all type B influenza viruses, a bioinformatics approach was employed, as previously described (Chun et al., 2008). In brief, approximately 8000 influenza B viral HA sequences from the NCBI influenza virus resource were downloaded. A multiple alignment was then performed, followed by extraction of the consensus sequences in all HA2 subunits of HA, in which the conservation index was sorted by the degree of variation and determined by calculation of the Shannon entropy for each position of amino acids. The resulting, highly conserved sequence represents a full HA2 region of 223 amino acids consistent with more than 90% conservation rate among all influenza B viruses (HA2 full sequences can be found in the Appendix AP1). Geneious 7.0.6 software was then used to confirm the bioinformatics findings and ensure the universal conservation in all HA2 subunits as previously described (Gravel et al., 2015).

2.4.3 Recombinant adenovirus (rAds)

As shown in Figure 2.1, constructs were designed to express the consensus HA2 in addition to a 114 amino acid portion of the HA1 subunit from influenza B/Florida/04/06 (B/Flo), followed by a 26 amino acid segment from the bacteriophage T4 fibritin trimerization motif,

under the control of a cytomegalovirus (CMV) promoter. Additionally, one construct was also designed to carry the ectodomain of mouse CD40L (amino acids 117-260) as described by Pereboev et al. (2004). Full sequences of engineered constructs can be found in Appendix AP2. The sequence was codon optimized for expression in mouse. Designed influenza B HA2 sequence was synthesized by Bio Basic Canada Inc. (Markham, ON, Canada) and cloned in pBluescript SK vector.

2.4.3.1 Generation of rAds

All PCR were done using the Platinum Pfx DNA polymerase kit (Life Technologies Inc., Burlington, ON, Canada). Primers used in cloning were synthesized by Life Technologies Inc. (Burlington, ON, Canada) and are listed in Appendix AP3. All transformations were done using Library Efficiency DH5α Chemically competent cells (Life Technologies Inc., Burlington, ON, Canada).

The rAd-vaccines were assembled in three steps. Briefly, using primers F1, F-R1 and CD40L-R1, the designed constructs were transferred into pENTR^{TM}/SD/D-TOPO vector. Product was recombined using the same primers, to be subsequently cloned into a pAd/CMV/V5-DEST Gateway Vector (Life Technologies Inc., Burlington, ON, Canada). The resulting recombinant plasmids were *Pac I* linearized and transfected into 293A cells to package the adenoviral vector. Cloning was confirmed at every step by DNA sequencing and restriction enzyme digestion.

2.4.3.2 Generation of adenovirus vaccine stocks

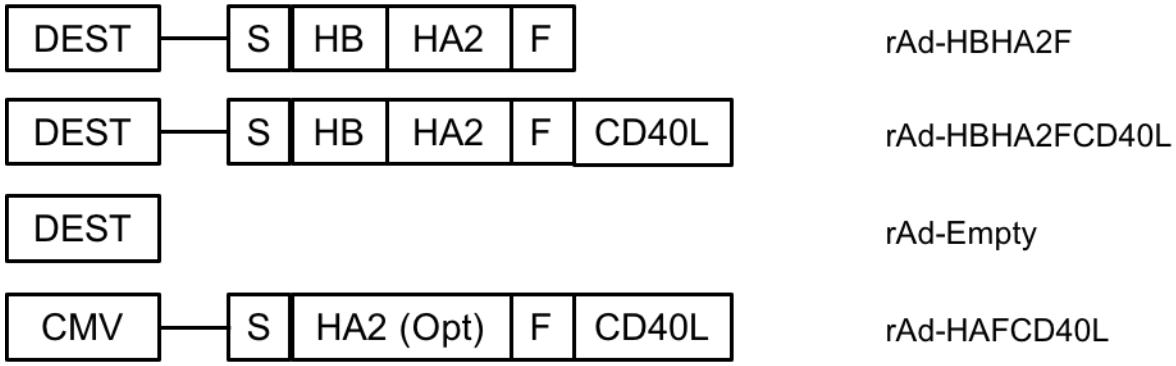
The generated rAds were used to infect 293A cells in 150 mm dishes at a MOI of 0.01. After 96 hours, supernatant of infected cells were collected and the infected cells were lysed by 3 cycles of freeze-thawing. Supernatant and cell lysate were clarified by filtration and purified by ultracentrifugation at 30,000 RPM for 90 min, at 4ºC, over a 30% sucrose layer. Stocks were titered using the Adeno-X rapid titer kit protocol (Takara Bio USA, Inc., CA, USA).

In addition to the two HA2 consensus sequence-containing constructs (rAd-HBHA2F and rAd-HBHA2FCD40L), two other vectors were grown to be used as controls: an empty vector (rAd-empty), consisting of the same backbone used for the adenovirus production and an additional adenoviral vaccine with a similar structure but originated from the HA2 of influenza A/California/7/2009 (rAd-HAFCD40L) designed by Fan et al (2015) and that proved promising protection results against diverse influenza A viruses.

Figure 2.1 Schematic representation of the rAd constructs.

Schematic representation of the generated rAd constructs, designed to evaluate protection in animal studies. **S** is an N-terminal leader sequence derived from human tyrosine signal peptide. **HB** is a 114 amino acid segment from the HA1 subunit of Influenza B/Florida/04/06, attached to the designed, conserved, influenza B **HA2**. **F** is a 26 amino acid fragment from the T4 bacteriophage fibritin trimerization motif fused with **CD40L** which is the ectodomain of the mouse CD40 ligand. **HA2 (Opt)** is the influenza A HA2 studied by Fan et al (2015) optimized for mouse and used as a control. Additional designed controls are also represented, where **HA2(Opt)** represents the HA2 of influenza A/California/7/2009 optimized for mouse.

Figure 2.1


DEST
S
HB
HA2
F
rAd-HBHA2F
DEST
S
HB
HA2
F
CD40L
rAd-HBHA2FCD40L
DEST
rAd-Empty
CMV
S
HA2 (Opt)
F
CD40L
rAd-HAFCD40L

2.4.3.3 Confirmation of protein expression from rAds in cell culture by Western blot

Generated rAds were used to infect HeLa cells in a 6 well plate at a MOI of 600. After forty-eight hours, infected cells were washed twice with PBS, lysed with RIPA buffer and slightly sonicated. Protein expression was confirmed by Western blot using a mouse monoclonal antibody against influenza B HA2.

2.4.3.4 Western Blot

Cell lysate samples were mixed with sample buffer containing 4% SDS and 20% β-mercaptoethanol and heated at 90ºC for 5 min. Proteins were fractionated on a 4% to 15 % gradient Mini Protean TGX polyacrylamide gel from Bio Rad, followed by transferring to polyvinylidene difluoride (PVDF) membrane (Millipore Ltd., Etobicoke, ON, Canada). The membrane was washed with 1X Tris buffered saline with 0.1% Tween 20 (TBST) twice for 5 min and blocked overnight with 3% BSA-TBST. Membranes were washed twice for 5 min in TBST, followed by incubation with a mouse monoclonal antibody to Influenza B HA2 for an hour at room temperature, at a 1:5,000 dilution. Subsequently, membranes were incubated with an anti-mouse IgG Horseradish Peroxidase (HRP) conjugated antibody (GE Healthcare Life sciences, Baie d'Urfe, QC, Canada) at a 1:15,000 dilution for one hour at room temperature. The detection was carried out using the Super Signal West dura Extended Duration Substrate (Millipore Ltd., Etobicoke, ON, Canada). The membrane was exposed on Kodak film and developed using a Kodak XOMAT film processor.

2.4.4 Animal Experiments

2.4.4.1 Mice

As mentioned above, there is no clear evidence indicating that influenza B viruses continuously circulate among animals. Therefore, there is currently no reliable animal model that shows clinical signs and illness symptoms following infection from influenza B strains as it would be observed in humans, such as fever, cough, runny or stuffy nose, muscle or body aches and/or fatigue. Additionally, the limited animal models available fail to show a wide range of measurable signs and symptoms of the illness. So far, DBA/2 mice provide the best animal model for human influenza B infection for a variety of reasons, with the main ones being: (a) the vaccine evaluation can be performed in this model in a cost and time effective manner, (b) depending on the challenge dose, DBA/2 mice respond better to infection by multiple strains of influenza B virus showing certain clinical symptoms before clearing the infection or succumbing to the infection, and (c) a wide range of immunological reagents are available for use in this model (Pica, N. et al, 2011 and Van de Sandt, C., 2015).

Six-week old DBA/2 mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were maintained in the animal facility of Health Canada (Food Directorate, Scientific Services Division, Ottawa, ON, Canada). All animal experiments were conducted in accordance with Health Canada Institutional Guidelines and with the approval of the Animal Care and Use Committee.

2.4.4.2 Determination of virus challenge dose

Before a protection study was performed, a small pilot study was done to determine an influenza B virus challenge dose that would allow observation of illness symptoms within 7

days. The tested doses range from $1x10^{3.2}$ PFU to $1.5x10^{4}$ PFU, approximately 10 times difference between the lowest and highest dose tested, which was based on the LD50 reported by Pica et al (2012). Mice were challenged intranasally with 25 µl of influenza B virus strain B/Vic virus diluted in PBS. Mice were weighed daily and observed for 14 days for signs of illness such as unresolved dehydration and marked respiratory distress, muscular atrophy, erratic feeding behavior, and low mobility and/or weight loss of >25%. LD50 was calculated as described by Hashem, A., et al., 2014.

2.4.4.3 Protection study

Mice were immunized intranasally twice with rAd vaccines at a concentration of $1x10^{9}$ PFU in 25 µl PBS on days 0 and 28 (Fan, X., et al. 2015; Hashem, A., et al., 2017). On day 56 after both immunizations, mice were challenged intranasally with 25 µl of influenza B/Vic virus, diluted in PBS at the dose previously determined. After challenge, mice were weighed daily and clinically monitored at least twice a day for 14 days for signs of illness as described in the pilot study (2.4.4.2 Determination of the viral dose).

2.4.4.4 Tissue collection and process

Mice were bled via cardiac puncture under isoflurane inhalation anesthesia on days 49, 59 and 71 after immunization. Serum was obtained by centrifugation of blood at 10,000 x g for 2 minutes, aliquoted and stored at -80ºC for antibody analysis.

For tissue viral titration, lungs and trachea were harvested from mice 3 days post challenge and stored at -80ºC until use. Frozen lungs and trachea tissues were thawed, weighed

and homogenized in 0.3 ml and 0.250 ml of cold PBS respectively, and used for viral titration by plaque assay in MDCK cells as described in section 2.4.5.

For histology, lungs were fixed with formalin and submitted to pathology analysis for microscopic lesion. Sections from multiple lung lobes from each mouse were trimmed, processed and embedded in paraffin. Five micron sections were cut and stained with Hematoxylin and Eosin (H&E) for analysis.

2.4.5 Plaque assay

Ten-fold serial dilutions of harvested viruses or homogenized tissue samples from processed tissues were prepared in complete DMEM medium supplemented with 0.2% bovine serum albumin (BSA) and 2 μg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin. Confluent monolayer of MDCK cells in 6 well plates were incubated with 500 μl of each dilution in duplicates at 37ºC for 2 hours. The inoculum was removed; cells were washed with same DMEM medium and overlaid with maintenance DMEM medium containing 0.8% agarose, 0.2% BSA and 2 μg/ml TPCK-treated trypsin. After incubation for 4 days at 35ºC in humidified atmosphere of 5% CO_2, cells were stained with 0.5% crystal violet and plaques were counted.

2.4.6 Data analysis

One-way analysis of variance (ANOVA) with Bonferroni post-test was used to compare data from the different groups. All statistical analysis was conducted using GraphPad Prism 7 software (San Diego, CA, USA).

2.5 RESULTS

2.5.1 rAds construction and protein expression

The first goal was to identify a consensus HA2 sequence, which represented the most conserved sequence from all the influenza B HA2 sequences reported to date. Bioinformatics analysis of the sequences resulted in a complete HA2 sequence of 223 amino acids (AP 1) representing more than 90% conservation rate. This sequence was then codon-optimized for mice and used to generate the rAd vector vaccine. As shown in Figure 2.1, due to the synthetic origin of the designed sequence, a portion of 114 amino acids derived from HA1 from influenza B/Flo was added to provide stability and aid in the formation of the active trimeric state of HA2 (HB). Additionally, this protein was fused to a secretion signal (S) at the N terminus to facilitate the secretion of the protein, thereby increasing the antigen load, and a trimerization motif (F) to help achieve its functional conformation (rAd-HBHA2F). To further study the role of CD40L, a rAd was designed with an additional CD40L (rAd-HBHA2FCD40L).

Before animal immunizations, we first studied the potential of the designed vaccines to infect mammalian epithelial cells. Towards this end, lysate from HeLa cells infected at a high MOI to increase expression levels was run through a gradient polyacrylamide gel, transferred to a PVDF membrane and detected with Influenza B HA2 monoclonal antibody. As shown in Figure 2.2 (A), the western blot confirms the expression showing bands of the expected sizes. rAd-HBHA2F (lane 2) was confirmed with a strong band at around 42 kDa and expression of rAd-HBHA2FCD40L (lane 1) with a lighter band at around 71 kDa. Control rAd-empty was also run and showed no signal when detected with an HA2 antibody, as expected.

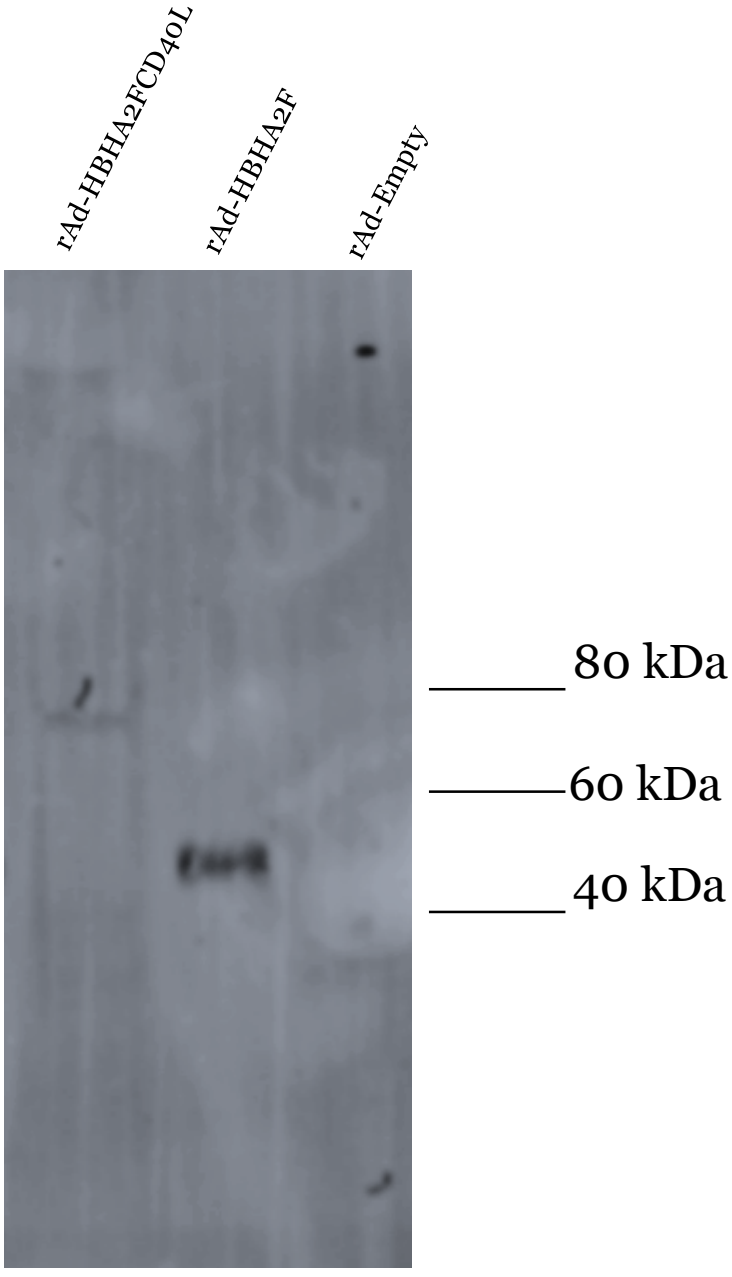
Although the use of anti-mouse CD40L antibody would have represented a better resource for the confirmation of CD40L in rAd-HBHA2FCD40L, the lack of availability of an

antibody with high specificity presented a challenge. Nevertheless, later confirmation of presence and function of CD40L was observed by performing flow analysis on splenocytes from immunized mice, which will be discussed later.

Figure 2.2 Recombinant Ad constructs and *in vitro* protein expression.

Protein expression was confirmed in HeLa cells from both rAd-HBHA2FCD40L (71.62 kDa) and rAd-HBHA2F (42.41 kDa). Cells infected with rAd-Empty were used as control. Cell lysates were mixed with reducing sample buffer containing 4% SDS and 20% β-Mercaptoethanol and detected by Western blot using mouse monoclonal antibody to influenza B HA2.

Figure 2.2



2.5.2 Establishing virus challenging dose

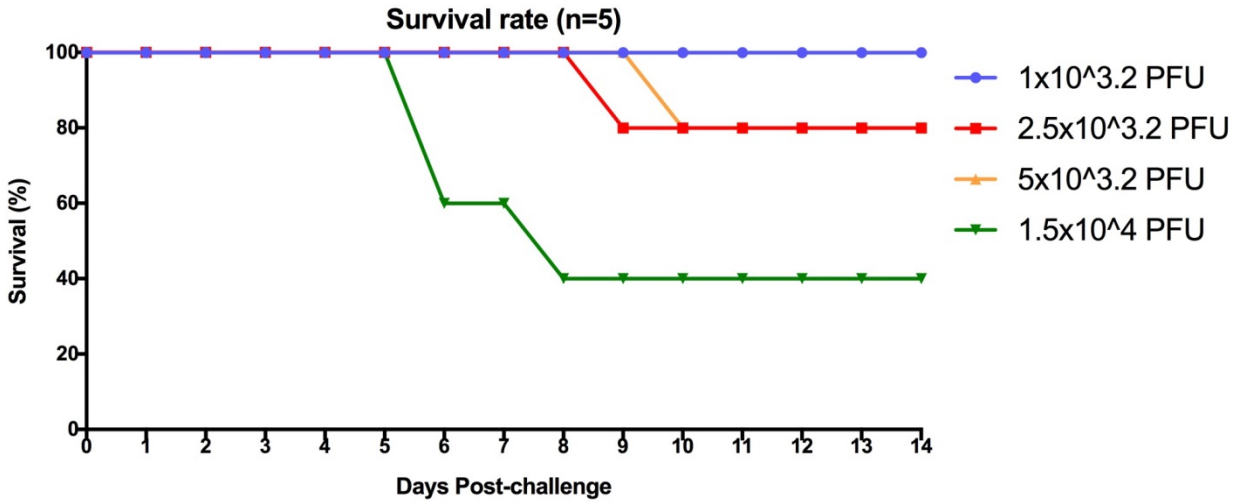
The first step before performing a protection study to evaluate the different designed vaccines was to establish the virus dose that caused clinical symptoms of illness within a period of 3 to 7 days but less than two weeks. These were established based on the known duration of the disease, ie., between 3 to 7 days the most severe symptoms would appear, while by 14 days it would be sufficient for the animals to clear the virus and fully recover if the animals did not succumb to infection (WHO, 2004). To this end, four virus doses were tested, starting at $10^{3.2}$ PFU up to $1.5x10^4$ PFU; the mice were weighed daily and monitored at least twice a day in order to determine the survival rate. As described above, the endpoint in the survival study was defined when an animal lost more than 25% of body weight, 72 hours of unresolved dehydration and marked respiratory distress, marked muscular atrophy, erratic feeding behaviour, and low mobility or a combination of lost weight, muscular atrophy and respiratory distress.

Results showed that although doses $2.5x10^{3.2}$ PFU and $5x10^{3.2}$ PFU resulted in 20% mortality after the 14-day observation period, the highest dose tested ($1.5x10^4$ PFU) caused 60% mortality. As shown in Figure 2.3, we would be able to observe the symptoms and mortality 6 to 8 days post challenge.

Figure 2.3 Mice infected with 1.5 x10^4 PFU showed highest mortality rate.

Four doses of influenza B/Victoria/2/87 were tested starting at 1x$10^{3.2}$ PFU up to 1.5x10^4 PFU. Mice were weighed and monitored daily for illness symptoms. Data shown from one experiment with n of 5 mice per treatment group.

Figure 2.3


Survival rate (n=5)
Survival (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post-challenge
1x10^3.2 PFU
2.5x10^3.2 PFU
5x10^3.2 PFU
1.5x10^4 PFU

2.5.3 Immunization with designed rAd vaccines containing consensus HA2 provides 100% protection against a high dose of Influenza B

To compare the protection capacity by the different vaccines, mice were immunized intranasally twice with the designed vaccines and challenged with a $1.5x10^4$ of B/Vic virus. Vaccines were evaluated for their ability to reduce mortality, disease symptoms or weight loss after challenge.

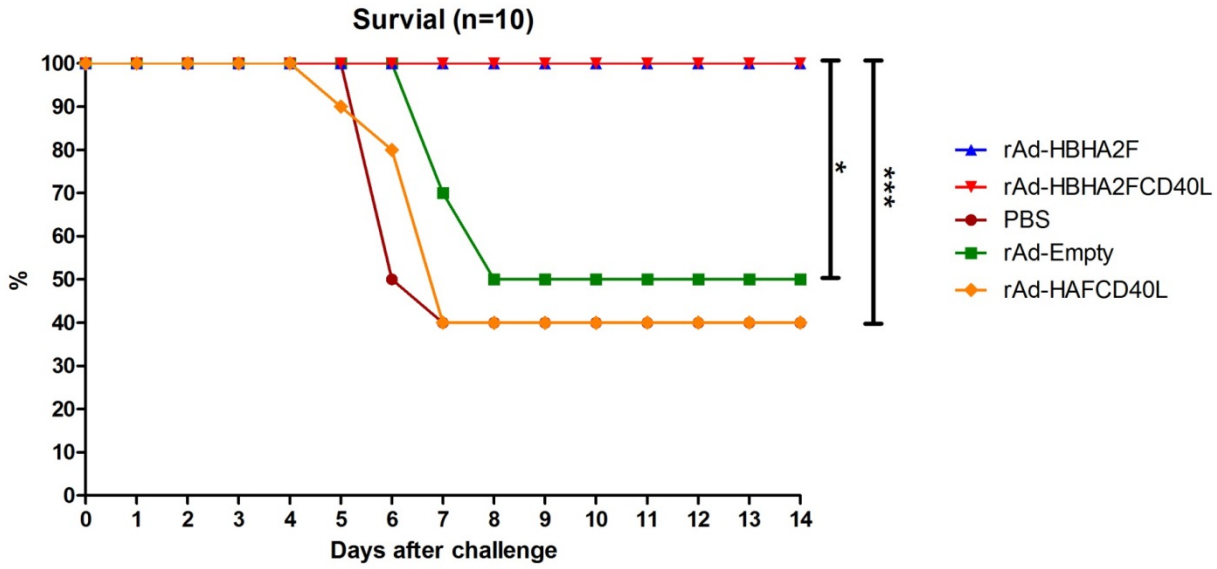
As shown in Figure 2.4 A, the control groups had the highest mortality rate after virus challenge. Specifically, mice immunized with PBS and the influenza A vaccine (rAd-HAFCD40L) had a higher mortality rate with only 40% survival, while mice immunized with rAd-empty had a 50% survival rate. In contrast, mice immunized with either rAd-HBHA2F or rAd-HBHA2FCD40L showed a survival rate of 100%. Additionally, mice from rAd-HBHA2F and rAd-HBHA2FCD40L were able to maintain their body weight during the entire observation period of 14 days, while mice from the control groups (PBS, rAd-empty and rAd-HAFCD40L) significantly lost body weight during the first week after infection (Figure 2.4 B). Nevertheless, in the control groups, ie. PBS, rAd-empty and rAd-HAFCD40L, there were still some animals which did not lose 25% of body weight, yet these animals were unable to fully regain their weight during the observation period. These results indicated that the synthetic HA2-based vaccines were able to fully protect the animals against a lethal dose challenge of influenza B/Vic virus in mice, while the addition of CD40L did not appear to be necessary in protecting the animals studied under this experimental condition.

Figure 2.4 Immunization with consensus HA2 protects mice from viral challenge

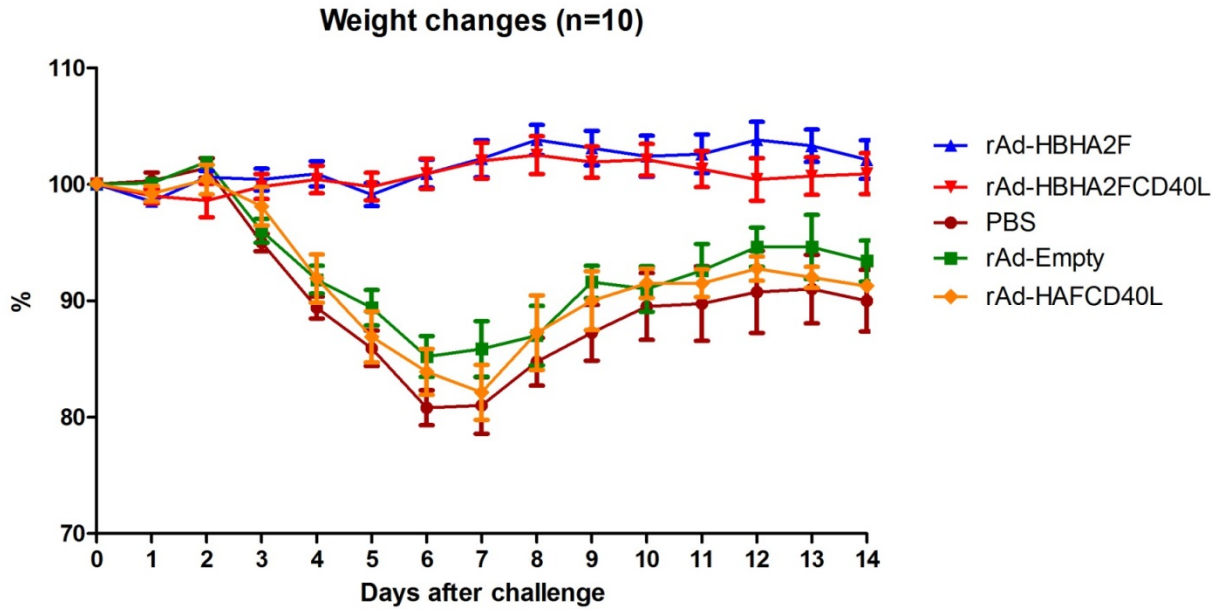
(A) Survival of immunized mice after challenge. Data of one experiment is presented as percentage of surviving mice at each time point compared to the initial number of animals in each group. *** represents $p<0.001$ and * represents $p<0.05$ (one-way ANOVA with Bonferroni post-test). **(B) Body weights of the challenged mice.** Weight loss is expressed as percentage of animal weight at each time point from their initial body weight. Data are shown from one experiment with n of 10 mice per treatment group. *** represents $p<0.05$ (One-way ANOVA with Bonferroni post-test).

Figure 2.4

A)


Survial (n=10)
100
90
80
70
60
50
40
30
20
10
0
%
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days after challenge
*
***
rAd-HBHA2F
rAd-HBHA2FCD40L
PBS
rAd-Empty
rAd-HAFCD40L

B)


Weight changes (n=10)
110
100
90
80
70
%
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days after challenge
rAd-HBHA2F
rAd-HBHA2FCD40L
PBS
rAd-Empty
rAd-HAFCD40L

In addition to survival and body weight analysis, quantification of the viral titer in tissue from challenge mice was performed in order to observe the damage in the upper and lower respiratory tract. Lung and trachea were collected three days after challenge and analysed by plaque assay as described in section 2.4.5.

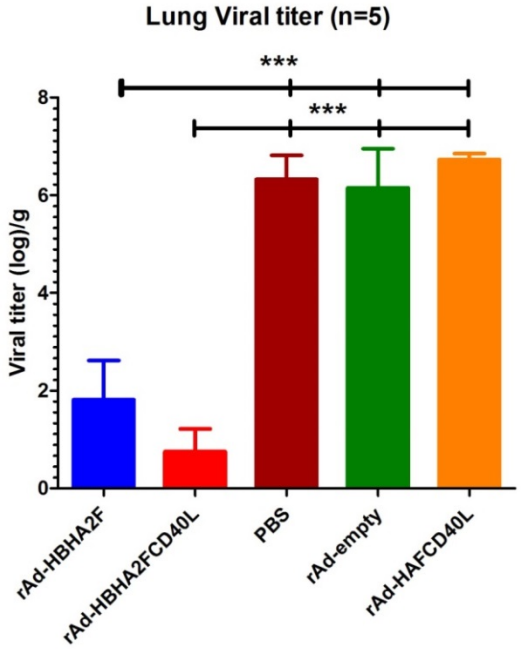
Consistent with data obtained from experiments on survival and body weight, we were able to observe the highest viral titers in the lungs and trachea (Figure 2.5) in the control groups including rAd-empty, PBS and rAd-HAFCD40L, whereas viral amounts in tissue from groups immunized with vaccines carrying the synthetic HA2 gene (rAd-HBHA2F and rAd-HBHA2FCD40L) were significantly lower. In addition, three days post challenge, the groups immunized with rAd carrying the consensus sequence HA2 were able to fully clear the virus from the upper respiratory tract and clear most of the virus in the lower respiratory tract.

Figure 2.5. Lung and trachea viral titer is reduced in mice immunized with consensus HA2

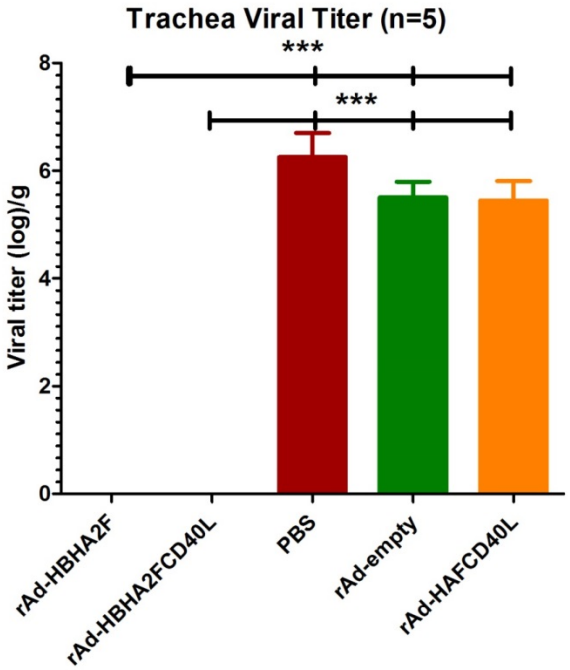
Viral titer in (A) lungs and (B) trachea was measured on day 3 post-challenge as described in material and methods. There is a statistical difference between the two groups immunized with the designed consensus HA2 and the control groups. Data are shown from one experiment with n of 5 mice per treatment group. *** represents $p<0.001$ (One-way ANOVA with Bonferroni post-test).

Figure 2.5

A)


Lung Viral titer (n=5)
***
***
Viral titer (log)/g
8
6
4
2
0
rAd-HBHA2F
rAd-HBHA2FCD40L
PBS
rAd-empty
rAd-HAFCD40L

B)


Trachea Viral Titer (n=5)
***
***
Viral titer (log)/g
8
6
4
2
0
rAd-HBHA2F
rAd-HBHA2FCD40L
PBS
rAd-empty
rAd-HAFCD40L

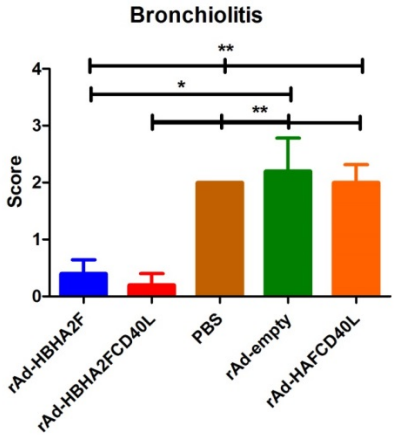
These results were confirmed by the histology analysis of lung tissue collected at the same time point as the tissue used for viral titer (Figure 2.6). H&E analysis of multiple sections from the lungs demonstrated that control mice immunized with PBS, rAd-empty and rAd-HAFCD40L have similar abrasions between them and show significantly higher broncho-interstitial pneumonia, represented by necrosis of the bronchiolar epithelial tissue, than mice immunized with the consensus HA2 (rAd-HBHA2F and rAd-HBHA2FCD40L). These lesions in DBA/2 mouse lungs were more severe than the mild changes found in Guinea Pigs infected with Influenza B virus, where symptoms such as mild alveolitis and mild neutrophil infiltration were observed in 1 out of 2 animals (Pica et al.2012). However, the DBA/2 infected with the influenza B virus demonstrated similar symptoms to those noted in Balb/c mice infected with Influenza A virus Sw31, in which moderate to marked necrosis of the bronchiolar epithelial was observed; in addition, there was also observed alveolitis and neutrophil inflammatory infiltration in the Balb/c infected by influenza A virus (Memoli et al.2009).

Figure 2.6 Pathology scoring of lung tissue 3 days after challenge

(A and B) Lungs from infected mice were harvested 3 days post-infection with influenza B/Victoria/2/87. For each lesion a numeric scoring was subjectively applied to indicate severity where 0 indicates no lesion present within normal limits; 1 means a minimal degree of severity; 2 means mild; 3 is moderate; 4 stands for marked and 5 means severe. Data are shown from one experiment with n of 5 mice per treatment group. ** represents $p<0.001$ and * represents $p<0.05$ (one-way ANOVA with Bonferroni post-test). **(C)** Representative lung tissue sections from rAd-Empty and rAd-HBHA2FCD40L mice. Arrows show necrotic tissue.

Figure 2.6

A)



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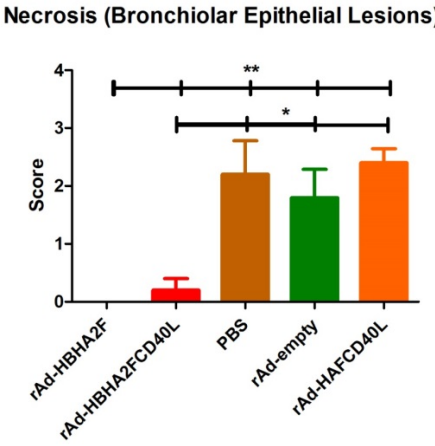
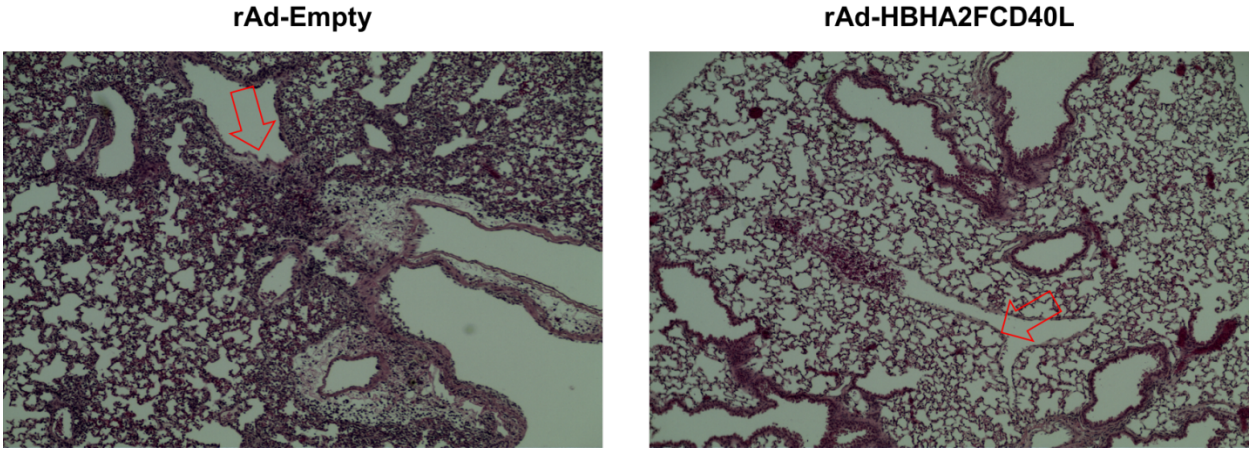


C)



2.6 DISCUSSION

Although the current influenza vaccines provide substantial protection against the circulating strains of influenza virus, they mainly induce strain-specific neutralizing Abs against the highly variable regions of HA1 (Epstein, S.L. 2006, Gerhard, W. et al. 2006; Sui, J. 2009). On the other hand, the HA2 domain is the most conserved region, making it an attractive target to induce a broadly protective activity (Hashem, A.M, et al. 2010; Wang, T.T. et al. 2010).

Using bioinformatics analysis, the most conserved sequence in the HA2 domain of over 8000 influenza B viruses was identified and used to design experimental vaccines to study its ability to provide protection against a lethal challenge by influenza B virus. Even though a similar technique has been used for studying NP or HA2 derived from a highly conserved strain from influenza A by us and others (Hashem, A.M, et al. 2010; Fan et al., 2015; Wang, T.T. et al. 2010), this is the first time influenza B has been used to design a synthetic HA2 and used to study a possible broad protection. Nevertheless, studies derived from influenza A were successful in demonstrating antibody-mediates protection against multiple strains of the same influenza type.

In this study we were able to demonstrate that this consensus HA2 sequence provides full protection in mice against a lethal challenge by influenza B virus. This protection was not only confirmed by the complete survival and reduction of illness symptoms, such as weight loss, in mice immunized with the vaccines containing the consensus HA2 sequence (rAd-HBHA2F and rAd-HBHA2FCD40L), but also by a significant decrease in their viral titer in lung and trachea tissues and the lesions to the tissue collected from the same mice.

These results also allowed us to conclude that there is an antigen-specific response to each influenza virus type A or B, confirmed by the lack of protection observed in mice immunized with rAd-HAFCD40L, which had proven to provide full protection against different multiple strains of influenza A (Fan et al., 2015), this could be explained by the 35% homology between influenza A and influenza B. Furthermore, this lack of protection in mice immunized with rAd-HAFCD40L, confirms that the observed protection is due to the synthetically designed consensus HA2 derived from influenza B and not from the CD40L ectodomain portion. It is also clear that the control group rAd-empty provided no protection against influenza B virus infection as demonstrated by the survival and pathology results similar to that in PBS and rAd-HAFCD40L control.

It is of interest to note that we observed no difference in protection between vaccines with and without CD40L. Given CD40L is known to play important roles in orchestrating humoral and cellular immune responses, our findings suggest that the two vaccines could afford protection in the animals through different mechanisms, which would need to be further investigated (Chapter 3).

CHAPTER 3 Mechanistic studies of protection afforded by CD40-targeted and non-targeted vaccines

3.1 ABSTRACT

The synthetic consensus HA2 sequence has been confirmed to induce protection in mice against a high dose of influenza B virus (Chapter 2). However, the mechanism behind this protection is still unclear as well as the potential effects of CD40L on modulating the type of antigen-specific immune response. Here we were able to characterize the type of immune responses induced by immunization with adenovirus-vectored vaccines encoding the designed HA2 consensus sequence, with or without a functional CD40L. Specifically, this was done by assessing the levels of various isotypes of serum and mucosal antigen-specific antibodies, as well as their neutralizing activities *in vitro*, and by measuring cytokine production by immune cells. We found that immunization with a CD40L-targeted HA2 consensus sequence results in higher levels of mucosal IgG and IgA antigen-specific antibodies, a higher TH1-skewed immune response demonstrated by higher levels of serum antigen-specific TH1-bias IgG antibodies and increased TH1 cytokine production in antigen-stimulated splenocytes. Additionally, Targeting the HA2 sequence with CD40L also resulted in a significant increase in the induction of germinal center formation and the up-regulation of CD40.

3.2 INTRODUCTION

HA is a homotrimeric protein which is composed of two subunits. The HA1 (head domain), with a higher mutation rate, contains the binding site of the virus. The second subunit, HA2 (stem domain) is highly conserved and contains the fusion peptide. Due to its critical role in the early stages of the virus replication, HA represents an attractive target against influenza (Chen, J., et al., 1995; Chun, S. et al., 2008; Fan, X., et al., 2015; Gerhard, W., et al., 1981; Skehel J.J., et al., 2000; Wiley, D.C., et al., 1987).

Among the identified co-stimulatory molecules are CD40 and its ligand (CD40L). CD40 is a type I transmembrane protein, member of the TNF receptor family and is constitutively expressed on all antigen presenting cells. CD40L is a type II transmembrane protein, mainly expressed by activated T cells as a cell surface or secreted protein. The potential of CD40L as a molecular adjuvant has been explored, with findings indicating enhanced immune responses and protection against various tumors and pathogens (Cao, J. et al., 2010; Fan et al., 2015; Hashem et al., 2014; Huang, D. et al., 2004; Lin, F.C. et al., 2009; Liu, J. et al., 2008; Tripp, R.A. et al., 2000; van Kooten and Banchereau, 2000). In 2015, our laboratory reported using CD40L as a targeting ligand for an adenovirus-vectored vaccine expressing the conserved HA2 subunit of influenza A; we showed that the use of CD40L as a molecular adjuvant was essential to increase HA2-specific mucosal IgA and serum IgG levels and afford protection against multiple strains of influenza A (Fan, et al., 2015).

As discussed in Chapter 2, we were able to observe full protection in mice immunized with adenoviral vectors expressing the consensus HA2 with or without CD40L. Questions remain as to whether there is a need for additional CD40L stimulation when the influenza B virus HA2 subunit is used to make prototype universal vaccine; how the protection afforded by the two different vaccines were working mechanistically.

3.3 OBJECTIVE

1. To better understand the mechanisms underlying the protection afforded by either CD40L-targeted or non-targeted vaccines.

3.4 MATERIAL AND METHODS

3.4.1 Viruses, recombinant proteins and flow markers.

The recombinant influenza B HA2 protein Fc Tag was purchased from Sino Biological Inc. (Beijing, P.R., China). The mouse monoclonal antibody to influenza B HA2 and rabbit polyclonal antibody to influenza B virus nucleoprotein (NP) were purchased from GeneTex, Inc. (Irvine, CA, USA).

Influenza B/Florida/04/06 (B/Flo) was obtained from Dr. Kramer's laboratory at the Icahn School of Medicine at Mount Sinai (New York, NY, USA) and was propagated at 33C in the allantoic cavities of 10-day old embryonated hen eggs (Canadian Food Inspection Agency, Ottawa, ON, Canada) for 72 hours. Allantoic fluid was clarified by ultracentrifugation, aliquoted and stored at -80 C. Virus was titrated by plaque assay in MDCK cells as described in section 2.4.5. All flow cytometry antibodies were obtained from eBioscience (San Diego, CA, USA).

3.4.2 Animal Experiments

3.4.2.1 Tissue collection and processing

Blood was collected as mentioned in section 2.3.4.4. Bronchoalveolar lavage fluid (BALF) was obtained by flushing the lungs with 2 mL of PBS, with the buffer collected and then clarified by centrifugation at 10,000 x g for 2 minutes.

For cellular immune response analysis in animals after immunization, mice were bled by cardiac puncture and spleen, lungs and axillary and mediastinal lymph nodes (LNs) were isolated. Spleen and LNs were teased between the frosted ends of two sterile glass microscopic slides in complete RPMI 1640 medium supplemented with 10% FBS. The cell suspension was

passed through a 70 μm cell strainer (BD Falcon, Chelmsfrod, MA, USA). Lungs were processed using the lung dissociation kit for mouse from Miltenyi Biotec (Auburn, CA, USA).

3.4.3 Antibody measurement by ELISA

End-point titers of antibodies in serum (total IgG, IgG1, IgG2a and IgG2b) and BALF (total IgG and IgA) collected from immunized mice were determined by performing indirect ELISA as described by Hashem et al. (2014). In brief, 96 well plates were coated with 0.5 μg/ml of recombinant influenza B HA2 protein overnight at 4ºC. The wells were washed 6 times with PBS with 0.05% Tween 20 (PBST), followed by blocking with PSBT with 3% BSA for 1 hour at 37ºC. Serum and BALF samples collected from mice one week before challenge were added in serial dilutions starting from 1:25 to 1:102,400 dilutions, for one hour at 37ºC. Subsequently, HRP-conjugated goat anti-mouse secondary antibodies, specific to each IgG subclass or IgA, (Jackson Immunoresearch Laboratories West Grove, PA) were added at a 1:2,000 dilution in blocking buffer for 1 hour at 37ºC. Plates were finally washed 6 times before tetramethylbenzidine (TMB) substrate (Cell signaling technology, Inc. Danvers, MA, USA) was added for 5 minutes for colorimetric development. The reaction was stopped with an equal volume of stop solution. Absorbance was measured spectrophotometrically at 450 nm using a Synergy ^{TM} 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA).

The antibody titers were calculated as reciprocals of the final detectable dilution with a cut-off defined as the mean of PBS samples plus three times standard deviation.

3.4.4 Microneutralization assay

Microneutralization assay was carried out as described in the WHO Manual for the laboratory diagnosis and virological surveillance of influenza (version 2011). Briefly, serum and BALF samples were treated with receptor-destroying enzymes (RDE) (SEIKEN Ref 370013 Denka Seiken Co. LBT) for 18 hours at 37ºC, followed by a 30-minute incubation at 56ºC. One hundred TCID50 of the tested influenza viruses were mixed with equal volume of 2-fold serial dilutions of the treated samples in 96 well plates and incubated for 1 hour at 37ºC. Subsequently, MDCK cell suspension was prepared by washing three times with DMEM media with no FBS and cells were then seeded at a density of $1.5x10^4$ cells/well. The plate was incubated for 20 hours at 37ºC in humidified 5% CO2 incubator. After incubation, media was removed and cells were washed twice with PBS and fixed with cold 80% acetone for 10 minutes. Viral NP antigen was then detected by indirect ELISA by first washing with PBS containing 0.1% Tween 20 (PBS-T) and then incubated with a rabbit polyclonal antibody to influenza B virus NP at a concentration of 0.5 μg/ml in PBS-T containing 5% skim milk at 37ºC for 1 hour. Cells were then washed again four times with PBS-T and incubated with a 1:2,000 dilution of HRP-conjugated anti-rabbit IgG (GE Healthcare Life sciences, Baie d'Urfe, QC, Canada) for 1 hour at 37ºC. After six more washes, TMB substrate was added to wells and incubated for 10 minutes at RT. The reaction was stopped with an equal volume of stop solution and absorbance was read at 450 nm using a Synergy TM 2 Multi-Mode Microplate Reader. Inhibition was calculated as a percentage of the average absorbance from triplicate wells from virus-antibody mixture relative to no antibody control.

3.4.5 Extracellular cytokine analysis

3.4.5.1 Selection of MHC class I peptide

Since immunodominant epitopes in influenza B HA remained to be defined, specifically in the HA2 subunit, we evaluated the designed sequence with IEDB Analysis resource, which allows the prediction of peptide binding to major histocompatibility complex (MHC) molecules (Lundegaard, C. et al., 2009). As a result, we were able to delineate 9-mer H-2K^{d}-restricted CD8 T cell epitopes from the MHC class I which were evaluated to determine their immunodominance in mice challenged with influenza B. The result was a nine amino acid sequence (YYSTAASSL), located at position 189 of the HA2 designed sequence, that possess the highest predicted binding affinity and the best capability of stimulating peptide-specific *ex-vivo* cytotoxicity against target cells (Muralidharan, A., et al. 2018).

3.4.5.2 Cytokine stimulation and analysis

Cells were collected from spleen as described in section 3.4.4, placed in a 12 well plate at a concentration of 2 million cells in 350 µl and stimulated with the selected peptide at a concentration of 22 µg/mL for 48 hours at 37ºC, 5% CO_2. After incubation, cells were centrifuged at 400 x g for 5 min at 4ºC; supernatant was then collected, aliquoted and stored at -80ºC until use. Analysis was done using a ProcartaPlex Multiplex Immunoassay Kit (Thermo Fisher Scientific, Ottawa, ON, Canada). Briefly, a black 96 well flat translucide bottom plate was coated with 50 µl of magnetic beads, followed by two washes with 1x Wash Buffer from kit prior to addition of 50 µl of the cell samples. Plate was shaken 30 minutes at RT at 500 rpm, followed by incubation overnight at 4ºC over a flat surface. After incubation, cells were shaken 30 minutes at RT at 500 rpm and washed three times with 150 µl of 1x Wash Buffer, before

adding 25 µl of detection antibody. Cells were washed and 50 µl of Streptavidin-PE was added. Plate was shaken and prepared for detection with 120 µl of reading buffer. Plate was read in a Luminex 200 (Luminex, Toronto, ON, Canada) and results were analysed using the Milliplex Analysit 5.1 Software (Millipore Ltd, Etobicoke, ON, Canada).

3.4.6 Flow cytometry analysis of B cells

Single-cell suspensions of LNs, lungs or spleen from immunized mice were washed twice with FACS buffer and stained with APC-conjugated anti-mouse CD11c (clone HL3); FITC-conjugated anti-mouse CD40 (clone HM40-3); PE-conjugated anti-mouse I-Ad (clone AMS-32.1); FITC-conjugated anti-mouse CD80 (clone 16-10A1); Alexa Fluor 647-conjugated anti-mouse Ly77/GL7 (clone GL7) and PE-Cy7-conjugated anti-mouse CD45R/B220 (clone RA3-6B2) for 30 minutes on ice in the dark. Cells were then washed again and fixed before acquiring data with a BD LSRFortessa flow cytometer. Data analysis of single and double B cells markers on live cell was completed with BD Facsdiva 6.2 software. Unstained cells and single stained compensation beads (BD Biosciences, San Jose, CA, USA) were used as control for background fluorescence and false positive due to fluorochrome bleeding.

3.4.7 Data analysis

One-way analysis of variance (ANOVA) with Bonferroni post-test was used to analyse data when comparing 3 or more groups. Un-paired t-test with 95% confidence interval was used to analyse data when comparing 2 groups. All statistical analysis was conducted using GraphPad Prism 7 software (San Diego, CA, USA).

3.5 Results

3.5.1 Addition of CD40L increases production of CD40

One of the first addressed questions was if the targeting CD40L ectodomain used in the construction of rAd-HBHA2FCD40L was indeed functional. To confirm this, we examined by flow cytometry the expression levels of CD40 marker in B cells from draining LNs isolated 1 week pre-challenge from the different groups. LNs were collected and washed before being stained with FITC-conjugated anti-mouse CD40, cells were washed and fixed before acquiring data.

As we have discussed before, CD40 is a member of the TNF receptor family and is constantly expressed at a basal level in the system. As expected, we were able to observe a certain amount of expression of CD40 in mice immunized with control groups PBS and rAd-empty (Figure 3.1); mice from another group (rAd-HBHA2F) showed a similar level of expression when compared to the control groups. In contrast, mice immunized with rAd-HBHA2FCD40L showed a significantly higher level of production of CD40 ($p < 0.01$). This result confirms the presence of a functional CD40L that activates CD40+ cells upon ligation and induces up-regulation of CD40.

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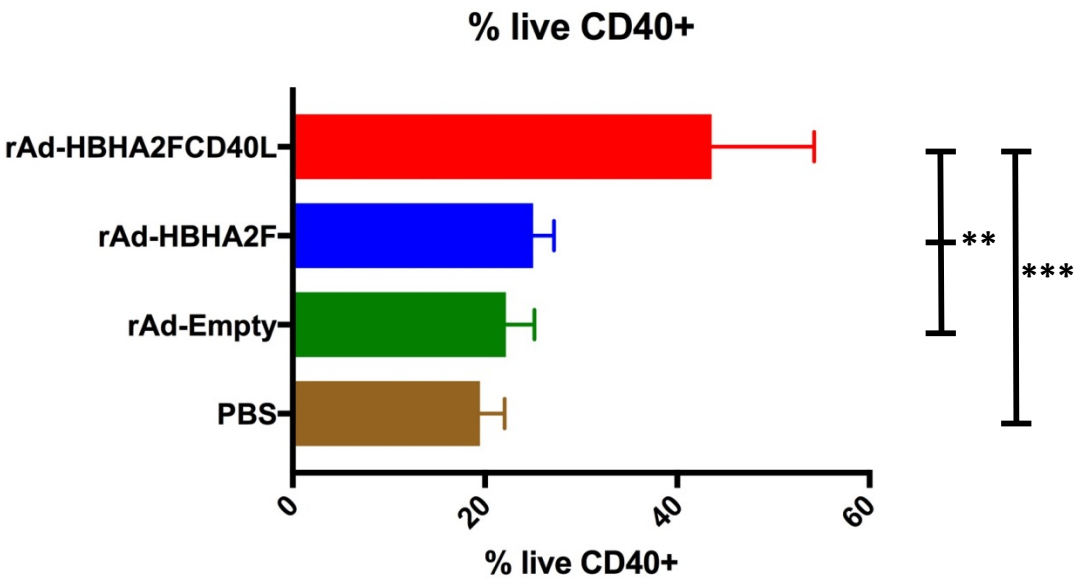
Figure 3.1 CD40L targeting increases CD40 expression.

3 days post-challenge draining inguinal LNs were excised from immunized mice with the designed rAd constructs. Data shown from one experiment with n of 5 mice per treatment group. *** $p<0.001$ and ** $p<0.01$ one-way ANOVA with Bonferroni post test.

Figure 3.1


% live CD40+
rAd-HBHA2FCD40L
rAd-HBHA2F
rAd-Empty
PBS
0
20
40
60
% live CD40+
**
***

3.5.2 CD40L induces a higher GC (germinal center) B cell formation

After confirming the functionality of CD40L ectodomain in our designed vaccine, we next investigated its effects on the germinal center formation. As we have discussed before, CD40 and its ligand have a major role in the development of humoral and cellular immunity. The humoral immune response is when B cells produce antibodies against extracellular microorganisms, preventing the spread of intracellular infections. In this early response, B cells form interactions with antigen-specific T cells in order to become fully activated. These activated B cell and T cells differentiate into GC-B cells (De Silva, N. 2015). GCs are present in the lymph nodes and spleen, and are the main site where antigen-activated B-cells proliferate, differentiate and undergo immunoglobulin gene hypermutation and selection (Zhang, Y., et al. 2016).

To observe its direct effect in GC formation, we analyzed by flow cytometry the expression levels of GCs in B cells from draining LNs isolated 3 weeks after the second immunization. Similar to the procedure followed to measure the levels of CD40, LNs were washed and double stained with Alexa Fluor 647-conjugated anti-mouse Ly77/GL 7 and PE-Cy7-conjugated anti-mouse CD45R/B220. GL7 is a marker for mouse GC- B cells and B220 is an epitope expressed by B cells in early and mature stages (eBioscience). The double staining allowed us to see the population of GC directly related to B cells.

As expected, both groups of mice immunized with the consensus HA2 (rAd-HBHA2F and rAd-HBHA2FCD40L) show significantly higher levels of expression of GC compared to both control groups PBS and rAd-empty (Figure 3.2), this can be related to the protection results we were able to observe in the previous chapter, where both groups show the same level of protection and similar viral titer and tissue damage, suggesting this is antigen-specific protection. Importantly, consistent to results observed in the CD40 expression (section 3.1), the expression

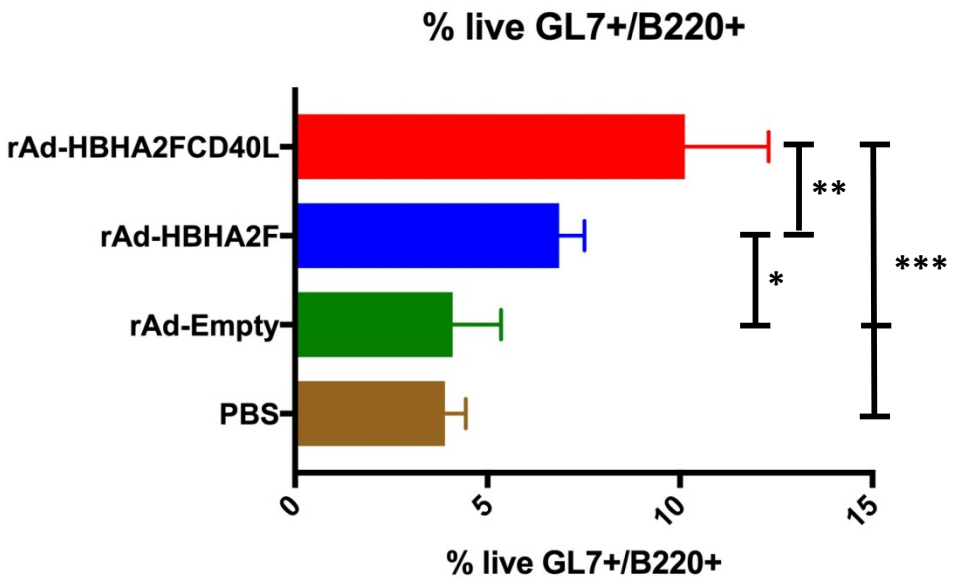
levels of GC in mice immunized with rAd-HBHA2FC40L is significantly higher than that observed in mice immunized with rAd-HBHA2F (** $p<0.01$). These results support the fact that the addition of CD40L has a direct impact on the up-regulation of GC-B cell formation.

Furthermore, previous studies have shown the importance of CD40L expressed on T cells in the regulation of apoptosis of GC-B cells, where stimulation of CD40 prevented premature apoptosis of B cells, resulting in long-lasting antibodies (Koopman, G., et al. 1997). This suggests that the addition of CD40L to the designed vaccine (rAd-HBHA2FCD40L) could result in long-lasting protection.

Figure 3.2 CD40L addition up-regulates B cell GC expression on lymph nodes

3 days post-challenge draining inguinal LNs were excised from immunized mice with the designed rAd constructs and stained to measure expression level of GL7+/B220+ cells. Data shown from one experiment with n of 5 mice per treatment group. *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ one-way ANOVA with Bonferroni post test.

Figure 3.2


% live GL7+/B220+
rAd-HBHA2FCD40L
rAd-HBHA2F
rAd-Empty
PBS
**
*
***
0
5
10
15
% live GL7+/B220+

3.5.3 Consensus HA2 induce T_H1 antibody isotypes against influenza B in DBA/2 mice

As we have previously discussed, GCs are where activated B-cells undergo immunoglobulin differentiation (Zhang, Y., et al. 2016). And since we were able to observe a higher level of GCs due to the stimulation of CD40L in rAd-HBHA2FCD40L, we decided to see its effect on the antibody profile of the different immunized groups.

To achieve this, blood samples were collected to analyze the antibody profile. Collected blood samples were processed to obtain the serum and analyzed by ELISA as described in section 3.4.3. The time point for sample collection was determined based on literature, where it has been shown that antibody production occurs after immunization and reaches the highest peak during the first 14 days before returning to a steady level (AGDHA, 2013), making the ideal time point for study 3 weeks after immunization. Also, these samples were collected before the challenge in order to be able to study the direct effect of the vaccine on the mice, without the influence of virus.

First, we analyzed the total IgG production of the different groups; our results showed that mice immunized with control groups, PBS and rAd-empty, have no detectable IgG, while mice immunized with the consensus HA2, rAd-HBHA2F and rAd-HBHA2FCD40L, showed similar antibody levels between them (Figure 3.3A). These results are consistent with the similar levels of protection observed before from both groups.

Then we proceeded to analyze specific isotypes, such as IgG2a, IgG2b and IgG1. These isotypes have the highest concentration in the bloodstream, making them detectable in serum analysis (Vidarsson, G., et al., 2014). As expected, mice immunized with PBS and rAd-empty, showed very little and non-detectable levels of every specific isotype, while mice immunized with designed consensus HA2 showed significantly higher levels of the specific isotypes (Figure

3.3B, C and E). Statistical analysis revealed that mice immunized with rAd-HBHA2FCD40L had significantly higher production of IgG2a and IgG2b ($p<0.05$), while mice immunized with rAd-HBHA2F had significantly higher production of IgG1 ($p<0.05$).

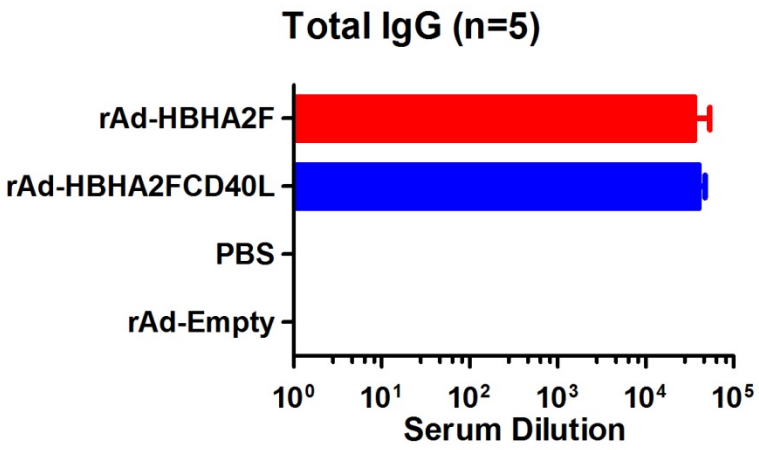
This difference in isotypes is important because these IgG isotypes have previously been investigated as type 1 T-helpers (TH1) and type 2 T-helpers (TH2) markers (Mountford, A.P., et al., 1994; Hashem, A., et al., 2014; Rostamian, M., et al., 2017; Sousa, A. O., et al. 1998), that can lead to enhanced susceptibility toward specific classes of pathogens (Vidarsson, G., et al., 2014). TH1 are known to activate macrophages and produce inflammatory responses against infections by intracellular parasites such as bacteria and some viruses, while TH2 cells are known to mediate phagocyte-independent protective responses (Berger, A., 2000; Mosmann, T.R., 1989; O'Garra, N., 2000; Perkel, J., 2011; Romagnani S.,1999). So by performing a IgG1:IgG2a ratio of mice immunized with and without CD40L we were able to observe a 7-fold difference between groups (Figure 3.3E), indicating that the addition of a functional CD40L has a significantly higher TH1-bias influence, giving us an idea of the possible resulting cytokine we could expect from these immunizations.

Figure 3.3 CD40 targeting favourably induces T_H1 antibody isotypes

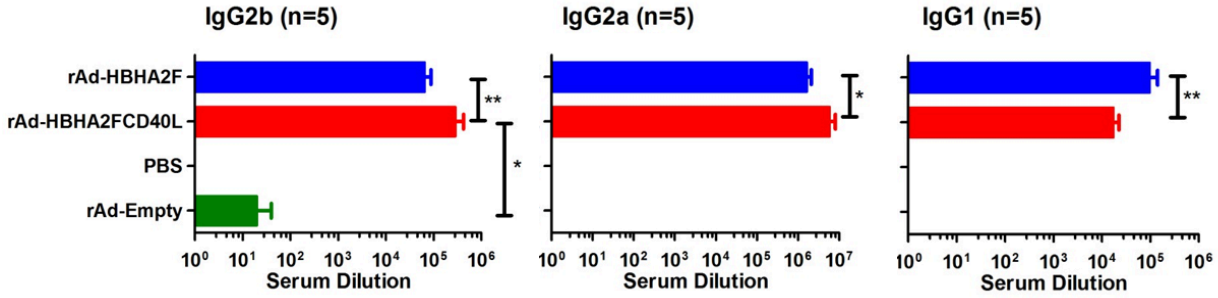
(A) Total antigen specific antibody titers after priming and boost is shown, along for **(B)** IgG2b**, (C)** IgG2a and **(D)** IgG1 isotypes. Data is shown for one experiment with n of 5 mice per treatment group. Antibody titer end-point was expressed as reciprocals of the final detectable dilution with cut-off defined as the mean of PBS samples +3SD. **(A) ***** represents $p<0.001$ (one-way ANOVA with Bonferroni post-test). **(B) *** represents $p<0.05$ (one-way ANOVA with Bonferroni post-test); ****** represents rAd-HBHA2F vs rAd-HBHA2FCD40L un-paired t test with 95% confidence interval. **(C) *** represents rAd-HBHA2F vs rAd-HBHA2FCD40L un-paired t test with 95% confidence interval. **(D) **** represents rAd-HBHA2F vs rAd-HBHA2FCD40L un-paired t test with 95% confidence interval. **(E)** IgG1: IgG2a ratio was measured after priming and boosting to determine the type of immune response induced (T_H2 vs T_H1). Numbers on the columns indicate the mean ratio. ****** represents rAd-HBHA2F vs rAd-HBHA2FCD40L un-paired t test with 95% confidence interval.

Figure 3.3

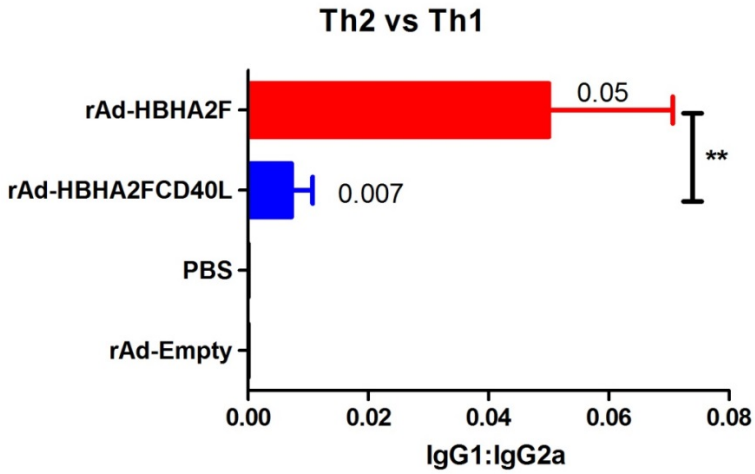
A)



B) **C)** **D)**



E)



3.5.4 Mucosal administration of HBHA2CD40L induces higher antibody level in mucosal antibodies

Since it has been confirmed, the mucosal tissue is part of the main portal entry of influenza, being the first line of defence against infection of inhaled virus (Cox, R.J., et la., 2003). Additional to the antibody profile in serum, we decided to evaluate the profile in the mucosal tissues. To this end, BALF samples were collected at the same time point as serum, aliquoted and frozen until analysis.

We first evaluate the production of antigen-specific total IgG; as expected, we found no detectable levels of antibody in mice immunized with PBS and rAd-Empty, whereas mice immunized with rAd-HBHA2FCD40L produced significantly higher levels compared to rAd-HBHA2F (Figure 3.4A). Next, we analyzed the levels of IgA, which is one of the major neutralizing antibodies against mucosal pathogens, preventing entry and inhibiting replication of the virus (Cox, R.J., et la., 2003). Similar to the results of total IgG in BALF, (Figure 3.4B) control groups showed no detectable levels of antibody, while mice immunized with the designed vaccine with consensus HA2 had higher values. Specifically, mice with CD40L had significantly higher levels than those without it. These results are consistent with our previous study conducted against influenza A using CD40L as a molecular adjuvant, with the observation that the addition of CD40L elicited >=2fold increase in the levels of mucosal antibodies (Fan 2014).

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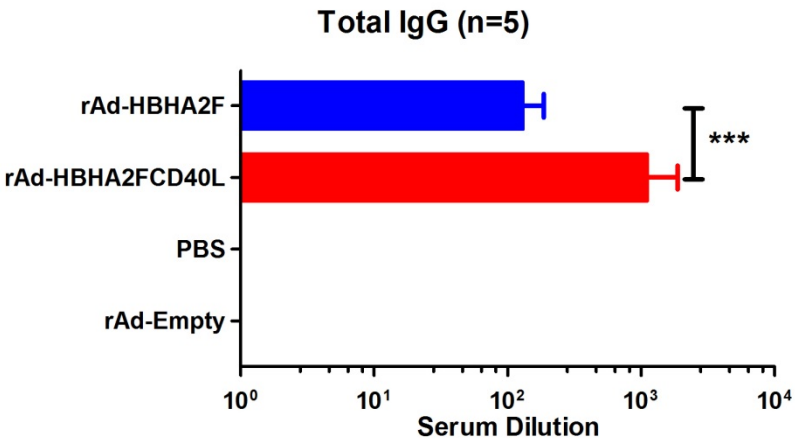
Figure 3.4 CD40L induces the highest mucosal antibody

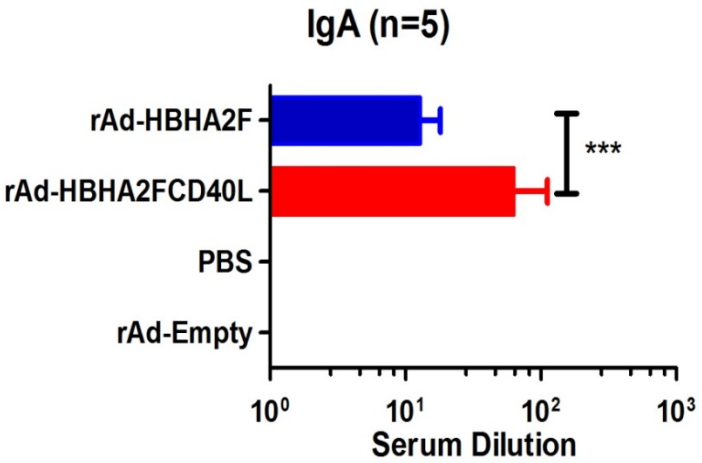
(A) Total antigen specific antibody titers after priming and boosting are shown, along with specific **(B)** IgA. Data is shown for one experiment with n of 5 mice per treatment group. Antibody titer end-point were expressed as reciprocals of the final detectable dilution with cut-off defined as the mean of PBS samples +3SD. *** represents rAd-HBHA2F vs rAd-HBHA2FCD40L un-paired t test with 95% confidence interval.

Figure 3.4

A)


Total IgG (n=5)
rAd-HBHA2F
rAd-HBHA2FCD40L
PBS
rAd-Empty
***
10⁰
10¹
10²
10³
10⁴
Serum Dilution

B)


IgA (n=5)
rAd-HBHA2F
rAd-HBHA2FCD40L
PBS
rAd-Empty
***
10⁰
10¹
10²
10³
Serum Dilution

3.5.5 Consensus HA2 doesn't show cross-neutralizing capacities with or without CD40L

Since we were able to confirm production of antibodies in mice immunized with the consensus HA2 we next aimed to determine whether sera or BALF from such mice could neutralize different subtypes of viruses. To analyze universal protection *in vitro*, we followed the protocol established by WHO in which samples were treated with RDE to increase the sensitivity and remove non-specific inhibitors. Treated samples were exposed to infected cells and left for 20-hour incubation before detection of viral inhibition. We decided to first test two influenza viruses with serum samples; one of the selected viruses was the same used for infection in mice from the Victoria lineage (B/Victoria/2/87) and the second one is the one used to obtain the HA1 portion sequence used in our vaccine which is from the Yamagata lineage (B/Florida/04/06). In addition to the negative controls we used for the animal study (PBS and rAd-empty), we also added a positive control which is polyclonal antibodies against B/Brisbane antisera (Ctrl), which allowed us to ensure the proper interpretation of the results.

Our results showed that the serum antibodies are unable to offer any protection in an *in vitro* neutralizing assay (Figure 3.5 A), this trend was consistent not only in the control groups and the designed vaccines against the same virus but also in the two genetically distinct viruses we tested in this study.

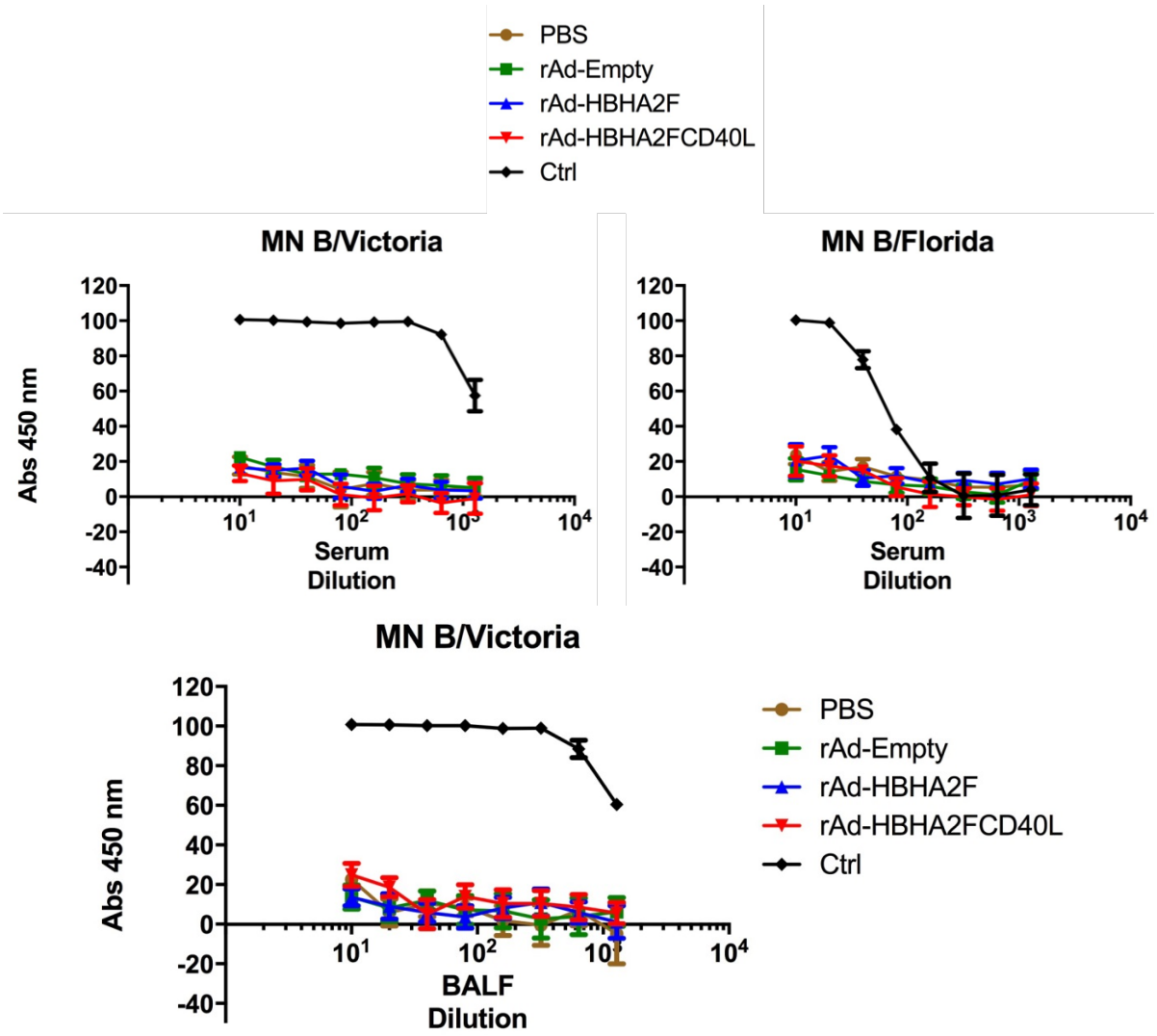
Since there was no visible neutralization from the tested serum samples, we decided to try BALF following the same protocol and using the same virus tested in the animal study. Results were consistent with the ones obtained from the serum analysis which showed none of the tested groups possessed any neutralizing capacity (Figure 3.5 B).

These data differed from the previous study which showed HA2 based antigen in conjunction with CD40L were able to induce neutralizing antibodies against influenza A virus (Fan et al 2014). The discrepancy in results remains to be fully understood. However, it is noted that the previous study employed a native HA2 stalk while ours is a synthetic HA2 stalk with an additional portion from the HA1 domain, which may not properly expose the neutralizing epitopes. However, since these two studies demonstrated a similar level of protection, ie., 100% protection, it would be reasonable to conclude that the protections were mediated by a different mechanism, where non-neutralising antibodies would be mainly involved in activation of complement or trigger antibody-mediated cytotoxicity (ADCC). Furthermore, induction of different cytokine profiles may also help elucidate the mechanisms underlying the protections afforded by the synthetic vaccines.

Figure 3.5 HA2 antibodies tested for cross-neutralizing influenza B strains

Serum and BALF HA2 antibodies don´t show neutralizing capacity against different subtypes of influenza B**.** Influenza B/Victoria/2/87 (Victoria) and B/Florida/04/06 (Yamagata) viruses were tested in microneutralization assay. Data are presented as percentage of neutralization from one experiment with an n of 5 per group.

Figure 3.5


PBS
rAd-Empty
rAd-HBHA2F
rAd-HBHA2FCD40L
Ctrl
MN B/Victoria
Abs 450 nm
Serum Dilution
MN B/Florida
Serum Dilution
MN B/Victoria
Abs 450 nm
BALF Dilution
PBS
rAd-Empty
rAd-HBHA2F
rAd-HBHA2FCD40L
Ctrl

3.5.4 HBHA2FCD40L upregulates T_H1 cytokine production

We have so far confirmed that the addition of CD40L has a direct effect on the humoral immune response by increasing the production of antibodies in serum and mucosal and eliciting a higher TH1-skewed response. Nevertheless, we weren't able to observe neutralization *in vitro* against diverse strains of influenza B. But as we had previously mentioned, CD40 plays a role in the humoral and cell-mediated immune response so we next proceeded to study its effects on the cell-mediated response.

The cell-mediated immune response, also known as T cell-mediated immunity because of the main role T cells play in it, occurs when activated T cells come across APCs, such as dendritic cells (DCs) or macrophages, displaying foreign antigens, resulting in the activation of antigen-specific cytotoxic T-lymphocytes, activating NK cells and stimulating the secretion of cytokines (Janeway, C.A., et al., 2001).

In order to study the differences between mice immunized with our designed HA2 vaccine with and without CD40L, we performed an analysis of the cytokine profile by Luminex assay on spleen cells collected 3 weeks after second immunization with the different vaccines. To this end, collected cells were stimulated with an MHC class I peptide for 48 hours before being analyzed with ProcartaPlex Multiplex Immunoassay kit, which uses magnetic beads loaded with target-specific capture antibodies and allows the detection by using a biotinylated analyte-specific detection antibody and quantification by using a fluorescent detection label.

Cytokines are produced in response to an antigen, acting as a messenger in the regulation of the innate and adaptive system. They can be divided into two groups according to their functional roles into TH1 or TH2 cytokines (Berger, A., 2000). The used multiplex assay allowed us to study multiple cytokines from both groups.

Our results showed very low to non-detectable levels of cytokines from the TH2 family such as IL-4, IL-5 and IL-13. In contrast, there were 3 cytokines from the TH1 family (IL-2, TNF-alpha and IFN-gamma) that not only showed detectable levels but showed a significant difference between the treatment with and without CD40L. IL-2 was detectable in lower concentrations in the control groups, PBS and rAd-empty, but had a significantly higher concentration in mice immunized with the consensus HA2 (Figure 3.6 A). Additionally, there was a significantly higher concentration, >=2 fold higher, in mice immunized with rAd-HBHA2FCD40L than rAd-HBHA2F. As expected, IFN-gamma and TNF-alpha didn't show detectable amounts in mice immunized with PBS and rAd-empty, while mice immunized with rAd-HBHA2FCD40L had a significantly higher level than rAd-HBHA2F (95% confidence) (Figure 3.6 B-C).

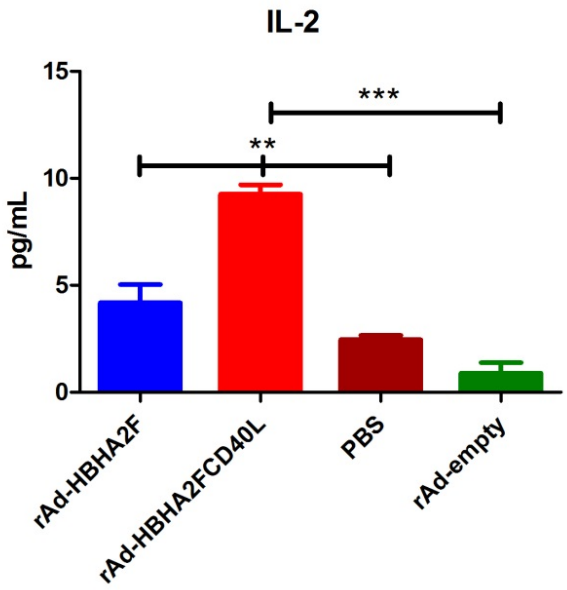
As we had discussed before a TH1 response is directly related to the activation of macrophages, the pro-inflammatory response and perpetuation of autoimmune responses against intracellular parasites such as bacteria and some viruses (Berger, A., 2000; Mosmann, T.R., 1989; O'Garra, N., 2000; Perkel, J., 2011; Romagnani S.,1999). But in particular, these cytokines are known to have a direct effect on the stimulation and activation of NK cells and CTLs (Berger, A., 2000; Hashem, A., et al., 2014; Kaiser, G.E., 2014), suggesting that the higher polarized immune response toward a TH1 phenotype using CD40L had been induced.

Figure 3.6 CD40L upregulates T_H1 cytokine production

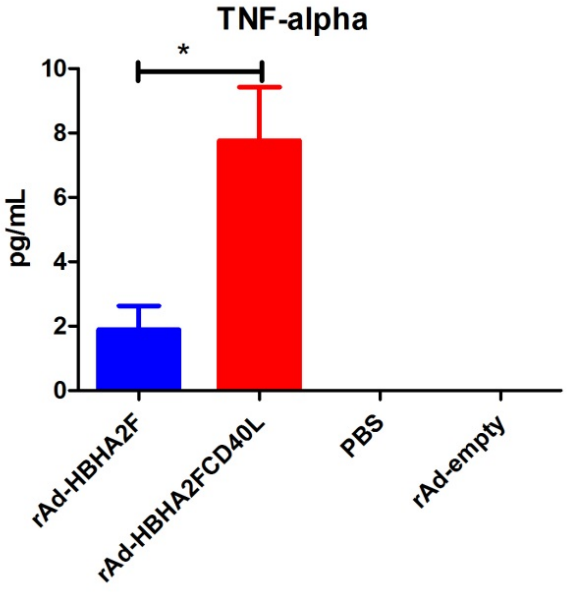
Data shown from one experiment with n of 3 to 5 mice per treatment group. pg/ml = (peptide stimulated sample) - (sample with no peptide). **(A)** *** represents $p<0.001$ and ** represents $p<0.01$ (one-way ANOVA with Bonferroni post-test). **(B)** * represents rAd-HBHA2F vs rAd-HBHA2FCD40L un-paired t test with 95% confidence interval. **(C)** *** represents rAd-HBHA2F vs rAd-HBHA2FCD40L un-paired t test with 95% confidence interval.

Figure 3.6

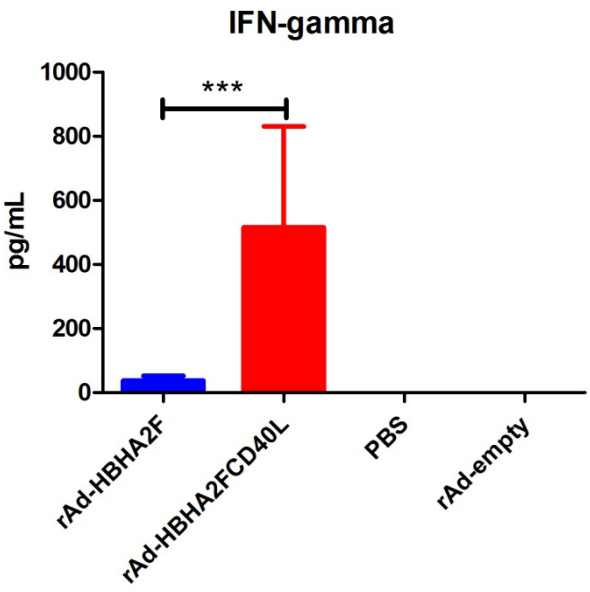
A)



C)



D)



3.6 DISCUSSION

The binding of CD40L to CD40 plays a significant role in orchestrating both the humoral and cellular immune responses. The use of CD40L as a molecular adjuvant with antigens of interest using viral vectors has been previously reported by several groups including our own (Cao, J. et al., 2010; Fan et al., 2015; Hashem et al., 2014; Huang, D. et al., 2004; Lin, F.C. et al., 2009; Liu, J et al., 2008; Tripp, R.A., et al., 2000). However, the potential of CD40L as a targeting and stimulatory molecule has never been studied in relation to the protection against influenza B virus. As is the case for influenza A viruses, the highly conserved stem region of the HA2 subunit of the HA protein is an attractive target for the development of a universal vaccine against influenza B viruses but it appears to have low immunogenicity and confer poor protection. It is therefore of great interest to study the potential enhancing effect of a costimulatory molecule, such as CD40L, on the protective immune response induced by vaccination targeting the HA2 region of influenza B.

In this study, immunization with rAd-HBHA2FCD40L resulted in a significantly higher number of CD40+ cells, indicating the functionality of the added CD40L by the up-regulation of expressed CD40. As mentioned above, this is an important costimulatory molecule involved in the amplification and regulation of inflammatory responses (Bennett, S.R., et al., 1998; Fan et al., 2015; Oxenius et al., 1996; Schoenber, S.O. et al., 1998; Schultze J.L. et al., 1997; van Kooten et al., 2000).

Based on this rationale, we first evaluated the effect of CD40L in the humoral immune response by analyzing its effect on the differentiation of GC-B cells, with results showing high levels of GL7+ B cells, which indicates the induction of GC formation in draining lymph nodes. This was supported by further analysis of the specific antibody isotypes on serum and BALF,

where higher levels of IgG2a but lower levels of IgG1 were observed on mice immunized with rAd-HBHA2FCD40L compared to mice immunized without CD40L and both control groups, suggesting a higher TH1-skewed immune response. Additionally, we were able to observe a higher production of specific mucosal antibody isotypes, IgA, on mice immunized with rAd-HBHA2FCD40L.

Secondly, we studied the effect of CD40L on the cellular immune response by performing analysis on the cytokine profile on splenocytes from mice immunized with the designed vaccine with and without CD40L. Results supported what we had been able to observe so far since there were no detectable levels of cytokines of the TH2 family, but there was a significant increase in the concentration of IL-2, TNF-alpha and IFN-gamma, cytokines of the TH1 family.

Collectively, these results indicate that CD40L incorporated in our vaccine constructs is functionally active and can enhance the HA2-specific cellular and humoral immune response.

Additionally, we investigated the potential of inducing broadly neutralizing antibodies. This is based on our previous studies that have shown that a vaccine designed with the HA2 domain of influenza A (rAd-HAFCD40L) was able to induce cross-reactive antibodies capable of neutralizing multiple strains of influenza A *in vitro* (Fan et al., 2015). Interestingly, the antibodies induced here using our consensus influenza B HA2 by both vaccines with and without CD40L didn't show any detectable *in vitro* inhibitory activity against influenza B virus of both lineages. Such difference in HA2 based vaccines between type A and type B viruses remain to be fully understood. However, the HA2-based type B vaccine reported here is different from the HA2-based type A vaccine in that the former has an additional sequence from HA1 for stability and is also based on a synthetic sequence derived from bioinformatics analyses. Nonetheless, our

results are in agreement with previous observations that most antibodies targeting the HA2 stem region are not capable of preventing viral entry, in contrast to the antibodies against the HA1 domain (Fan et al., 2015).

Importantly, our findings reinforce the notion that non-neutralising antibodies and cell-mediated immune responses may play key roles in HA2 elicited immune responses. This was confirmed by the observed antibody profile that could be working by activating a complement cascade and the proliferation of cytokines directly involved in the stimulation and activation of NK cells and CTLs. For future studies, ADCC effects should be analyzed while a passive transfer of serum and adoptive transfer of T cells isolated from immunized mice into naïve animals could also provide valuable information on the role of the humoral and cellular response in these two vaccine constructs. Finally, it would be essential to conduct more animal studies in which multiple viruses would be used to challenge the animals so as to further determine the protection against diverse strains of influenza B viruses.

CHAPTER 4 General Discussion and Conclusion

Even though influenza A virus is known to be more infectious and contagious, and well documented to transmit between species, influenza B cases have been increasing over the years. Indeed, approximately 25% of influenza cases are caused by influenza B viruses. While the current vaccination strategy has helped decrease the number of severe cases when the vaccines match the circulating viruses, the tendency of the virus to unpredictably undergo mutations and gene re-assortment, present tremendous challenges in the development of universal vaccines.

As discussed above, the current influenza vaccines mainly provide strain-specific protection, targeting the highly variable regions of the globular head in the HA1 subunit. On the other hand, the HA2 subunit, part of the stem domain of the HA protein, has a higher conservation rate between the different subtypes of influenza, making it an attractive target to induce broadly protective antibodies, and has recently been the focus of some research groups, including our own (Chai, N. 2016; Fan, X. et al., 2015; Hashem et al., 2015).

This current work is novel in that it is the first time a synthetic sequence of HA2 was identified through a comprehensive bioinformatics analysis of all influenza B viruses; the sequence represents the highest degree of conservation in all influenza B virus, i.e., over 95% of conservation rate. Moreover, the recombinant adenoviral vector strategy was employed for antigen delivery because of its relatively low cytotoxicity, ability to infect both proliferating and non-proliferating cells and most importantly, its reported efficiency in mucosal immunization (Fan, et al., 2015: Huang, D., et al., 2008; Santosuosso, M. et al., 2005).

The second novelty of this work is that we studied the possibility of using CD40L as a molecular adjuvant and targeting ligand to improve the influenza B vaccine-induced immune response. Our results presented here are consistent to a similar strategy we previously used to target the influenza A virus, in which the addition of CD40L resulted in long-lasting protection

and a skewed TH1 response (Fan et al., 2015; Hashem et al., 2014). Collectively, our work provides strong evidence that additional stimulation of CD40 can substantially enhance immune responses against both type A and B viruses.

Specifically, we found that mice immunized with rAd vectors expressing the HA2 consensus sequence were fully protected against a high dose of influenza B/Victoria/2/87, as demonstrated not only by a 100% survival rate after viral challenge but also by observation of minimal clinical symptoms such as weight loss, respiratory distress and muscle atrophy in the surviving animals. Additionally, this was supported by the minimal damage observed in the lower respiratory tissue.

Similar to what was observed with a CD40L targeting HA2 influenza A vaccine, we found that the addition of CD40L with rAd vector expressing the HA2 consensus sequences from influenza B was able to promote a higher production of specific mucosal antibody and a higher TH1-skewed response as demonstrated by the higher production of TH1 antibody isotypes like IgG2a and stimulation of cytokines from the TH1 family which are involved in the activation of NK cells and CTLs. As mentioned earlier, it is important to note that the full protection afforded by the synthetic HA2-based influenza B vaccines was achieved even in the absence of CD40L, an observation that is different from that observed in our previous type A influenza vaccine studies, which showed that CD40L-targeting/ HA2-based vaccine protects better than vaccine without CD40L (Fan et al., 2015).

While the discrepancy in terms of the need for CD40L between type A- and type B- HA2 vaccines remains to be fully understood, it is likely several factors could have contributed to the differences which might be worth being investigated further in future studies.

First, much larger amounts of virus inocula would have been needed in challenge studies to detect the difference in protection between the HA2 based-vaccines with or without the CD40L. This proved to be a technical difficulty in the current influenza B infection animal models because the mouse model for influenza B virus infection is less sensitive than that for influenza A virus. Specifically, the animals were less susceptible to infection from influenza B virus infection as shown in Chapter 3; in the challenge studies, the infection of influenza B virus resulted in only 60% death in all the animals tested while 100% of mortality could be achieved in influenza A virus infection. As much larger amounts of influenza B virus could have been needed to achieve 100% mortality, the viscosity of the virus inocula could be challenging as it is much more difficult for the virus to be inhaled intranasally by the animals. Future studies should employ more animals in the groups and explore more mouse-adapted influenza B viruses; if possible different species of animals should be explored. Second, although we did not observe a significant difference in protection between CD40L-containing and CD40L-free vaccines, the two vaccines may induce protection through different mechanisms. Specifically, targeting the secreted HA2 to CD40+ cells with CD40L resulted in a significantly skewed immune response towards a TH1 immune response, as evidenced by increased production of TH1-bias cytokines by antigen-stimulated splenocytes and higher levels of HA2-specific TH1-bias serum IgG antibodies. As cytotoxic T-lymphocytes (CTL) responses may be favoured in rAd-HBHA2FCD40L vaccinated mice, a more in-depth analysis of antigen-specific cell-mediated cytotoxicity will be needed to better understand how CD40L may affect the effector function of antigen-specific CD8+ T cells and their ability to kill target cells. Additionally, to further investigate whether CD40L targeting influences the role of CD4+ and CD8+ T cells in

protection, adoptive transfer studies of cells isolated from mice immunized with each construct should also be considered, as well as evaluating the protection induced by the CD40L-targeted or non-targeted HA2 vaccines in CD4-/- and CD8-/- knockout mice. On the other hand, a passive transfer study of the serum TH1-skewed anti-HA2 antibodies would help elucidate the role of CD40L-targeting HA2 vaccine, while the potential of CD40L targeting approach to induce long-lasting immune responses in vaccinated animals against influenza B virus should be explored (Hashem, A., et al., 2012).

In summary, the findings reported in this thesis lead us to a better understanding of the potential of using a conserved consensus HA2 sequence to induce protection against influenza B virus, providing insight towards the development of a potential universal vaccine. To our knowledge, this is the first report on the prototype universal vaccine based on the most highly conserved HA2 sequence found in all influenza B viruses in conjunction with CD40L stimulation. While more studies would obviously be needed to fully understand the scope of protection and mechanism, the results obtained so far are very promising in that the prototype vaccine could afford 100% protection in animals with no detectable clinical signs and symptoms of toxicity.

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