

Targeting the Highly Conserved Sequences in Influenza A Virus

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for a doctoral degree in Microbiology and Immunology

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ABSTRACT

All challenges associated with influenza A viruses including antigenic variation in hemagglutinin (HA) and neuraminidase (NA), the evolving drug resistance and the drawbacks of current vaccines hinder our ability to control this constant threat. Furthermore, gene reassortment as well as the direct transmission of highly pathogenic avian viruses to humans can result in an occasional emergence of novel influenza strains with devastating pandemic potential. Therefore, it is crucial to investigate alternative approaches to better control these viruses and to develop new prophylactic and treatment options.

Targeting highly conserved epitopes or antigens among the different subtypes of influenza A virus could offer protection against broad range of influenza viruses, including emerging strains. In my research, I have investigated the potential of broadly neutralizing antibodies against HA and conducted mechanistic study of a prototype vaccine based on the highly conserved nucleoprotein (NP).

We recently found that the 14 amino acids of the amino-terminus of the fusion peptide of influenza HA2 subunit is the only universally conserved sequence in all HA subtypes of influenza A and the two lineages of influenza B viruses. Here, I show that universal antibodies targeting this linear sequence in the viral HA (Uni-1 antibodies) can cross-neutralize multiple subtypes of influenza A virus by inhibiting the pH-dependant fusion of viral and cellular membranes.

It is noted that the influenza NP is a highly conserved antigen and has the potential to induce heterosubtypic immunity against divergent subtypes of influenza A virus. However, NP-based vaccination only affords weak protective immunity compared to HA. This is mostly due to the non-sterilizing immunity induced by NP. Using CD40 ligand (CD40L), a key regulator of the immune system, as both a targeting ligand and a molecular adjuvant, I show that single immunization with recombinant adenovirus carrying a fused gene encoding the secreted NP-CD40L fusion protein provided robust and long-lasting protection against influenza in normal mice. It enhanced both B-cell and T-cell responses and augmented the role of both NP-specific antibodies and CTLs in protection. Importantly, it afforded effective protection in CD40L and CD4 deficient mice, confirming that the induced protection is CD40L-mediated and CD4⁺ T cell-independent.

The rapid evolution of the influenza A viruses necessitates the development of new alternatives to contain this medically important pathogen. The results of these studies could significantly contribute to future vaccine development and avert the necessity of yearly vaccine updates.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Sean Li for his mentorship and patience which were shown through his encouragement, guidance and valuable advice. He was always there for me with his help and support. I really feel privileged for the scientific freedom that he provided me in pursuing my research in his laboratory. His faith in me was the driving force behind all the work that we have done together.

I would like to thank my co-supervisor Dr. Eillean Tackaberry for her guidance and support. I am very grateful for all the times she came to congratulate me on any paper I had published. I would like also to extend my appreciation to the members of my thesis advisory committee, Dr. Harold Rode, Dr. Karen Copeland, Dr. Michel Girard and Dr. Kylie Scoggan for their support, guidance and helpful suggestions. I am very thankful to Dr. Runtao He and Dr. Aaron Farnsworth for their constructive criticism and scientific discussions throughout my years of study at Health Canada.

I am greatly indebted to my wonderful colleagues Caroline Gravel, Bozena Jaentschke, Monika Tocchi, Louise Larocque and Marsha Russell for their technical assistance, support and friendships. They have spent a lot of their time and energy to help me with my research and they were always there for me. I could not complete my work without their invaluable help and support. A thank you also goes out to my fellow graduate student Tracey Doyle as well as all the former lab members for their valuable help and input.

A special word of appreciation for Dr. Martha Navarro and all the staff in the animal care facility at Health Canada for their great assistance with the animal experiments. I would also like to acknowledge Dr. Michael Rosu-Myles and Emily Chomyshyn for their assistance with flow cytometry analysis, and Michelle Lemieux for her help with the sequencing. They were always accommodating and helpful.

I would like to thank all the co-authors in all the papers we published together for their collaboration. Their contribution helped me not only to publish my work but also to advance my knowledge.

I am very grateful to everybody at the Centre for Vaccine Evaluation at Health Canada for their warm feelings and kindness. They all made me feel like I am home.

Special thank you goes to all my friends and relatives in Saudi Arabia, Canada and the US for their support and understanding.

I would like also to thank King Abdulaziz University and the Saudi Arabian Cultural Bureau in Canada for granting me a full scholarship to pursue my graduate studies. Their support made my dream come to reality.

I am forever grateful to my parents and my siblings for their unconditional love and support throughout my life. My parents have always been the true inspiration in my life. Their dedication, support and sacrifice are the true reasons behind my success in every aspect of my life. My brothers and sisters were always there when I needed them.

Last but not least, I am very fortunate to have my wife Ashwaq and son Nawaf in my life. Ashwaq's support, tolerance and love were undeniably the bedrock upon which the past years of my life have been built. Thank you Ashwaq and Nawaf for all the nights that you have spent without me or waiting for me to come home early. Thank you for your unwavering love, patience and understanding over all these years.

DEDICATION

To my parents, wife Ashwaq and son Nawaf

You are the true inspiration in my life

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
APC	Allophycocyanin
ASC	Antibody-secreting cells
ATCC	American Type Culture Collection
BGTD	Biologics and Genetic Therapies Directorate
BnAbs	Broadly-neutralizing antibodies
BPL	β -propiolactone
bp	Base pairs
BSA	Bovine serum albumin
CAR	Coxsackie-adenovirus receptor
CD	Cluster of designation
CDC	Complement-dependent cytotoxicity
cDNA	Complementary DNA
CD40L	CD40 ligand
CD4 ^{-/-}	CD4 deficient mice
CD8 ^{-/-}	CD8 deficient mice
CD40L ^{-/-}	CD40L deficient mice
cRNA	Complementary RNA
CTLs	Cytotoxic T lymphocytes
C-terminus	Carboxyl-terminus
CVE	Centre for Vaccine Evaluation
DCs	Dendritic cells
DTT	Dithiothreitol
Δ FCD40L	Mouse CD40L with truncated trimerization motif
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
dsRNA	Double stranded RNA
E. coli	Escherichia coli
ELISA	Enzyme linked immunosorbent assay
F	Trimerization motif
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcR	Fragment crystallizable receptor
FITC	Fluorescein isothiocyanate
FPLC	Fast performance liquid chromatography
GC	Germinal centers
GST	Glutathione S-transferase
HA	Hemagglutinin
HA0	Hemagglutinin precursor protein
HA1	Hemagglutinin subunit 1
HA2	Hemagglutinin subunit 2
HCL	Hydrogen chloride

HEK-293	Human Embryonic Kidney-293 cells
HI	Hemagglutination-inhibition
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HPAI	Highly pathogenic avian influenza
H5-TH04	Influenza A/Thailand/2-SP-33/2004(H5N1)
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IgG2b	Immunoglobulin G2b
IgG2c	Immunoglobulin G2c
IgM	Immunoglobulin M
IL	Interleukin
IP-10	Interferon induced protein 10
IPTG	Isopropyl- β -D-galactopyranoside
Kb	Kilobase
kDa	Kilodalton
kg	Kilogram
LAIV	Live-attenuated influenza vaccine
LB	Luria Broth
LC MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LDH	Lactate dehydrogenase
LD ₅₀	Median lethal doses
LN	Lymph node
M	Molar
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein 1
MDCK	Madin-Darby canine kidney cells
MEM	Minimum Essential Medium
MF59	Microfluidized Emulsion 59
μ g	Microgram
mg	Milligram
MHC	Major histocompatibility complex
μ l	Microliter
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger RNA
Mx	IFN-induced large guanosin triphosphatases proteins
M1	Matrix 1 protein
M2	Matrix 2 protein
M2e	Matrix 2 protein extracellular domain
NA	Neuraminidase
NCBI	National Center for Biotechnology Information

NEP	Nuclear export protein
Ni-NTA	Nickel-nitrilotriacetic acid
NIBSC	National Institute for Biological Standards and Control
nm	Nanometers
NP	Nucleoprotein
NP-6xHis	Influenza NP tagged with six histidine residues
NS1	Nonstructural protein 1
NS2	Nonstructural protein 2
N-terminus	Amino-terminus
ORF	Open reading frame
PA	Acidic polymerase protein
PA-X	Acidic polymerase X polypeptide
PB1	Basic polymerase 1 protein
PB1-F2	Basic polymerase 1 open reading frame 2 polypeptide
PB1-N40	Basic polymerase 1-N40 polypeptide
PB2	Basic polymerase 2 protein
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.05% Tween-20
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFU	Plaque forming units
Pe-Cy5	Phycoerythrin-Cyanine 5
Pe-Cy7	Phycoerythrin-Cyanine 7
PerCP	Peridinin-chlorophyll protein
pH1N1	Swine-origin 2009 pandemic H1N1 virus
pNP	pcDNA3.1 vector expressing influenza NP
pQE2-NP	pQE2 prokaryotic plasmid expressing influenza NP
PrP	Human prion protein
pUC19	pUC19 vector
pUC19-NP	pUC19 vector containing influenza NP
pUC19-CD40L	pUC19 vector containing mouse CD40L
PVDF	Polyvinylidene difluoride membrane
rAd	Recombinant adenovirus
rAd-Control	rAd empty control vector
rAd-NP	rAd expressing unsecreted influenza NP
rAd-NP40L	rAd expressing unsecreted influenza NP-CD40L fusion protein
rAd-SNP	rAd expressing secreted influenza NP
rAd-SNP40L	rAd expressing secreted influenza NP-CD40L fusion protein
RAF-2p48	RNA polymerase activating factor-2 48 kDa polypeptides
RANTES	Regulated on activation, normal T-cell expressed and secreted
RdRp	RNA-dependent RNA polymerase
RIG-I	Retionic acid inducible helicase I
RNA	Ribonucleic acid
rNP	Recombinant influenza NP
RT	Room Temperature
S	Secretion signal
SA	Sialic acid

SA α 2,6-linked Gal	Sialic acid attached to the galactose by an α 2,6 linkage
SA α 2,3-linked Gal	Sialic acid attached to the galactose by an α 2,3 linkage
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNP Δ F	secreted influenza NP with truncated trimerization motif
SP-A	Surfactant proteins A
SP-D	Surfactant proteins D
SRID	Single radial immunodiffusion
svRNAs	Negative-sense small vRNAs
tAd	Transfer vector
tAd-NP	tAd expressing unsecreted influenza NP
tAd-NP40L	tAd expressing unsecreted influenza NP-CD40L fusion protein
tAd-SNP	tAd expressing secreted influenza NP
tAd-SNP40L	tAd expressing secreted influenza NP-CD40L fusion protein
Tat-SF1	Tat specific factor 1
TBS-T	Tris buffered saline with 0.1% Tween-20
TCID ₅₀	Median tissue culture infectious dose
TFA	Trifluoroacetic acid
T _H 1	Type I helper CD4 ⁺ T cells
T _H 2	Type II helper CD4 ⁺ T cells
TIV	Trivalent inactivated vaccine
TLRs	Toll-like receptors
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor superfamily
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
T4	Bacteriophage T4
UPLC	Ultra-performance liquid chromatography system
vRNA	Viral RNA
vRNPs	Viral ribonucleoprotein complexes
v/v	Volume per volume
WHO	World Health Organization
WV	Whole virus
w/v	Weight per volume
x g	Centrifugal force x gravity

CHAPTER 1

General Introduction

1.1 INFLUENZA VIRUSES

Influenza viruses cause highly contagious respiratory tract infections associated with high morbidity and mortality rates. Common symptoms of influenza infection include fever, myalgia, sore throat, nasal congestion, cough, headache and malaise. Pneumonia as well as exacerbation of underlying medical conditions are the most common complications associated with influenza infection. Although influenza viruses cause disease among persons from all age groups, complications, hospitalization and associated death most directly impact young children, individuals with chronic diseases and the elderly (Fiore et al., 2008). Each year, seasonal influenza epidemics affect up to 500 million people, causing 3 to 5 million cases of severe illness, death of up to 500,000 people and debilitating economic costs worldwide (World Health Organization, 2003). More than 90% of influenza-related deaths, however, occur in adults older than 65 years (Fiore et al., 2008).

In addition to the seasonal epidemics, antigenically novel strains of influenza virus can emerge and spread rapidly, causing global pandemics such as the 1918 Spanish pandemic which resulted in up to 50 million deaths or the recent swine-origin 2009 H1N1 pandemic (pH1N1) which spread globally within a few weeks (Garten et al., 2009). Furthermore, the direct transmission of highly pathogenic avian influenza (HPAI) viruses to humans and their persistent circulation in domestic poultry (Lin et al., 2000) highlight the potential for the emergence of a novel human virus with devastating results.

1.1.1 Virus isolation

In 1892, Richard Pfeiffer suggested that *Bacillus influenzae* (now known as *Haemophilus influenzae*) was the causative agent of influenza after his isolation of the bacteria from flu-infected patients (Pfeiffer et al., 1892). However, this suggestion was

partially rejected when nasal secretions from patients infected with the 1918 Spanish virus were filtered to exclude the bacteria but still caused lung disease in rabbits (Olitsky and Gates, 1921). It was not until 1931 that influenza viruses were first isolated from infected pigs by Richard Shope (Shope, 1931). Soon after, the first human influenza virus was isolated at the National Institute for Medical Research, in the United Kingdom in 1933 by Wilson Smith, Christopher Andrewes and Patrick Laidlaw (Smith et al., 1933). Subsequently, this virus was named influenza A because it was the first human influenza virus. In 1940, an antigenically distinct virus that causes similar disease was isolated and named influenza B virus (B/Lee/40) (Francis, 1940). Influenza C virus on the hand was isolated in 1947 (Briody, 1948).

1.1.2 Classification and nomenclature

All influenza viruses belong to the *Orthomyxoviridae* family which encompasses five different genera including *influenzavirus A*; *influenzavirus B*; *influenzavirus C*; *Thogotovirus* and *Isovirus* (Kawaoka et al., 2005). The three types of influenza virus (A, B and C) are classified according to differences between their internal proteins; nucleoprotein (NP) and matrix protein (M1) (Zambon, 1999). Only influenza A viruses are further categorized into different subtypes based on surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins. To date, there are at least 16 HA (1-16) and 9 NA (1-9) subtypes (Naffakh et al., 2008). Recently, a highly divergent influenza A virus was isolated from little yellow-shouldered bats (designated as H17N10). However, this virus cannot infect avian species or grow in mammalian cells (Tong et al., 2012). Phylogenetic analysis shows the 16 subtypes of HA fall into two distinct groups. Specifically, HA group 1 contains H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16, while HA group 2 contains H3, H4, H7, H10, H14 and H15 (Sui et

al., 2009; Nabel and Fauci, 2010). Similarly, the 9 subtypes of NA cluster into two separate groups where subtypes N1, N4, N5 and N8 make up NA group 1, and the remaining subtypes (N2, N3, N6, N7 and N9) compose group 2 (Nabel and Fauci, 2010).

The current nomenclature system for influenza viruses includes the genus, the host species from which the virus was isolated (except for human isolates), the geographical location of isolation, the isolate number and the year of isolation. This is followed by the HA and NA subtypes in parenthesis (for influenza A viruses only). For example, A/swine/Ontario/23866/2004(H1N1) represents influenza A virus isolated from pigs in Ontario in 2004 with an isolate number of 23866, and it is an H1N1 subtype.

1.1.3 Influenza A virion structure and morphology

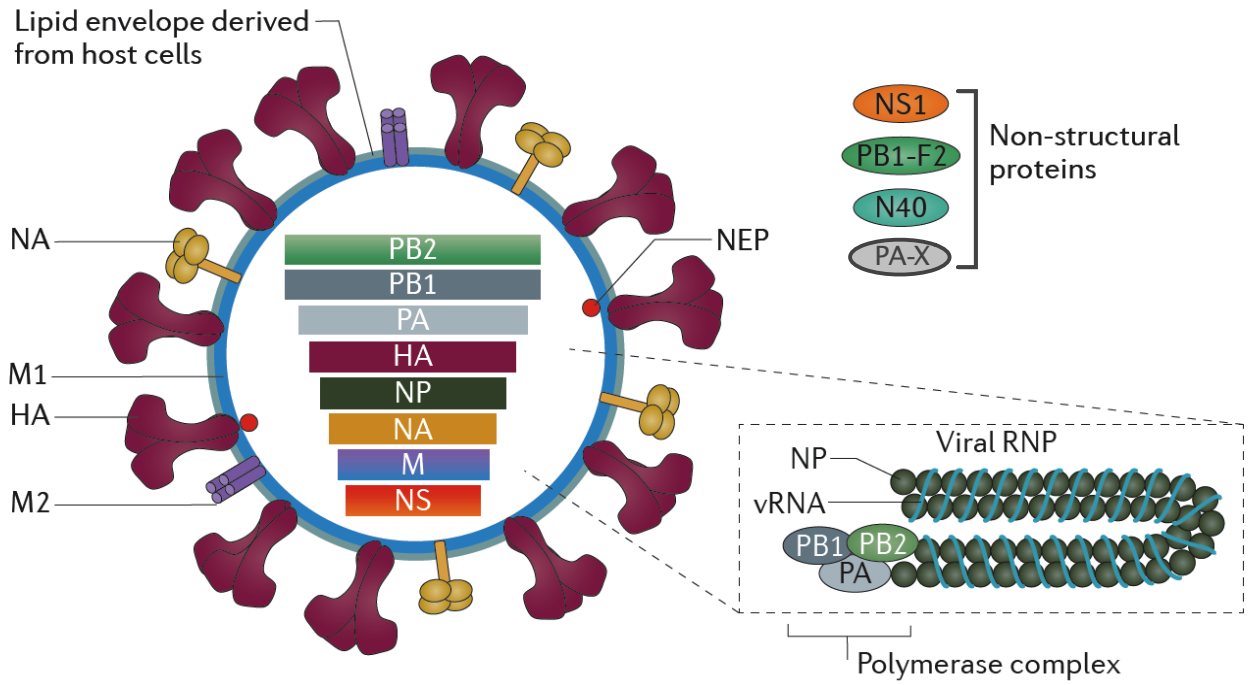
As shown in Figure 1.1, influenza A viruses have an outer lipid bilayer envelope derived from the host plasma membrane during viral budding and release. The HA and NA, are embedded in the viral envelope as spike proteins. The low abundance matrix 2 (M2) protein is also inserted in the viral envelope and as an ion channel involved in viral uncoating step during replication (Naffakh et al., 2008). Underlying the envelope is the viral M1 protein which is the most abundant structural component of the virion. Internally, the 8 viral RNA (vRNA) segments are encapsidated by the virally encoded RNA binding NP, and their partially complementary ends associate with the three RNA-dependent RNA polymerase (RdRp) subunits; polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA), to form the viral ribonucleoprotein complexes (vRNPs) (Compans et al., 1972; Steinhauer and Skehel, 2002). Virus particles also contain small amounts of the nuclear export protein (NEP), originally identified as nonstructural protein 2 (NS2) (Steinhauer and Skehel, 2002; Naffakh et al., 2008).

Figure 1.1

Schematic diagram of influenza A virus particle

The influenza A virus genome consists of 8 single-stranded negative sense RNA segments that encode 11-13 proteins. These RNA segments are associated with the RdRp subunits (PB1, PB2 and PA) and the NP to form vRNP complexes. The M1 protein underlies the lipid bilayer and interacts with the vRNP complexes. Three membrane proteins HA, NA and M2 are inserted in the lipid bilayer of the viral membrane. NEP/NS2 can also be found in the virion. The influenza A genome also encodes at least 4 nonstructural proteins including the host antiviral response antagonist nonstructural protein 1 (NS1), the pro-apoptotic protein PB1-F2 and the newly identified PB1-N40 and PA-X proteins. Reprinted by permission from Macmillan Publishers Ltd: [Nat. Rev. Microbiol.] (Medina and García-Sastre), copyright (2011).

Figure 1.1



Influenza viruses are pleiomorphic ranging from spherical particles that are ~ 100 nm in diameter to long filamentous forms that are ~ 100 nm in diameter and ~ 20 µm in length (Horne et al., 1960). It is believed that laboratory strains are mostly spherical virus particles, while fresh clinical isolates predominantly show filamentous forms (Choppin et al., 1960; Nakajima et al., 2010). A distinctive feature of influenza A virus particles are the HA and NA glycoproteins which project from the viral membrane as rod-shaped and mushroom-shaped spikes, respectively.

1.1.4 Influenza A genome structure and organization

Influenza A viruses have a genome composed of 8 single-stranded negative-sense RNA segments (Palese, 1977). The size of these segments ranges from 890 to 2341 nucleotides (Chan et al., 2006). The protein coding sequence in each strand is flanked by 23-61 and 20-45 non-coding nucleotides at the 5'- and 3'-ends, respectively (Steinhauer and Skehel, 2002). The terminal 12-13 nucleotides at these regions are highly conserved among all the segments and usually followed by a segment-specific non-coding region. In addition, these conserved 5'- and 3'-terminal sequences show partially inverted complementarity (Robertson, 1979; Desselberger et al., 1980).

While the influenza vRNA promoter is mapped to these terminal domains, several models have been proposed for the promoter secondary structure required for the interaction with viral RdRp. Electron microscopic studies demonstrated that both complementary ends of the vRNA form double-stranded secondary “panhandle” structures (Hsu et al., 1987), which was supported by additional biochemical studies (Baudin et al., 1994; Pritlove et al., 1995). However, other work suggested an “RNA fork” structure in which the 5'- and 3'-terminal nucleotides do not base pair to each other entirely, leaving the terminal 9-10

nucleotides at the 5'- and 3'-ends unpaired (Fodor et al., 1995). Another secondary structure based on “corkscrew” conformation was also proposed in which the vRNA promoter sequence within the 5'- and 3'-terminal single-stranded regions folds into local 5'- and 3'-hairpin loops (Flick et al., 1996). Although it is not clear whether only one or more of these structures occur during RNA transcription, it is evident that the overall secondary conformation, i.e. base-pairing within the promoter region, is critical not only for promoter recognition by RdRp (Fodor et al., 1995), but also for vRNPs packaging into progeny virions, polymerase endonuclease activity, messenger RNA (mRNA) polyadenylation and polymerase stability (Pritlove et al., 1999; Leahy et al., 2001; Brownlee and Sharps, 2002).

The 8 viral RNA segments of influenza A viruses encode at least 13 known viral proteins. These proteins are categorized as 9 structural and 4 nonstructural proteins based on their presence in complete virions. Structural proteins determine the host range, tissue tropism, and efficiency of transmission, antigenicity and, to a large extent, the pathogenicity of influenza viruses. On the other hand, nonstructural proteins are present only in infected cells but not as components of assembled viruses. These proteins are involved in virulence and they contribute to evasion of host defenses (Yewdell and García-Sastre, 2002).

1.1.5 Influenza A virus proteins

The first segment of the genome encodes the PB2 subunit, which plays an essential role in the initiation of transcription of viral mRNA. It is responsible for the recognition and binding to the 5' cap structures of host cellular pre-mRNAs (Blaas et al., 1982; Li et al., 2001) which are cleaved after 10-15 nucleotides to be used as primers for the initiation of viral mRNA synthesis (Bouloy et al., 1978). It also plays an important role in influenza virus replication as a single amino acid mutation in the amino-terminus (N-terminal) region of PB2

affected replication but not transcription (Gastaminza et al., 2003). Furthermore, PB2 contains two binding sites for NP and PB1 (Poole et al., 2004).

Segment 2 encodes the PB1 subunit which is the structural backbone of the polymerase complex, containing the binding sites for both PB2 and PA subunits (Digard et al., 1989). It contains the catalytic core of the polymerase responsible for elongation during negative and positive sense RNA synthesis (Braam et al., 1983; Biswas and Nayak, 1994; González and Ortín, 1999). This segment further encodes two nonstructural proteins, PB1-F2 (Chen et al., 2001b) and PB1-N40 (Wise et al., 2009). The PB1-F2 is an 87-residue polypeptide encoded by a +1 open reading frame (ORF) in the PB1 gene. Analysis of GenBank sequences showed different lengths of PB1-F2 ranging from truncated (57 residues) to variants longer than 87 amino acids (Zell et al., 2007). It is a pro-apoptotic protein which interacts with mitochondrial proteins and alters the mitochondrial membrane potential leading to the release of cytochrome c and eventually to apoptosis (Chen et al., 2001b; Gibbs et al., 2003; Zamarin et al., 2005), which has been suggested to contribute to virulence (Gibbs et al., 2003; Zamarin et al., 2005). PB1-N40, on the other hand, is encoded by an alternative ORF within PB1 starting at codon 40, and appears to be nonessential for virus viability although sometimes important for virus replication (Wise et al., 2009).

RNA segment 3 encodes the PA subunit which is the third member of the RdRp complex. PA has been implicated in multiple functions, including polymerase stability, endonuclease activity, cap and promoter binding and replication of influenza virus (Fodor et al., 2002; Lee et al., 2002; Hara et al., 2006). Although the endonuclease activity of the RdRp has been previously proposed to be in either the PB1 or the PB2 subunit (Shi et al., 1995; Li et al., 2001), resolving the crystal structure of the N-terminal domain of the PA subunit revealed an endonuclease active site with similarity to type II restriction

endonucleases (Dias et al., 2009; Yuan et al., 2009). Recently, a second ORF (named X-ORF), accessed via a +1 ribosomal frame-shifting, was identified in the PA mRNA (Jagger et al., 2012). The X-ORF product (PA-X) which consists of the N-terminal 191 amino acids of PA, i.e. the endonuclease domain, fused to 61 residues from the C-terminal domain encoded by the X-ORF was found to suppress cellular gene expression (Jagger et al., 2012).

Influenza A virus RNA segment 4 encodes the HA. It is a classical type I membrane glycoprotein which functions as both as a sialic acid (SA)-binding and membrane fusion protein during virus entry into target cells (Skehel and Wiley, 2000). X-ray crystallographic studies show the HA molecule as a homotrimer in its neutral pH form which projects from the viral envelope to form a rod-shaped structure (Cross et al., 2009). Each monomer in this trimer is initially synthesized as a single polypeptide precursor (HA0) in infected cells, which is later cleaved by host trypsin-like proteases into two subunits, HA1 and HA2, linked by a single disulfide bond (Skehel and Wiley, 2000). Cleavage of HA0 is a prerequisite for virus infectivity (Lazarowitz and Choppin, 1975) and a crucial determinant in pathogenicity and host range (Steinhauer, 1999; Bertram et al., 2010) (discussed in section 1.1.9.1). The cleavage site is a predominant surface loop near a deep cavity in HA0 (Cross et al., 2009). This cleavage event results in structural rearrangements in which the nonpolar N-terminus amino acids of HA2 (the fusion peptide) is repositioned to the interior of the trimer, thereby burying the ionizable residues in the cleaved HA and generating a fusion competent structure (Cross et al., 2009). Upon acidification in the endosome, HA undergoes irreversible conformational changes that result in extrusion of the HA2 N-terminal fusion peptide domain from its buried position to the end of a long coiled-coil domain. This allows its interaction with the target membrane and ultimately results in membrane fusion and release of the vRNPs into the cytoplasm (Gething et al., 1986; Cross et al., 2009). The HA1 subunit forms

a membrane-distal globular head that contains the receptor-binding site and most of the antigenic regions recognized by neutralizing antibodies (Webster and Laver, 1980; Gerhard et al., 1981; Wiley and Skehel, 1987). On the other hand, HA2 forms a stem-like structure that anchors the globular domain to the viral membrane (Chen et al., 1995).

Segment 5 of the viral genome encodes the NP which is the 2nd most abundant viral protein after M1 in infected cells (Webster et al., 1992). NP is a major structural protein which binds to and encapsidates newly synthesized vRNA and positive-sense complementary RNA (cRNA) to form vRNPs and cRNPs, respectively (Kobayashi et al., 1994; Portela and Digard, 2002). RNA binding by NP is essential for transcription and replication as only RNPs but not RNA serve as template for transcription and replication (Bishop et al., 1971). According to its crystal structure from influenza H1N1 (Ye et al., 2006) and H5N1 (Ng et al., 2008) viruses, NP has a curved crescent-like shape with head and body domains formed by non-contiguous regions. Each NP polypeptide has a highly flexible tail loop which is inserted into the body domain of the neighboring subunit for homo-oligomerization to maintain the RNP structure (Prokudina-Kantorovich and Semenova, 1996). It was shown that RNA binding domain is formed by basic amino acid residues distributed along the NP in a deep groove between these domains at the exterior of the molecule (Ye et al., 2006). Upon RNA binding, it melts all the secondary structures and exposes RNA to solvent to make it accessible to the viral RdRp (Baudin et al., 1994). Also, NP interacts with the PB1 and PB2 subunits of the RdRp (Biswas et al., 1998) as well as with the M1 protein (Ye et al., 1999). Proteomic studies revealed the importance of NP as a multifunctional adaptor protein which interacts with a number of cellular proteins such as importin- α , F-actin, and exportin-1 which are important in vRNP trafficking in and out of the nucleus (Reviewed in Portela and Digard, 2002). Furthermore, free NP interacts with host

splicing factors including Tat-specific factor 1 (Tat-SF1) (Naito et al., 2007) and RNA polymerase activating factor-2 (RAF-2p48) (Momose et al., 2001), which act as chaperones to prevent non-specific aggregation and to facilitate RNA-NP association. NP has been proposed to be the major switching factor from transcription (mRNA synthesis) to replication (cRNA synthesis) (Beaton and Krug, 1986; Skorko et al., 1991), however the exact mode of action is not clear. It was suggested that direct interaction between NP and PB1 and PB2 subunits results in this switch in a “polymerase modification model”. However, several studies support the direct role of NP-RNA interaction in the switch through either protection of nascent cRNA from degradation “stabilization model” or binding to the vRNA template to alter its structure and favor cRNA synthesis “template modification model” (Portela and Digard, 2002).

Influenza RNA segment 6 encodes the NA protein which is the second major integral membrane glycoprotein of the influenza virion. NA is a homotetramer with four identical subunits, each of which is composed of six, four anti-parallel β -sheets where the enzymatic site is located within a pocket on the surface of each protein (Varghese et al., 1983; 1988). Within this pocket NA binds SA and cleaves the ketosidic linkage between a terminal SA and the adjacent galactose residue (Colman, 1994). Previous reports suggest that the breaking of this bond manifests the predominant role of NA, which is to facilitate the release of the virus particles and the spread of the virus to neighboring cells (Palese et al., 1974; Air and Laver, 1989). Indeed, NA-defective virus or wild-type viruses in the presence of NA inhibitors are found to form aggregates on the apical surface of cells (Griffin et al., 1983; Air and Laver, 1989; Liu et al., 1995; Hashem et al., 2009). Other than viral release, NA has also been implicated in mucus break down and viral diffusion throughout the respiratory tract (Klenk and Rott, 1988). Recently, it has been proposed to play a role in an early stage of

infection, probably by facilitating virus entry (Matrosovich et al., 2004b; Ohuchi et al., 2006), fusion with target cells (Su et al., 2009) and/or enhancing late endosome/lysosome trafficking (Suzuki et al., 2005). Similar to HA, NA's highly variable regions contains 2-3 antigenic sites targeted by the host immune defenses (Gulati et al., 2002). This includes two major sites located on the upper surface of the molecule around the SA binding sites and a possible third site at the bottom of the head (Air and Laver, 1989).

RNA segment 7 of influenza A codes for at least two functional proteins; the M1 and M2 proteins. Unspliced mRNA encodes the M1 protein, while alternative splicing gives rise to three spliced mRNA products which share a common 3' splicing site at position 739 but have alternative 5' splice sites (Chiang et al., 2008; Robb and Fodor, 2012). The 5' splice sites at positions 12 and 146 of the M1 mRNA gives rise to mRNAs with potential 9-residue and 54-amino acids polypeptides, respectively (Lamb et al., 1981; Shih et al., 1998). The 5' splice site at position 52 results in M2 mRNA, which encodes for the M2 ion channel protein (Winter and Fields, 1980). The M1 is the most abundant protein which underlies the lipid envelope and acts as an adaptor between the lipid envelope and the vRNPs (Bui et al., 1996; Zhang and Lamb, 1996). It interacts with the cytoplasmic tails of HA, NA, and M2 and associates with the NEP to regulate vRNPs transport between the cytoplasm and the nucleus (Steinhauer and Skehel, 2002; Nayak et al., 2004). Therefore, it was proposed that M1 plays a central role in virus morphogenesis and assembly by recruiting the viral components to the site of assembly at the plasma membrane. Indeed, M1 is necessary and sufficient for the formation of virus like particles (Gómez-Puertas et al., 2000; Latham and Galarza, 2001; Pushko et al., 2005). The ion channel M2 protein is a 97 amino acid polypeptide associated with the membrane as a homotetramer (Holsinger and Lamb, 1991). Each monomer consists of a short N-terminal extracellular domain, a transmembrane domain and a cytoplasmic tail.

The transmembrane domain forms the pore of the ion channel which is involved in the viral uncoating process (Lamb et al., 1985). In addition, recent evidence suggests an important role for the M2 cytoplasmic tail in virus assembly and budding (Hughey et al., 1995; Schroeder et al., 2005; Chen et al., 2008; Rossman et al., 2010).

Collinear mRNA derived from vRNA segment 8 expresses NS1 (Lamb and Choppin, 1979) as a 230–237-residue polypeptide depending on the strain (Palese and Shaw, 2007). Alternative splicing of this same mRNA results in the synthesis of the 121 amino acid NEP/NS2 protein (Inglis et al., 1979; Lamb and Choppin, 1979). NS1 is a nonstructural homodimer protein with an N-terminal RNA-binding domain, which binds nonspecifically to several RNA species (Hatada and Fukuda, 1992; Qian et al., 1995; Chien et al., 2004), and a C-terminal ‘effector’ domain, which interacts with host-cell proteins and stabilizes the RNA-binding domain (Nemeroff et al., 1995; Wang et al., 2002). NS1 is involved in a variety of cellular processes during viral infection and contributes to efficient virus replication and virulence. Specifically, it temporally regulates viral RNA synthesis, controls viral mRNA splicing, enhances viral mRNA translation and regulates virus particle morphogenesis (reviewed in Hale et al., 2008). Importantly, NS1 is an essential protein to antagonize type 1 interferon (IFN)-dependent antiviral responses by interfering with both pre-transcriptional and/or post-transcriptional processes (Garcia-Sastre et al., 1998; Katze et al., 2002). On the other hand, the NEP/NS2 is found in influenza A virions after purification (Richardson and Akkina, 1991). It associates with the M1 protein (Richardson and Akkina, 1991; Yasuda et al., 1993) and acts as an adaptor between vRNP complexes and the nuclear export machinery of the cell (O’Neill et al., 1998) for the export of the new vRNPs from the nucleus. Moreover, NEP was proposed to play a role in the regulation of influenza virus transcription and replication (Robb et al., 2009).

1.1.6 Influenza A replication

As shown in Figure 1.2, the initial step in influenza A virus life cycle starts with the attachment of viral HA to the terminal SA of host cell receptors (Skehel and Wiley, 2000). Upon binding, the attached virus is then internalized into the cell by receptor-mediated endocytosis. Both clathrin-dependent and independent endocytosis pathways have been reported for influenza virus entry (Lakadamyali et al., 2004). As indicated in the previous section, the low pH inside the endosome triggers the cleaved HA to undergo a conformational change which exposes and extends the fusion peptide at the N-terminus of HA2 subunit. This allows the fusion peptide to interact with the endosomal membrane and leads to the subsequent fusion between endosomal and viral membranes (Stegmann, 2000). In addition, the M2 ion channel permits proton entry into virus particles causing acidification of the virus interior and dissociation of the M1 protein from vRNPs, thus finishing the uncoating process by releasing the vRNPs into the cytoplasm (Martin and Helenius, 1991; Shimbo et al., 1996).

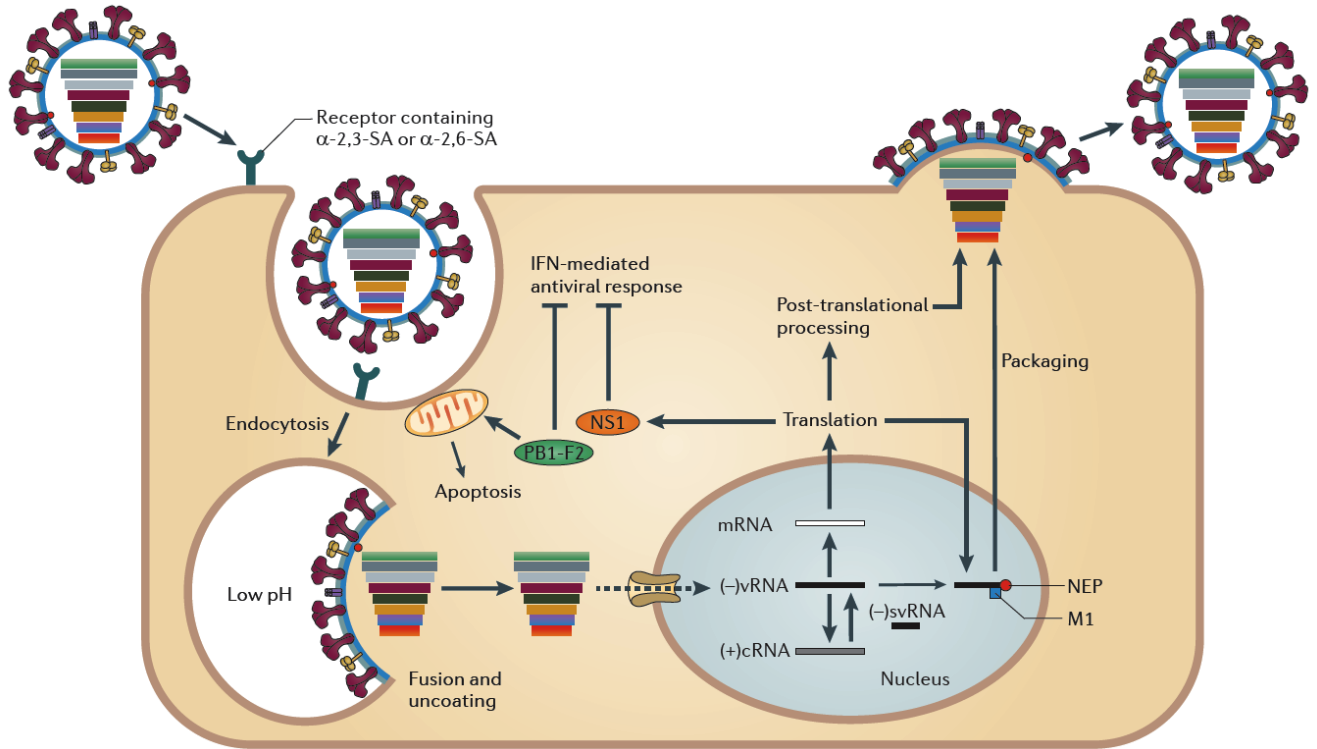
Viral RNP complexes then translocate to the nucleus for viral transcription and replication (Herz et al., 1981). In the nucleus, the RdRp transcribes the negative-sense vRNA and generates three different RNA species. Early during infection, host-derived cap-dependent transcription results in the synthesis of 5' capped and 3' polyadenylated mRNAs which are exported to the cytoplasm for translation by the cellular machinery into viral proteins (Hay et al., 1977). Viral polymerase subunits and NP are imported back to the nucleus for the synthesis of uncapped, non-polyadenylated positive-sense cRNA which serves as a template for vRNA replication (Huet et al., 2010). Newly synthesized vRNAs are then encapsidated in NP and serve as templates for secondary transcription of viral mRNAs. Recently, a third RNA molecule was identified as negative-sense small vRNAs (svRNAs),

Figure 1.2

Influenza A virus life cycle

Influenza A HA binds to target cell receptors (SA α -2,6-linked or SA α -2,3-linked Gal), and the virus enters the cell by receptor-mediated endocytosis. The low endosomal pH causes irreversible conformational changes of the cleaved HA which result in extrusion of the HA2 N-terminal fusion peptide domain to initiate membrane fusion. Furthermore, the low pH inside the endosome opens the M2 ion channel and results in acidification of the virion interior and dissociation of vRNPs from the M1 protein for the viral uncoating step. Then, vRNPs translocate to the nucleus, where the RdRp transcribes and replicates the negative-sense vRNA, giving rise to three types of RNA molecules: the intermediate positive-sense cRNA, which acts as a template for vRNA generation; negative-sense small viral RNAs (svRNAs), which are proposed to regulate the switch from transcription to replication; and the viral mRNAs, which are exported to the cytoplasm for translation. Viral proteins that are necessary for replication and transcription are translocated back to the nucleus, and progeny vRNPs are then exported to the cytoplasm for packaging, assisted by M1 and NEP proteins. Viral HA, NA and M2 are transported by the trans-Golgi secretory pathway to the plasma membrane, where M1 assists in the formation of virus particles. Release from the host cells is mediated by the sialidase activity of NA. Reprinted by permission from Macmillan Publishers Ltd: [Nat. Rev. Microbiol.] (Medina and García-Sastre), copyright (2011).

Figure 1.2



which are proposed to be involved in the regulation of the switch from transcription to replication (Perez et al., 2010; Umbach et al., 2010).

Late in infection, newly synthesized vRNAs associate with the NP and the polymerase complex proteins to form new progeny vRNPs. Then, these complexes are exported to the cytoplasm and transported to the apical surface of the plasma membrane with the help of NS2/NEP and M1 proteins for packaging and assembly (O'Neill et al., 1998; Whittaker and Helenius, 1998). Newly synthesized HA and NA accumulate at the plasma membrane, where M1 binds to their cytoplasmic tails and serves as a docking site for the vRNPs. Polymerization of the M1 protein elongates the budding virion and results in a polarized localization of the vRNPs. Eventually, M2 interaction with M1 results in its recruitment to the periphery of the budding virus and in membrane scission as insertion of the M2 amphipathic helix at the lipid phase border alters the membrane curvature at the neck of the budding virus. Finally, the release of progeny virions from host cells is mediated by the sialidase activity of the NA (Compans et al., 1974; Rossman and Lamb, 2011).

1.1.7 Influenza A genetics

1.1.7.1 Reassortment

Reassortment is the rearrangement of viral gene segments in cells infected with more than one virus. Reassortment resulted in 3 pandemics including 1957, 1968 and the recent swine-origin 2009 pandemic where reassortant viruses emerged and spread globally. The 1957 and 1968 pandemics resulted from reassortant human viruses containing HA, PB1 and NA or HA and PB1 segments from avian viruses, respectively (Scholtissek et al., 1978;

Kawaoka et al., 1989). The 2009 pH1N1 virus resulted from a triple avian, human and swine reassortant virus (Garten et al., 2009).

1.1.7.2 Recombination

Recombination has also been detected in influenza A virus gene segments. For example, insertion of nucleotide segments from the 28S ribosomal RNA or NP gene into the HA gene enhanced the cleavability of HA (Khatchikian et al., 1989; Orlich et al., 1994) and increased the pathogenicity of low pathogenic avian viruses (Suarez et al., 2004). Similarly, insertion of 21 nucleotides from the M gene into HA converted low pathogenic avian virus to highly pathogenic virus (Bowes et al., 2004; Hirst et al., 2004). Recombination in other gene segments such as NA and NP has also been observed (Rohde and Scholtissek, 1980; Mitnaul et al., 2000).

1.1.7.3 Dominance

Some influenza A strains are more dominant than others when co-infecting the same cell. For example, the cold adapted influenza A/Ann Arbor/6/60 interferes with the growth of wild-type virus (Whitaker-Dowling et al., 1990).

1.1.7.4 Reverse genetics

Development of reverse genetic systems for negative strand viruses such as influenza A was challenging because the negative-sense vRNAs are not infectious on their own. Only vRNAs encapsidated with the three subunits of the viral RdRp (PB1, PB2, and PA) and the NP as viral vRNPs can initiate viral replication and transcription (Huang et al., 1990).

Therefore, all reverse genetics methods for negative-sense RNA viruses rely on intracellular reconstitution of vRNP complexes into cells by a variety of techniques.

The first reverse genetics method for influenza A virus was based on transfection of cells with *in vitro*-reconstituted vRNPs (Luytjes et al., 1989). Here, vRNAs were *in vitro* transcribed from plasmid DNA in the presence of purified polymerase protein subunits and NP isolated from influenza virus which eventually led to the rescue of infectious viruses containing specified mutations (Enami et al., 1990). Alternatively, methods involved the use of viral complementary DNA (cDNA) inserted between the polymerase-I promoter and hepatitis delta virus genomic ribozyme sequences allowed for intracellular synthesis of vRNA molecules that lack both a 5' cap and the 3' polyadenylated tail (Neumann et al., 1994). Eventually, transfection of cells with such a plasmid together with plasmids expressing the three polymerase subunits and the NP under the control of polymerase-II promoters led to intracellular production of functional vRNP complexes (Pleschka et al., 1996). However, these systems required helper influenza virus to generate transfectant influenza viruses which requires highly efficient selection methods to isolate these viruses from the helper virus (Pleschka et al., 1996; Zhou et al., 1998).

Soon after, efficient generation of influenza A viruses entirely from cloned cDNAs was achieved. In this system, each of the eight viral cDNA segments was cloned into a plasmid between the human RNA polymerase I promoter at the 5' end and the mouse RNA polymerase I terminator (Neumann et al., 1999) or a hepatitis delta virus genomic ribozyme sequence (Fodor et al., 1999) at the 3' end. Transfection of these 8 plasmids together with 4 protein expression plasmids encoding for viral NP and the PB2, PB1, and PA viral polymerases under the control of polymerase-II promoter into Human Embryonic Kidney cells (HEK-293T) or Vero cells resulted in highly efficient rescue of recombinant viruses in

cell supernatants (Fodor et al., 1999; Neumann et al., 1999). Subsequently, this system was modified to reduce the number of plasmids to eight by placing the viral cDNA segments which are flanked by the polymerase-I promoter and terminator sequence between polymerase-II promoter and a polyadenylation signal in the opposite orientation allowing for the synthesis of both vRNA and mRNA from one viral cDNA template (Hoffmann et al., 2000). More recently, this 8 plasmid system was improved to rescue recombinant viruses with high efficiency by combining the RNA polymerase I and/or II transcription units to reduce the number of plasmids to as few as one plasmid (Neumann et al., 2005). These systems have allowed the generation of up to 5×10^7 plaque forming units (pfu)/ml entirely from plasmids without the need for helper viruses.

1.1.8 Epidemiology of influenza A viruses

Influenza A viruses infect many animal species including humans, pigs, horses, dogs, cats, sea mammals and birds (Zambon, 1999; Capua and Alexander, 2004b). All combinations of HA and NA subtypes have been isolated from aquatic birds, which serve as a natural reservoir for influenza A viruses (Webster et al., 1992; Capua and Alexander, 2004b; Palese, 2004). In these hosts, influenza A viruses replicate in the epithelial cells of the intestinal tract in contrast to mammalian viruses which mostly replicate in the respiratory tract (Webster et al., 1978). Influenza A viruses in wild aquatic birds are usually benign and evolutionarily stable indicating their optimal adaptation to these hosts. On the other hand, these viruses are in continuous evolution in mammalian hosts and land-based poultry (Ludwig et al., 1995; Sturm-Ramirez et al., 2004). The evolution rate of influenza A viruses in humans differs among the different segments. The surface proteins, especially HA, are evolving faster than the internal proteins mostly due to the selective immune pressure

imposed by the host's immune system on these surface glycoproteins as well as the structural restrictions on the internal proteins (Webster et al., 1992). Phylogenetic analyses of influenza A genes revealed host-specific lineages for most of the gene segments except for HA and NA (Kawaoka et al., 1989; Ito et al., 1991; Webster et al., 1992).

Until 1997, only H1N1, H2N2 and H3N2 subtypes circulated in humans with limited cases of direct transmission of avian viruses to humans. It was believed that the differences in receptor specificity between human and avian viruses represented a host range barrier. However since 1997, direct transmission of the HPAI H5N1 virus from poultry to humans has increased and resulted in high mortality rate (Subbarao et al., 1998). Other avian viruses such as H9N2 (Lin et al., 2000) and H7N7 (Fouchier et al., 2004) have also been isolated from humans. Although human-to-human transmission of these viruses has been limited so far, the ability of these avian viruses to infect humans and cause disease has raised concern about their potential to cause devastating pandemics.

1.1.8.1 Antigenic drift

Antigenic drift occurs due to gradual accumulation of point mutations in influenza genes especially those encoding HA and NA as a result of the error-prone nature of the RdRp (Tulloch et al., 1986; Wiley and Skehel, 1987). Most of these mutations do not affect the conformation of the proteins; nevertheless, some mutations can lead to emergence of novel strains to which most people have no immunity. Specifically, accumulation of mutations at the antigenic sites in HA, NA, or both proteins due to the intense immune pressure imposed by neutralizing antibodies results in selection of new variants which can cause annual epidemics because they can no longer be neutralized by antibodies generated against parental or previously circulating strains (Capua and Alexander, 2004a; Shih et al., 2007).

1.1.8.2 Antigenic shift

Unpredicted pandemics might also occur due to a less frequent major antigenic change known as antigenic shifting (Steinhauer and Skehel, 2002). Because of the segmented nature of the influenza genome as well as the ability of these viruses to infect humans and other animals, co-infection with influenza A viruses can result in genetic reassortment. This could replace the HA or any other gene segment with novel ones from a virus previously restricted to a different host, resulting in a new virulent subtype, that has never been associated with humans nor recognized by the immune system (Steinhauer and Skehel, 2002). Shifting also can occur due to the direct transmission and establishment of avian or swine influenza viruses in the human population.

During the last century, 4 pandemics have occurred due to antigenic shift. In 1918, the H1N1 Spanish flu is believed to have killed up to 50 million people (Ludwig et al., 2003), in 1957, the H1N1 virus was replaced by the H2N2 virus causing the Asian flu, in 1968, H3N2 replaced the H2N2 subtype and resulted in the Hong Kong flu, and in 1977, the H1N1 subtype reemerged again causing the Russian flu pandemic (Scholtissek et al., 1978; Kawaoka et al., 1989). More recently, the outbreak of the pH1N1 influenza virus in 2009 resulted from reassortment of viruses originated from human, avian and swine hosts in pigs and resulted in the first pandemic of the 21st century (Belshe, 2009; Garten et al., 2009).

1.1.9 Host range restriction and pathogenesis

Influenza A viruses display host specificity and usually cannot cross the species barrier. Recent data indicates that host specificity is rather multigenic where multiple gene segments play a crucial role in host adaptation and in crossing the species barrier. While HA, PB2 and NS1 have been identified as major factors of host range restriction and viral

pathogenesis, other proteins including NA and NP have also been suggested to play a role in host range restriction (Scholtissek et al., 1985; Goto and Kawaoka, 1998).

1.1.9.1 HA protein

The cleavage of precursor HA0 by host proteases is critical for infectivity because it exposes the N-terminus (fusion peptide) of the HA2 subunit which mediates the viral-cellular membrane fusion. In addition, HA cleavability by host proteases is a key determinant of virulence and tissue tropism (Horimoto and Kawaoka, 1994). HPAI H5 and H7 viruses have polybasic amino acid linker at the cleavage site which renders them cleavable by ubiquitous intracellular proteases. Thus, these viruses can cause systemic infection in poultry and can form plaques in cell culture without exogenous proteases. On the other hand, low or nonpathogenic avian and non-avian influenza A viruses except for equine H7N7 viruses (Kawaoka, 1991) can only be cleaved by extracellular host proteases because they contain a single arginine residue at the cleavage site which in turn limits their spread in hosts to tissues where the appropriate proteases are encountered (Bosch et al., 1979).

Furthermore, the receptor binding specificity of HA proteins determines the host range of influenza A viruses. Human and classical H1N1 swine influenza viruses preferentially bind to SA attached to the galactose by an α 2,6 linkage (SA α 2,6-linked Gal) receptors, whereas avian and equine HA preferentially bind to SA with an α 2,3 linkage (SA α 2,3-linked Gal) (Rogers et al., 1983; Connor et al., 1994; Ito et al., 1997). In humans, most of the cells in the upper respiratory tract (nonciliated cells) express SA α 2,6-linked Gal receptors and only minor proportion of cells (ciliated cells) express SA α 2,3-linked Gal receptors (Matrosovich et al., 2004a). On the other hand, SA α 2,3-linked Gal receptors are found on most cells in the lower respiratory tract (Shinya et al., 2006). These findings not

only explain the sporadic transmission of avian viruses to humans but also the limited transmission of avian viruses between humans (Claas et al., 1998; Fouchier et al., 2004). In contrast, avian viruses replicate in epithelial cells in the intestine of ducks, which contains SA α 2,3-linked Gal receptors (Ito et al., 2000). Similarly, equine viruses replicate in the upper respiratory tract of horses which contains SA α 2,3-linked Gal oligosaccharides (Ito et al., 1997). Importantly, cells in the upper respiratory tract of pigs contain both receptors which explains their susceptibility to avian and human viruses (Ito et al., 1998).

HA receptor specificity is determined by amino acids within the receptor-binding domain. Glutamine at position 226 and glycine at position 228 in H2 and H3 confer SA α 2,3-linked Gal specificity for avian viruses, while leucine and serine at these positions are responsible for SA α 2,6-linked Gal preference in human viruses (Rogers et al., 1983; Connor et al., 1994). For H1 viruses, amino acid at position 190 determines the specificity of HA. Specifically, for human and swine viruses aspartate at position 190 determines the specificity for SA α 2,6-linked Gal and glutamate for avian viruses confers specificity for SA α 2,3-linked Gal receptors (Matrosovich et al., 2000; Kobasa et al., 2004). Other residues at positions 136, 195 and 225 have also been implicated in receptor binding specificity (Vines et al., 1998; Glaser et al., 2005). In addition, the number and the location of glycosylation sites in HA protein affect the virulence as well as the host range of influenza A viruses (Ohuchi et al., 1997; Matrosovich et al., 1999).

1.1.9.2 PB2 protein

The amino acid at position 627 of the PB2 subunit plays an important role in virus pathogenicity (Hatta et al., 2001) and host range (Subbarao et al., 1993; Foeglein et al., 2011). In particular, HPAI H5N1 viruses contain lysine at this position while low pathogenic

H5N1 viruses have glutamic acid (Hatta et al., 2001). Moreover, H7N7 viruses isolated from fatal cases in the Netherlands in 2003 contain lysine at this position (Fouchier et al., 2004). The presence of lysine residue at this position confers better adaptation to mammalian cells and improves replication at 37 °C compared to viruses with PB2 protein containing glutamic acid (Naffakh et al., 2000; Crescenzo-Chaigne et al., 2002).

1.1.9.3 NS1 protein

NS1 protein is an IFN antagonist which helps efficient viral replication by interfering with the double stranded RNA (dsRNA)-dependent activation of transcription factors. NS1 protein activity differs between influenza A viruses. For example, the NS1 protein of the 1997 and 2003 HPAI H5N1 viruses antagonize IFN response but at the same time induces high level of proinflammatory cytokines (tumor necrosis factor (TNF)- α and IFN- α/β which correlates with the high virulence and pathogenicity (Cheung et al., 2002; Seo et al., 2002). Similarly, NS1 protein of the Spanish flu virus played an important role in the pathogenicity and virulence of this virus (Geiss et al., 2002).

1.1.9.4 NA protein

NA has also been suggested to have a role in host range and pathogenicity (Goto and Kawaoka, 1998). For example, the NA protein from some avian viruses shows more resistance to the low pH of the digestive tract compared to human and swine viruses (Takahashi et al., 2001). Moreover, NA shows preferential specificity for SA receptors similar to HA. NA from avian viruses have specificity for SA α 2,3-linked Gal receptors, while NA from human viruses have acquired the ability to cleave both receptors to adapt with HA specificity (Baum and Paulson, 1991). This preference in specificity is linked to the

amino acid at positions 275 in NA protein (Kobasa et al., 2001).

1.1.9.5 NP protein

Phylogenetic analysis studies have shown that NP falls into divergent host-specific lineages, thus it was suggested to be involved in host range restriction (Scholtissek et al., 1985). It was proposed that NP evolves in response to host specific factors and it reflects host specific adaptation. Indeed, reassortant human influenza viruses with NP from avian viruses are attenuated and restricted in their replication in both seronegative children and squirrel monkeys (Tian et al., 1985; Murphy et al., 1989; Treanor et al., 1991). Similarly, replacement of NP in avian influenza viruses with NP from a human virus resulted in attenuated viruses in ducks (Hatta et al., 2002). Interestingly, a single substitution (N319K) in avian NP increased its binding to importin- α 1 and import into the nucleus in mammalian but not avian cells (Gabriel et al., 2008). Therefore, it was proposed that adaptation of NP interaction with importin- α 1 is important for host adaptation.

1.1.9.6 Other proteins

Other viral proteins such as PB1 and M1 have also been implicated in host range restriction (Almond, 1977; Scholtissek et al., 2002; 1978; Snyder et al., 1987).

1.2 IMMUNE RESPONSE TO INFLUENZA A VIRUS

Host non-specific protective mechanisms in the respiratory tract including the mucin layer, ciliary action, and protease inhibitors represent the first line of defense against respiratory infections. They help in preventing infection of respiratory epithelial cells from most pathogens including influenza A viruses (Wright et al., 2007). In addition, lung and

salivary scavenger macromolecules and receptors such as glycoprotein-340, surfactant proteins A (SP-A) and D (SP-D) and mannose-binding lectins contribute to host innate defense against influenza A viruses by neutralizing, aggregating the viruses or enhancing phagocytosis (Hartshorn et al., 2003; van Eijk et al., 2003; Gomi et al., 2004; Hawgood et al., 2004). Furthermore, resistance to influenza A virus infection involved innate and adaptive immunity with both local and systemic responses.

1.2.1 Innate immune response

The mammalian innate immune system recognizes pathogens by Toll-like receptors (TLRs) (Takeda and Akira, 2005). Infection of epithelial cells with influenza A virus initiates innate immune responses by triggering intracellular signaling through at least two TLRs, TLR-3 and TLR-7 which are located within endosomal compartments. TLR3 is expressed in alveolar and bronchial epithelial cells, the primary targets for influenza virus infection, and recognizes the replicative intermediate dsRNA (Guillot et al., 2005). TLR7 is found in professional antigen-presenting cells (APCs) such as dendritic cells (DCs) and B cells and recognizes influenza viral single-stranded RNA (Diebold et al., 2004; Barchet et al., 2005). TLR3 and TLR7 signaling induces secretion of various inflammatory cytokines including interleukin (IL)-8, IL-6, regulated on activation, normal T-cell expressed and secreted (RANTES) and type I IFN which can effectively limit viral replication and spread (Lund et al., 2004; Guillot et al., 2005; Takeda and Akira, 2005). The importance of the type I IFN response in the control of infection is evident as it activates many host genes including the Mx proteins, IFN-induced large guanosine triphosphatases, which interfere with viral replication and induce an antiviral state (Garcia-Sastre, 2001; Kochs et al., 2005).

Influenza A viruses infect epithelial cells as well as alveolar macrophages and result in their necrosis and apoptosis, respectively (La Gruta et al., 2007). Cell necrosis and apoptosis induce the production of various chemokines and cytokines including TNF- α , IL-1, IL-8, monocyte chemoattractant protein (MCP)-1, RANTES and interferon induced protein (IP)-10 (La Gruta et al., 2007). Such a response results in the recruitment of other innate immune cells such as macrophages, natural killer cells, and neutrophils from the peripheral blood across the endoepithelial barrier into infected lung tissue. These cells have been suggested to participate in viral clearance through phagocytosis, IFN- γ production and cytotoxic activity (He et al., 2004; Tumpey et al., 2005).

1.2.2 Adaptive immune response

Following the presentation of viral peptides to T cells via APCs, antigen-specific adaptive systemic and mucosal immune responses (humoral and cellular) can be initiated. Influenza-specific neutralizing antibodies and cytotoxic T lymphocytes (CTLs) generated by adaptive responses contribute to viral clearance, recovery from the disease and complete or partial protection against re-infection.

1.2.2.1 Humoral immunity

Following exposure to influenza A virus, the host immune system generates antibodies against most of the viral proteins including surface glycoproteins and internal proteins which participate in viral clearance and may provide protection against future infections (Potter and Oxford, 1979). Specifically, neutralizing antibodies targeting the antigenic sites in the globular head domain of the HA can directly block virus attachment to

the target cell receptors and prevent influenza infection (Gerhard et al., 2006). However, the neutralization activity of these antibodies is mostly strain-specific because of the high variability of HA. Other antibodies directed against other regions or proteins such as the stem region of HA or NA could also inhibit the invading virus by interfering with the low-pH induced conformational change in the HA molecule and ultimately inhibiting membrane fusion (Prabhu et al., 2009; Sui et al., 2009; Hashem et al., 2010) or binding to budding viruses and preventing release of virions from the infected cells (Huber et al., 2010), respectively. Also, antibodies targeting the M2 protein have been shown to be protective in mice and to be induced in humans upon infection (Treanor et al., 1990). Indirect antibody-mediated mechanisms such as Fragment Crystallizable Receptor (FcR)-mediated phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) can also contribute to an earlier recovery from the infection (Marasco and Sui, 2007; Staneková and Varečková, 2010).

Interestingly, individuals can be re-infected with antigenically related influenza A strains, indicating incomplete immunity upon single infection. This has been shown to correlate with the gradual loss of serum immunoglobulin (Ig)G, mucosal IgA antibodies as well as the antigen-specific B cells within a year after infection (Wright et al., 1983). In addition, the host immune system usually generates antibodies targeting dominant epitopes in the HA from previously encountered strain rather than novel epitopes from circulating strain in a phenomenon known as “original antigenic sin”. It is only after multiple infections that antibodies with broad range of specificities are induced (Wang et al., 1986).

It has been long believed that during natural infection and vaccination, the host immune system mainly recognizes the bulky and highly variable-immunodominant globular head domains in HA and NA which shield the more conserved regions such as HA2 (Nabel

and Fauci, 2010; Steel et al., 2010); thus, it does not lead to the development of universal protection, so called heterosubtypic immunity, against drifting strains. Furthermore, it has always been thought that such immunity is mainly mediated by cross-reactive CTLs directed against the highly conserved internal proteins but not surface glycoproteins (Rimmelzwaan et al., 2007). However, recent studies have shown that B cell-dependent heterosubtypic immunity can also be conferred in mice by mucosal delivery of vaccines (Tumpey et al., 2001; Nguyen et al., 2001; Takada et al., 2003; Quan et al., 2008) and infection with influenza virus (Kreijtz et al., 2007). More recently, it was reported that immunization with plasmid DNA encoding HA from H1N1 influenza virus and boosting with seasonal vaccine or replication-defective recombinant adenovirus (rAd) vector encoding HA could protect nonhuman primates against different viral subtypes (Wei et al., 2010). Such immunity is mediated via broadly-neutralizing antibodies (BnAbs) targeting highly conserved epitopes within the HA protein (see Chapter 2 for details).

1.2.2.2 Cellular immunity

The cellular response to influenza infection is mediated by antigen-specific CD4⁺ and CD8⁺ T cells. Overall, during any viral infection, CD4⁺ T cells act mainly as helpers and CD8⁺ T cells as cytotoxic/killer cells (Doherty et al., 1997).

CD4⁺ cells recognize antigens presented via major histocompatibility complex class II (MHC-II) molecules. These cells are important in the development of adaptive immunity against infectious pathogens as they provide help to both antigen-specific B cells for antibody production and to CD8⁺ T cells for their proliferation (Castellino and Germain, 2006; Simpson, 2008). Depending on the cytokines in their microenvironment, i.e. either IL-12 or IL-4, naïve CD4⁺ T cells can differentiate into Type I (T_H1) or Type II (T_H2) effector

CD4⁺ T cells, respectively (Doherty et al., 1997; Murtaugh and Foss, 2002). T_H1 cells secrete IFN- γ , IL-2 and TNF- α , which promote the clonal expansion of antigen-specific CTLs and the production of IgG2a and IgG2b in mice (Germann et al., 1995; Lefeber et al., 2003). On the other hand, T_H2 cells produce IL-4, IL-5 and IL-13 to provide help for an effective B-cell response and to drive IgG1 production (Brown et al., 2004). While it has been shown that CD4⁺ T cells can be dispensable in the primary CTL response to influenza infection since DCs can also elicit a delayed CD8⁺ T cell response (Allan et al., 1990; Diebold et al., 2004), these cells are critical for the induction of memory and secondary CTLs responses (Mintern et al., 2002; Thomas et al., 2006). Interestingly, some CD4⁺ T cell subsets have been found to have a direct cytotoxic effector function during viral infections (Brown et al., 2006; 2012). Specifically, IFN- γ and perforin producing CD4⁺ T cytotoxic cells mediate antibody-independent protection early in infection, whereas a CD4-dependent B cell antibody response acts at a later stage in infection. However, in the absence of B cells these cells are insufficient to control influenza infection (Topham and Doherty, 1998; Mozdzanowska et al., 2000).

On the other hand, antigen-specific CD8⁺ T cells, which recognize peptides presented on MHC-I molecules, are important in the elimination of infected cells and clearance of influenza viruses (Ennis et al., 1978; McMichael et al., 1986). These cells show specificities to epitopes from several viral antigens including surface and internal proteins (Jameson et al., 1998). CD8⁺ T cell-mediated protection involves direct cytotoxicity of infected cells or secretion of cytokines (i.e. IFN- γ and TNF- α) (Carding et al., 1993; Barry and Bleackley, 2002; Xu et al., 2004).

Generation of immunological memory is dependent on the induction of adaptive immune responses. Both naïve B and CD8⁺ T cells can differentiate into short-lived effector

cells during primary infection (Westermann et al., 2001) and long-lived memory cells with the help of CD4⁺ T cells (Belz et al., 2002; Swain, 2005). Here, memory CD8⁺ T cells can mediate an accelerated and enhanced recall response to secondary virus infection (Kambayashi et al., 2003). Compared with memory B cells which provide sterilizing immunity, the protection mediated by memory CTLs is infection-permissive but with a more efficient cross-protective immune responses against influenza viruses (Epstein et al., 1998; Flynn et al., 1998). Specifically, influenza virus-specific CD8⁺ T cells that recognize internal proteins such as NP and PA are highly cross-reactive and are the primary contributors to heterosubtypic immunity (Yewdell et al., 1985; Bennink et al., 1987).

1.2.2.3 Mucosal immunity

During primary and secondary influenza infection, neutralizing antigen-specific secretory IgA, IgM and low levels of IgG comprise the local humoral immune response in the respiratory tract (Murphy et al., 1982; Fokkens and Scheeren, 2000; Renegar et al., 2004). These antibodies mainly target the HA and NA proteins. While both IgA and IgM are actively secreted in the upper respiratory tract, local IgG antibodies accumulate mostly through passive transudation from serum to the lower respiratory tract (Murphy et al., 1982; Wagner et al., 1987; Renegar et al., 2004). The mucosal IgA response is also associated with serum IgA production and memory IgA response (Wright et al., 1983).

In addition, influenza infection results in a local T cell response in which antigen-specific CD8⁺ T cells found in the airways and the lung parenchyma can provide local cellular responses against infection (Baumgarth and Kelso, 1996; Ely et al., 2003; Marsland et al., 2004). Importantly, memory CTLs in the respiratory mucosa are associated with local protection against heterosubtypic challenge (Haanen et al., 1999; Nguyen et al., 1999).

1.3 ANTIVIRAL DRUGS

Presently, only two classes of antiviral agents have been developed and approved for prophylaxis and treatment of human influenza virus A infection.

1.3.1 Ion channel blockers

Amantadine hydrochloride and its derivative rimantadine, are ion channel protein (M2) blockers which interfere with replication of influenza A viruses only. Early in infection, they inhibit hydrogen ion flow from the acidified endosome into the virion interior, thereby inhibiting the low pH-induced dissociation of vRNPs from M1 protein and their subsequent release into the cytoplasm; i.e. uncoating of incoming viruses (Kato and Eggers, 1969; Bukrinskaya et al., 1982). Late in infection, it reduces the pH inside the trans/post-Golgi vesicles causing premature HA conformational changes during transport from endoplasmic reticulum to the cell surface (Ciampor et al., 1992). Recently, crystal structure (Stouffer et al., 2008) as well as solution NMR structure (Cady et al., 2010) showed a single amantadine molecule physically blocking the pore of the channel. On the other hand, another solution NMR structure of M2 in the presence of rimantadine suggested allosteric inhibition of M2 through the binding of four rimantadine molecules to the lipid-facing region of the channel and stabilization of a closed pore conformation (Schnell and Chou, 2008).

1.3.2 NA inhibitors

The second class is viral NA inhibitors, i.e. oseltamivir and zanamivir, which are effective against both influenza A and B viruses (Itzstein et al., 1993; Kim et al., 1997). These inhibitors interfere and inhibit established infection by binding to the active site of NA more strongly than SA, thus preventing efficient viral release from infected cell (Varghese,

1999). Both compounds zanamivir and oseltamivir are well tolerated and effective prophylactically and therapeutically (Gubareva et al., 2000).

1.3.3 Drug resistance

Unfortunately, resistance to both classes of drugs has been reported, and can be conferred by a single amino acid substitution in the protein (Baranovich et al., 2011; Medina and García-Sastre, 2011). For example, single amino acid substitution at positions 26, 27, 30, 31, or 34 in the M2 protein can grant resistance of influenza A viruses to both amantadine and rimantadine (Bright et al., 2005). The significant increase in adamantane-resistant influenza A viruses since the 2005/2006 influenza season, (91-92% of the tested strains showed S31N substitution) (Bright et al., 2006; Regoes and Bonhoeffer, 2006) led to a recommendation by the Center of Disease Control against using amantadine and rimantadine for the treatment or chemoprophylaxis of influenza A infections (Bright et al., 2006; Fiore et al., 2008). Resistance to these drugs remains high among all tested seasonal influenza A H3N2 and 2009 pH1N1 viruses (Fiore et al., 2011; Medina and García-Sastre, 2011). Amantadine and rimantadine-resistant H5N1 viruses containing S31N substitution have also been isolated (Li et al., 2004; Medina and García-Sastre, 2011).

Emergence of resistance against NA inhibitors can arise from changes in residues involved in either NA enzymatic activity, such as D198N, R292K or R152K, or in the structural stability of the active site, such as E119V or H275Y, (Gubareva, 2004). All of these substitutions confer oseltamivir resistance except for R292K, which is associated with low resistance to zanamivir (Gubareva, 2004; Fiore et al., 2011). Mutations such as H275Y can reduce viral fitness (Regoes and Bonhoeffer, 2006), thus additional mutations in HA can decrease its binding affinity and compensate for these defects in NA (Yen et al., 2005).

While the rate of H275Y oseltamivir-resistant viruses increased rapidly in H1N1 isolates during the 2007-2008 season, 2009 pH1N1 viruses are oseltamivir-susceptible (Fiore et al., 2008; Baranovich et al., 2011). Resistance due to H275Y in 2009 pH1N1 and H5N1 viruses are sporadic so far and may emerge in patients undergoing treatment or prophylaxis (Medina and García-Sastre, 2011).

1.4 VACCINES

Several vaccination strategies have been evaluated for prevention against influenza; however, inactivated virus vaccine is the most widely used approach (Palese, 2006). More recently, the live-attenuated influenza vaccine (LAIV) has been approved for use in Russia and the USA (Bergen et al., 2004; Belshe et al., 2004). These vaccines typically are trivalent containing two influenza A strains (H1N1 and H3N2) and one influenza B strain (Fiore et al., 2008). Recently, a quadrivalent influenza vaccine containing two influenza B strains from both the B/Yamagata/16/88 and B/Victoria/2/87 lineages in addition to the two influenza A strains was approved for use in the USA (Ambrose and Levin, 2012).

1.4.1 Vaccine strain surveillance and selection

The high antigenic variability of HA and NA as well as the uncertainty about the actual strains that will be circulating require annual reformulation of seasonal vaccines to ensure that the vaccine strains match the circulating ones and to achieve sufficient protection of the population against the changing threat (Wood, 2002; Gerhard et al., 2006; Palese, 2006). Current global surveillance of influenza relies on the serum hemagglutinin-inhibition (HI) assay to identify antigenic variants (Smith, 2006; Barr et al., 2010). Thus, seasonal vaccines can be produced using strains recommended by the World Health Organization

(WHO) 9 to 12 months ahead of the targeted season (Carrat and Flahault, 2007). However, vaccine manufacturing, testing for effectiveness, approval by regulatory authorities and distribution is a slow process, requiring at least 6 months (Drape et al., 2006), which, in addition to the short shelf life of these vaccines (Fedson, 2003), can render the vaccine fairly ineffective. Furthermore, complex egg adaptation and growth characteristics required for some viruses such as the HPAI H5N1 viruses (Wood et al., 2002) might further delay vaccine production. Most importantly, any mismatch between the strains selected for the vaccine and those circulating due to either inaccuracy of prediction or introduction of a completely new strain during this lengthy production period might result in reduced efficacy and could be potentially devastating (Bridges et al., 2000; De Filette et al., 2005). For instance, mismatch between vaccine and circulating strains in 1997-1998 (Aymard et al., 1999; Klimov et al., 1999; de Jong et al., 2000) or the emergence of the H3N2 A/Fujian/411/2002-like virus, which replaced the circulating A/Panama/2007/99 in 2003–2004 season (Yohannes et al., 2004) resulted in severe disease outbreaks even in vaccinated individuals. Also, as witnessed in the 2009 pH1N1 outbreak, completely new strains can unexpectedly emerge. Spread of new pandemic strains is difficult to contain because of the time required to engineer and manufacture effective vaccines, and to prepare reagents required for vaccine lot release. Indeed, these limitations were evident in 2009 when pH1N1 virus spread globally in few weeks, and when a vaccine was produced, its impact on disease prevention was very low.

1.4.2 Vaccine potency determination

For more than 30 years, single radial immunodiffusion (SRID) has been used to standardize influenza vaccine potency and for vaccine lot release by most jurisdictions

around the globe (Wood et al., 1977; Hardy et al., 2011). SRID is a relatively simple, cheap and reproducible method which quantifies the antigenic HA in vaccine preparations against a homologous HA reference antigen (Williams, 1993). However, there are inherent disadvantages associated with SRID including the need to generate annual reference reagents (homologous antigens and corresponding subtype-specific antisera) by the WHO collaborating centers. This is a complex and time-consuming process representing a regulatory hurdle for timely release of vaccine lots as was witnessed during the 2009 pH1N1 outbreak (Hardy et al., 2011). In addition, SRID is a low throughput assay with low sensitivity for very low-dose vaccines (Fiore et al., 2008; Hardy et al., 2011). Furthermore, several studies have highlighted the role of NA in protection (Johansson et al., 1993; Sandbulte et al., 2007; Takahashi et al., 2009; DiNapoli et al., 2010; Marcelin et al., 2011). Yet, the content of NA in current influenza vaccines is not quantified (Huber et al., 2010).

Several research groups have reported various alternative physiochemical methods for HA quantification (Garcia-Cañas et al., 2006; Kapteyn et al., 2006; Luna et al., 2008; Creskey et al., 2010). Recently, we have developed different alternative immunoassays for the quantification of both HA and NA antigens in vaccine preparations (Chun et al., 2008; Li et al., 2009; Gravel et al., 2010; Gravel et al., 2011, Hashem et al., 2013).

1.4.3 Inactivated vaccines

Trivalent inactivated vaccine (TIV) is produced by direct inactivation of viruses purified from the allantoic fluid of infected embryonated hens' eggs using either formalin or β -propiolactone (BPL) (Goldstein and Tauraso, 1970). Inactivated vaccine is available as a whole virus (WV), chemically disrupted and partially purified split vaccine or highly purified surface glycoproteins subunit vaccine. TIV vaccines are usually unadjuvanted vaccines

administered intramuscularly for individuals aged more than 6 months including those with chronic medical conditions (Fiore et al., 2009; Ambrose et al., 2011). Each dose contains 15 µg of HA per strain for adults and older children or two doses of 7.5 µg HA for children younger than 3 years (Fiore et al., 2008; 2009). Recently, Microfluidized Emulsion 59 (MF59), an oil-in-water microemulsion adjuvant was approved for use with a subunit vaccine, Fluvad® in Europe (Podda and Del Giudice, 2003; Lee et al., 2011). In general, TIV cause minimal local and systemic adverse reaction which might include pain, redness at the injection site, fever, malaise, myalgia and headache. WV vaccines are usually associated with more adverse reactions than split or subunit vaccines especially in children (Ruben, 2004). Residual egg proteins in these preparations can also cause some allergic reactions in persons with egg allergies (Murphy and Strunk, 1985).

1.4.4 Live attenuated vaccines

LAIV are produced using genetic reassortment between two influenza viruses to generate reassortant virus containing HA and NA coding genes from epidemic virus strain and the remaining attenuating genes from attenuated donor virus such as the cold adapted influenza A/Ann Arbor/6/60(H2N2) and B/Ann Arbor/1/66 (Belshe et al., 1998). The resulting attenuated reassortant is a cold adapted and temperature sensitive virus with limited replication in upper and lower respiratory tract (Belshe et al., 1998). LAIV vaccines are administered intranasally for nonpregnant individuals between the ages of 2 and 49 years (Fiore et al., 2009; Ambrose et al., 2011). Each dose contains $10^{6.5-7.5}$ 50% tissue culture infectious dose (TCID₅₀) of each of the three strains (Fiore et al., 2008; 2009). LAIV

vaccines can cause mild adverse reactions including symptoms such as runny nose, sore throat or fever (Stein et al., 1999).

1.4.5 Vaccine immunogenicity and efficacy

These vaccines provide substantial protection by predominantly inducing HA and NA strain-specific neutralizing antibodies (Epstein, 2006; Gerhard et al., 2006). LAIV are usually more effective in eliciting a broad immune response including mucosal, systemic and cell-mediated responses compared to TIV which do not induce mucosal immunity (Belshe et al., 2000). Many factors can influence the efficacy of TIV including the antigenic match between circulating and vaccine strains, the age of the recipients and their history of influenza exposure (Ambrose et al., 2011). When the vaccine and circulating viruses are antigenically matched, TIVs show 70% – 90% efficacy in healthy adults aged <65 years. Effectiveness against culture-confirmed influenza illness among children aged >6 months to 18 years varies between 50-90% depending of their age. However, it is 20% – 70% effective in preventing hospitalization in the elderly (Fiore et al., 2008). On the other hand, LAIV was reported to have up to 93% overall efficacy against culture-confirmed influenza and 86% against a mis-matched H3N2 strain (Belshe et al., 1998; 2000; Ambrose et al., 2011). LAIV was shown to be more protective than TIV in children 6 months to 18 years of age (Ambrose et al., 2011). However, two doses separated by at least 4 weeks are usually recommended for children younger than the age of 9 who are receiving either vaccine for the first time (Fiore et al., 2008). Both LAIV and TIV have similar efficacy in individuals between 17–49 years of age (Fiore et al., 2008). However, in the elderly, both vaccines have reduced immunogenicity and efficacy. Thus, a combination of both TIV and LAIV might be required to increase vaccine efficacy (Ambrose et al., 2011).

1.4.6 Alternative vaccination strategies

Several strategies have been investigated to improve current vaccines or to generate novel ones. For example, the use of adjuvants such as liposomes (Glück et al., 1994), Microfluidized Emulsion 59 (MF59) (Podda and Del Giudice, 2003; Lee et al., 2011) or aluminium hydroxide (Hehme et al., 2004) with inactivated vaccines, or intranasal administration of these vaccines (Kuno-Sakai et al., 1994; Muszkat et al., 2000; Greenbaum et al., 2001) have been shown to boost antibody response. Interestingly, intranasal immunization with inactivated vaccines adjuvanted with cholera toxin B subunit or *Escherichia coli* (*E. coli*) heat-labile toxin B subunit provided a stronger antibody response in the upper respiratory tracts and effective cross-protection (Tamura et al., 1988; 1992; 1994). Unconventional routes of administration such as epidermal immunization have also been investigated and found to be effective in eliciting immune response (Chen et al., 2000; 2001a; Dean and Chen, 2004).

On the other hand, alternative vaccination strategies which can provide broad immunity against various strains and subtypes, i.e. heterosubtypic immunity, are under extensive research. Such immunity has been suggested to be induced by conserved viral components such as the NP during influenza A virus infection in humans (Epstein, 2006). Immunization with NP-based vaccines (plasmid DNA or recombinant viral vectors) has been shown to induce heterosubtypic immunity in many animal species (Epstein et al., 2002; 2005; Saha et al., 2006; Breathnach et al., 2006; Laddy et al., 2009; Hashem et al 2012). However, some studies have revealed that NP based vaccines can only elicit limited immune response and protection in animals (Patel et al., 2009; Rao et al., 2010). Similarly, vaccination with the highly conserved extracellular domain of the M2 protein, M2e, (Frace et al., 1999; Neiryneck et al., 1999) provided cross-protection against variety of influenza A

subtypes but with lower magnitude compared to HA or NA (De Filette et al., 2005; Tamura et al., 2005; Tompkins et al., 2007; Du et al., 2010; Grandea et al., 2010;).

Although the epitopes in the more conserved HA2 could be less accessible to antibodies in intact viruses, HA2-specific antibodies are induced during natural infection in humans (Styk et al., 1979; Kreijtz et al., 2007) as well as in mice (Kostolansky et al., 2002). Moreover, it was previously demonstrated that immunization of mice with the HA2 subunit of A/Puerto Rico/8/34 (H1N1) HA or truncated HA2 generated cross-protective CTLs against virus strains from H1 and H2 subtypes (Kuwano et al., 1988). Collectively, these reports reinforce the notion that induction of protective heterosubtypic immunity is not restricted to internal proteins only, and can also be generated by conserved regions in HA. Thus, protective potential of the highly conserved fusion peptide or HA1-HA2 cleavage site of influenza A viruses was investigated by several groups, and found to cause milder illness and fewer deaths upon virus challenge (Okuno et al., 1993; Horváth et al., 1998; Gocnik et al., 2008). Furthermore, it was proposed that induction of cross-protective antibodies against the stem region of HA are more likely to be elicited by a molecule missing the immunodominant head-domain or by HA in the neutral pH conformation but not the low-pH conformation (Chen et al., 1995; Steel et al., 2010). While immunization of mice with the conserved HA stalk domain expressed as synthetic peptide comprising amino acids 76–130 of HA2 (Wang et al., 2010), headless HA in HIV gag viral-like particles (Steel et al., 2010) or HA2 subunit with regions (7–46) and (290–321) from HA1 in *E. coli* (Bommakanti et al., 2010) elicited broadly cross-reactive antibodies against HA subtypes, it only provided limited heterosubtypic protection against lethal influenza challenge.

In chapter 2 of this thesis, I will describe universal antibodies targeting the highly conserved fusion peptide in the HA protein and their potential to cross-protect against

divergent influenza A subtypes. In chapter 3, I will describe a prototype vaccine based on the highly conserved NP and I will try to elucidate the underlying immune responses involved in protection in different mouse models. Finally, the significance of these studies as well as their impact on future vaccine development will be discussed in chapter 4.

CHAPTER 2

Universal Antibodies against the Highly Conserved Influenza Fusion Peptide Cross-neutralize Several Subtypes of Influenza A Virus

2.1 ABSTRACT

The fusion peptide of influenza viral HA plays a critical role in virus entry through facilitating membrane fusion between the virus and target cells. Using a bioinformatics approach, the first 14 amino acids of the N-terminus of the fusion peptide was found to be the only universally conserved epitope in all 16 subtypes of influenza A and two genetic lineages of influenza B viruses. Thus, it could serve as an attractive target for antiviral and vaccine development. This peptide was modified and conjugated to overcome serious technical hurdles such as the high hydrophobicity and weak immunogenicity of the viral fusion peptides and used to generate universal anti-influenza antibodies (Uni-1 antibodies). These antibodies demonstrated remarkable specificity against virtually all subtypes of influenza viruses. Most importantly, they bound to the viral HA presented in infected cells and cross-neutralized multiple subtypes of influenza A virus by inhibiting the pH-dependant fusion of viral and cellular membranes rather than blocking the binding of HA to its receptor on target cells. Taken together, these results suggest that this unique, highly conserved linear sequence in viral HA is exposed sufficiently to be attacked by the antibodies during the course of infection. Furthermore, antibodies targeting the fusion peptide merit further investigation for their value in protection against diverse strains of influenza viruses.

2.2 INTRODUCTION

The HA protein is an attractive target for preventive and therapeutic intervention because it plays critical roles in the early stages of virus infection by binding to the viral receptor (SA) on the target cells and mediating the fusion of viral and cellular membranes (Skehel and Wiley, 2000). While vaccines offer the best protection by inducing neutralizing antibodies against HA, they only protect against specific influenza strains (Gerhard et al., 2006; Epstein, 2006). Development of antibodies with broad reactivity against diverse viral strains could be challenging as influenza A HA varies among not only the different subtypes 17 subtypes (H1-H17) but also among the different strains from the same subtype (Capua and Alexander, 2004; Shih et al., 2007). Nonetheless, several groups have isolated or generated a family of cross-subtype BnAbs that bind to highly conserved epitopes in either group 1 or group 2 HA proteins. For example, CR6261 (Throsby et al., 2008) or F10 (Sui et al., 2009) antibodies target a conformational dependant pocket-like epitope formed by two elements critical for the pH-induced conformational change, the “fusion peptide” and the α -helix in the stem region of HA from group 1 influenza A viruses only. Similar BnAb, CR8020 (Ekiert et al., 2011), was also identified against group 2 HA but targeting a distinct epitope from that recognized by group 1 antibodies. More recently, BnAbs against both group 1 and group 2 subtypes were described (Corti et al., 2011; Dreyfus et al., 2012). Interestingly, most of these antibodies shared their binding to parts of the fusion peptide as an element of their epitopes. Also, their mechanism of cross-neutralization depends on inhibiting the fusion step in the virus life cycle.

Through comprehensive bioinformatics analysis of the publicly available HA sequences, our lab has recently shown that the N-terminal 14 amino acids of the fusion peptide (GLFGAIAGFIEGGW) represent the most conserved peptide in all 16 subtypes of

influenza A and two genetic lineages of influenza B viruses (Figure 2.1 and 2.2) (Chun et al., 2008). This peptide was modified and conjugated to overcome serious technical hurdles such as the high hydrophobicity and weak immunogenicity of the viral fusion peptides (Figure 2.3). Then, it was used to generate rabbit mono-specific universal anti-influenza antibodies (designated as Uni-1 antibodies). These antibodies are capable of binding to virtually all influenza HA subtypes with high specificity (Chun et al., 2008). Therefore, I postulated that these antibodies could cross-neutralize multiple influenza viruses through inhibiting cellular and viral membranes fusion. To test this hypothesis, I examined the ability of these antibodies to bind native HA proteins in cell culture and to neutralize different influenza A strains. Furthermore, I confirmed that these universal antibodies cross-neutralized multiple subtypes of influenza A virus by inhibiting the pH-dependant fusion step rather than blocking the binding of HA to its receptor on target cells.

2.3 OBJECTIVES

1. To examine the neutralization activity of Uni-1 antibodies against multiple influenza A subtypes.
2. To determine the mechanism of inhibition.

Figure 2.1

Shannon entropy of Uni-1 peptide (amino acids 1-14 at the N-terminus of HA2 subunit)

All known HA sequences of influenza A and B viruses, regardless of host, from the National Center for Biotechnology Information (NCBI) influenza virus resource (<http://www.ncbi.nih.gov/genomes/FLU/FLU.htm>) were retrieved separately for each type and subtype, and a non-redundant dataset for each subtype was generated by removing identical sequences. A total of 7595 and 298 sequences for influenza A and influenza B, respectively were subsequently analyzed. Specifically, a separate multiple alignment was performed for each subtype using CLUSTALW-MPI, followed by the extraction of the target consensus sequence from the full-gene alignment. GLFGAIAGFIEGGW peptide, denoted Uni-1 peptide, within the fusion peptide (N-terminus of HA2 peptide of HA protein) was identified as the most conserved sequence in all HA sequences analyzed. The degree of variation for each position of amino acid in the identified sequences was then calculated using Shannon entropy which is a standard measure of the degree of conservation or variability in a multiple alignment. Entropy for each amino acid is calculated as: $E = -\sum (P_x \log P_x)$, where P_x is the probability of finding that amino acid in that position. Entropy is summed over all amino acids in that column.

Figure 2.1

Subtype	Consensus Sequence	Number of Sequences	Amino Acid Position														Normalized Total Entropy	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	Entropy	Total
H1	GI.FGAIAGFTEGGGW	1877	0.005	0.009	0.016	0.009	0.010	0.009	0.014	0.005	0.005	0.005	0.032	0.005	0.010	0.005	0.137	0.010
H2	GI.FGAIAGFTEGGGW	138	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.597	0.043
H3	GI.FGAIAGFTEGGGW	1596	0.005	0.494	0.005	0.021	0.010	0.011	0.005	0.010	0.005	0.010	0.010	0.015	0.005	0.005	0.609	0.043
H4	GI.FGAIAGFTEGGGW	260	0.025	0.029	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.356	0.025
H5	GI.FGAIAGFTEGGGW	2022	0.009	0.009	0.013	0.013	0.009	0.021	0.009	0.017	0.009	0.009	0.017	0.012	0.004	0.015	0.163	0.012
H6	GI.FGAIAGFTEGGGW	430	0.016	0.269	0.033	0.019	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.019	0.016	0.016	0.505	0.036
H7	GI.FGAIAGFTEGGGW	429	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.230	0.016
H8	GI.FGAIAGFTEGGGW	37	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	1.704	0.122
H9	GI.FGAIAGFTEGGGW	497	0.028	0.041	0.029	0.016	0.016	0.014	0.043	0.014	0.014	0.029	0.014	0.057	0.014	0.031	0.364	0.026
H10	GI.FGAIAGFTEGGGW	88	0.062	0.062	0.062	0.062	0.062	0.062	0.062	0.062	0.062	0.123	0.062	0.062	0.062	0.062	0.924	0.066
H11	GI.FGAIAGFTEGGGW	107	0.053	0.053	0.053	0.053	0.053	0.053	0.053	0.053	0.053	0.123	0.053	0.053	0.053	0.053	0.736	0.053
H12	GI.FGAIAGFTEGGGW	44	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	1.535	0.110
H13	GI.FGAIAGFTEGGGW	47	0.101	0.202	0.202	0.101	0.101	0.101	0.101	0.101	0.101	0.101	0.101	0.101	0.101	0.101	1.619	0.116
H14	GI.FGAIAGFTEGGGW	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H15	GI.FGAIAGFTEGGGW	6	0.518	0.518	0.518	0.518	0.410	0.518	0.410	0.410	0.410	0.410	0.410	0.410	0.410	0.410	6.283	0.449
H16	GI.FGAIAGFTEGGGW	16	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	G.FFGAIAGFTEGGGW	298	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.314	0.022

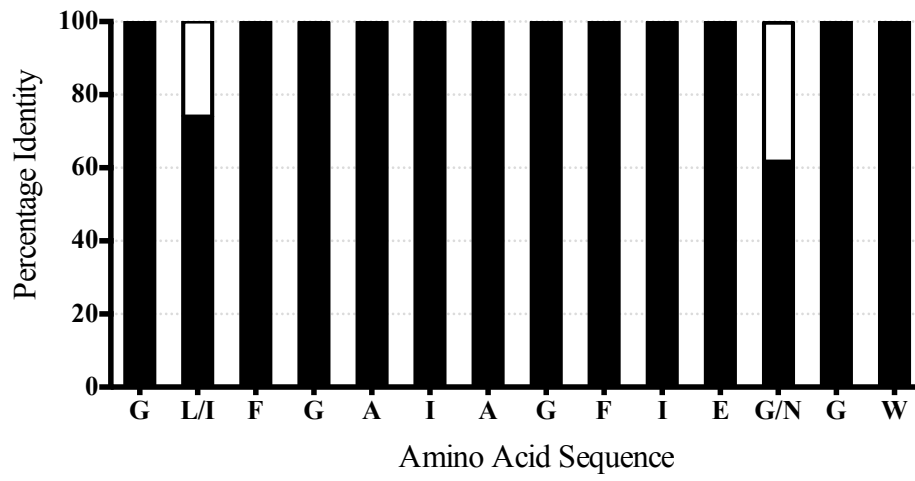
Figure 2.2

Sequence homology of Uni-1 peptide

Sequence homology of the identified region from **(A)** 7595 influenza A HA sequences and **(B)** 298 influenza B HA sequences were analyzed. The sequence represents the N-terminal 14 amino acids of the fusion peptide in the HA2 subunit (1–14). In influenza A viruses, minor substitutions were observed at two positions; position 2 from leucine (L) → isoleucine (I) with a frequency of 26% and at position 12 from glycine (G) → asparagine (N) occurred at a rate of 38.3%. While the consensus sequence of the fusion peptide in influenza B viruses is completely conserved without substitutions, but it differs from influenza A viruses at position 2 and position 12.

Figure 2.2

A



B

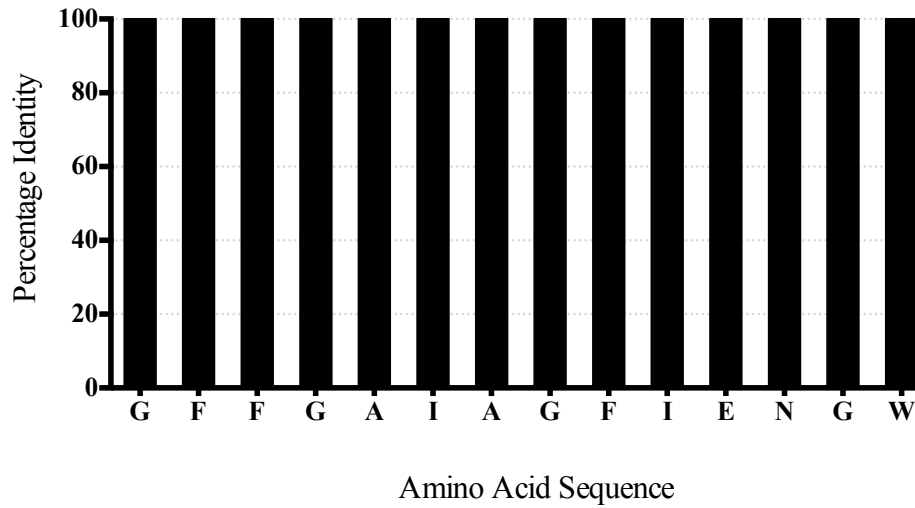
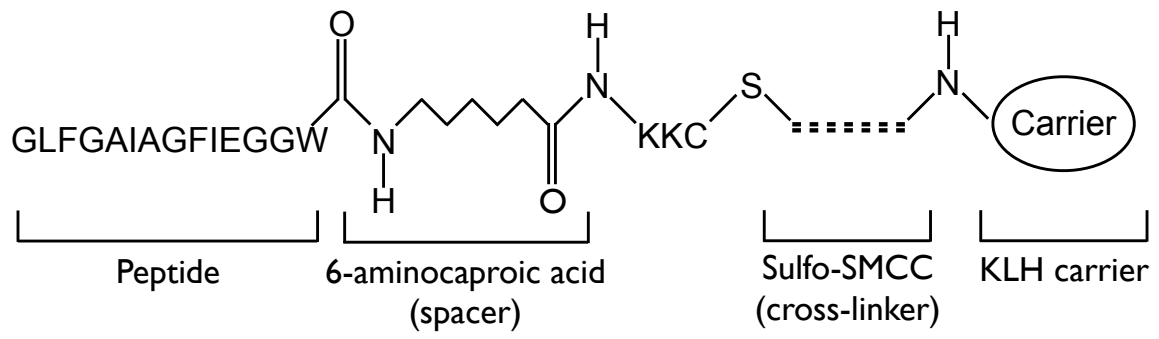


Figure 2.3

Conjugation and modification of Uni-1 peptide

Uni-1 peptide was synthesized by routine solid phase procedure and linked to 6-aminocaproic acid, followed by (KKC) tripeptide to expose and solubilize the hydrophobic peptide. The modified peptide was then conjugated to KLH using sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) as cross-linking reagent. Finally, it was purified and used to generate universal antibodies (Uni-1) targeting the N-terminus of HA2 peptide of HA proteins of influenza A and B viruses in rabbits.

Figure 2.3



2.4 MATERIALS AND METHODS

2.4.1 Cell lines, viruses and recombinant HA proteins

Madin-Darby canine kidney (MDCK) cells, HeLa cells and HEK-293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDCK and HEK-293T cells were cultured 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 2 mM L-glutamine, 25 mM HEPES, 1.5 g/l sodium bicarbonate (pH 7.2), 0.1 mM non-essential amino acids, 110 mg/l of sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin.

Influenza A strains A/Puerto Rico/8/34(H1N1), A/New Caledonia/20/99(H1N1), and A/New York/55/01(H3N2) were propagated at 37 °C in the allantoic cavities of 10-day-old embryonated hen eggs (Canadian Food Inspection Agency, Ottawa, ON, Canada) for 36 hours. Allantoic fluid was clarified by centrifugation, aliquoted and stored at -80 °C. All viruses were titrated by plaque assay in MDCK cells.

The recombinant proteins; H1 (A/New Caledonia/20/1999), H3 (A/Wisconsin/67/2005), H5 (A/Vietnam/1203/2004), H7 (A/Netherlands/219/03), H9 (A/Hong Kong/1073/99) and HB (B/Malaysia/2506/2004), were purchased from Proteins Sciences Corporation (Meriden, CT, USA).

2.4.2 Plaque assay

Viruses were prepared in ten-fold serial dilutions 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 monolayers of MDCK cells in 6 well plates were incubated with 1 ml of each dilution in duplicates at 37 °C for 2 hours. The inoculums were removed, cells were washed twice with phosphate buffered saline (PBS) and overlaid with maintenance DMEM medium containing 0.8% agarose, 0.2% BSA and 2 µg/ml TPCK-treated trypsin. After incubation for 3 days at 37 °C in a humidified atmosphere of 5% CO₂, cells were either directly stained with 0.01% neutral red (Sigma, Saint Louis, MO, USA) or fixed with 10% formaldehyde, followed by staining with 0.5% crystal violet and plaques were counted.

2.4.3 Uni-1 antibodies

Mono-specific universal antibodies generated in rabbits against the N-terminal 14 amino acids of the fusion peptide were purified from antisera using the Uni-1 peptide as a binding ligand in affinity column in a procedure described previously (Wu et al., 1993). Briefly, the antisera were incubated with the peptide-column for 10 minutes at RT, followed by washing for 5 times with PBS with 0.05% Tween-20 (PBS-T). The antibodies were then eluted with acetate buffer (pH 2.0), followed by immediate addition of sodium hydroxide to bring the pH to 7.2. Purified antibodies were then extensively dialyzed against PBS, concentrated, filter purified and stored at -80 °C until use.

2.4.4 GST-Uni1 fusion protein

Uni-1 peptide conjugated to Glutathione S-transferase (GST) as GST-Uni1 protein was synthesized in the laboratory of Dr. Yves Aubin (Centre for Vaccine Evaluation (CVE), Biologics and Genetic Therapies Directorate (BGTD), HPFB, Health Canada, Ottawa, ON,

Canada) as previously described (Gravel et al., 2010) (see Appendix for details). Human prion protein (PrP) conjugated to GST (GST-PrP) was used as a control.

2.4.5 Enzyme linked immunosorbent assay (ELISA)

Indirect ELISA was performed as described before (Gravel et al., 2010). In brief, 4 µg/ml of recombinant HA proteins or GST-peptides were coated onto 96-well plates (Nunc/VWR, Mississauga, ON) at 4 °C for overnight. The wells were then washed 6 times with PBS-T, followed by blocking with PBS-T containing 5 % skim milk for 1 hour at room temperature (RT). Purified Uni-1 antibodies were then added in a serial dilution starting from 100 µg/ml for 1 hour at RT. Subsequently, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies (Sigma, Oakville, ON, Canada) were added at 1:2000 dilution in blocking buffer for 1 hour at RT. Plates were finally washed 6 times before tetramethylbenzidine (TMB) substrate (Cell Signaling Technology, Inc., Danvers, MA, USA) was added for 30 minutes for colorimetric development, the reaction was stopped with equal volume of stop solution. Absorbance was measured spectrophotometrically at 450 nm using a Synergy™ 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

2.4.6 Immunoprecipitation

MDCK cells were infected with influenza A/Puerto Rico/8/34(H1N1) at MOI of 0.01 for 24 hours at 37 °C. Supernatant was then removed and cells were lysed using sodium dodecyl sulfate (SDS)-lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 0.5% SDS, 0.5% NP-40, 0.5% deoxycholate, 5% 2-β-mercaptoethanol, 1 mM EDTA, and Complete Mini PMSF protease inhibitors (Roche, Indianapolis, IN, USA). Five hundred µg of cell lysates were then incubated with 2 µg of Uni-1 antibodies, the positive control F10

antibodies (cross-react with all group 1 HAs (Sui et al., 2009)), or normal rabbit IgG antibodies for 2 hours. The antigen-antibody complexes were then immunoprecipitated using Catch and Release Reversible Immunoprecipitation kit (Millipore, Billerica, MA, USA) according to instructions.

2.4.7 Mass spectrometric analysis and protein identification

The proteins precipitated by immunoprecipitation were identified by liquid chromatography-mass spectrometry/mass spectrometry (LC MS/MS) analysis in the laboratory of Dr. Terry D. Cyr (CVE, BGTD, HPFB, Health Canada, Ottawa, ON, Canada) as previously described (Lu and Zhu, 2005) (see Appendix for details). The mass spectrometric data were searched against the National Center for Biotechnology Information (NCBI) database using Mascot Server (Matrix Science, Boston, MA) specifying tryptic digestion and up to 2 missed cleavage sites, fixed modifications of carbamidomethylation of cysteines, and variable modifications of deamidation of asparagine and glutamine, and methionine oxidation. Mass tolerances were set to 10 ppm for the FT MS peaks and 1 Da for ion trap MS/MS fragment ions.

2.4.8 Plaque reduction assay

Approximately 100 pfu of influenza A/Puerto Rico/8/34(H1N1) virus were incubated alone or with 100 µg/ml of Uni-1 or control rabbit IgG antibodies at 37 °C for 30 minutes. The virus-antibody mixture was then transferred onto confluent MDCK cell monolayers in 6-well plates, incubated at 37 °C for 1 hour and subjected to plaque assay as described in 2.4.2.

2.4.9 Microneutralization assay

Microneutralization assay was carried out as described previously (Sui et al., 2009) with slight modifications. Briefly, 100 TCID₅₀ of influenza A viruses were mixed with equal volume of log₂ serial dilutions of antibody stock solution (200 µg/ml) in 96-well plates and incubated for 1 hours at 37 °C. The virus-antibody mixture was then transferred onto a confluent monolayer of MDCK cells in 96 well plates and incubated at 37 °C for 20 hours. Cell monolayers were then washed twice with PBS, fixed with ice-cold 80% acetone for 10 minutes and viral NP antigen was detected by indirect ELISA using mouse monoclonal antibodies. Fixed cells were washed thrice with PBS-T and incubated with mouse anti-NP antibody (SouthernBiotech, Birmingham, AL, USA) diluted 1:4,000 in PBS-T containing 5% skim milk at 37 °C for 1 hour. Cells were then washed again for four times and incubated with 1:2,000 dilution of HRP-conjugated goat anti-mouse IgG (Sigma, Oakville, ON, Canada) at 37 °C for 1 hour. After 6 washes, TMB substrate was added to wells at RT for 30 minutes. Finally, the reaction was stopped with equal volume of stop solution and absorbance was read at 450 nm using a Synergy™ 2 Multi-Mode Microplate Reader. Inhibition was calculated as a percent of the average absorbance from triplicate wells of virus-antibody mixture relative to no antibody (virus infected) control.

2.4.10 Neutralization of H5N1-pseudotyped virus

The plasmids for the single-round HIV luciferase reporter virus pseudotyped with surface antigens of influenza A/Thailand/2-SP-33/2004(H5N1) (H5-TH04) were kindly provided by Dr. Jianhua Sui (Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA). H5N1 pseudovirus was

generated by co-transfection of HEK-293T cells using lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions with 4 plasmids: H5-expressing plasmid pcDNA3.1-H5, HIV packaging vector pCMVΔR 8.2 encoding HIV-1 Gag-Pol; transfer vector pHIV-Luc encoding the firefly luciferase reporter gene under control of the HIV-1 LTR; and N1-expressing plasmid pcDNA3.1-N1. The ratio of H5- to N1-expressing plasmids was 4:1. Viral supernatants were then harvested at 36 hours post-transfection and stored in +4 °C until use. The neutralization assay was performed as previously described (Sui et al., 2009). HEK-293T cells (6×10^3) were seeded in 96-well plates in DMEM media for overnight at 37 °C. H5N1-pseudotyped virus was mixed with equal volume of log₂ serial dilutions of antibody stock solution (200 μg/ml) in 96-well plates and incubated for 1 hour at 37 °C. The virus-antibody mixture was transferred onto HEK-293T cells and incubated at 37 °C for 48 hours. Viral entry level was evaluated by measuring luciferase activity in target cells. Neutralization was calculated as a percent of the average luciferase activity from triplicate wells of virus-antibody mixture relative to no antibody (H5N1-pseudotyped virus infected) control.

2.4.11 Viral binding inhibition assay

The assay was performed as previously described (Sui et al., 2009). HEK-293T (5×10^5) cells were incubated with H5-TH04-pseudotyped HIV viruses (500 ng of p24) in the absence or presence of different concentrations of Uni-1 or F10 antibodies (known to inhibit fusion but not binding), in PBS buffer containing 0.5% (w/v) BSA and 0.02% (w/v) NaN₃ at 4 °C. After 1 hour of incubation, cells were spun down, supernatant was collected and cells were washed twice with PBS and lysed. Then, p24 levels in supernatant and cell lysates were

quantified using an HIV-1 p24 CA capture ELISA kit (National Cancer Institute, US National Institutes of Health) to quantify unbound and cell-bound virus, respectively. Results were calculated as a percent of binding inhibition comparing the average of bound viruses in virus-antibody mixture relative to that in untreated wells.

2.4.12 Cell fusion inhibition assay

Cell fusion inhibition assay was conducted as described previously (Sui et al., 2009). Approximately 90% confluent HeLa cells in 6-well plates were transfected with 3 μg pcDNA3.1-H5 plasmid using lipofectamine 2000 according to manufacturer's instructions. After ~30 hours of transfection, the culture medium was replaced with 1 ml of medium containing Uni-1 or control antibodies at 100 $\mu\text{g}/\text{ml}$ or 10 $\mu\text{g}/\text{ml}$ for 2-3 hours at 37 °C. Cells were then washed twice with PBS and incubated with low-pH fusion buffer (150 mM NaCl plus 10 mM HEPES, adjusted to pH 5.0) for 4-5 minutes. Cells were then returned to the standard culture medium for 2-3 hours at 37 °C, and finally fixed with 0.25% (v/v) glutaraldehyde and stained with 0.1% crystal violet. Photomicrographs were taken at 10X magnification.

2.5 RESULTS

2.5.1 Uni-1 antibodies bind to native HA protein from infected cells

Binding specificity of Uni-1 antibodies was determined previously in our lab (Chun et al., 2008). As shown in Figure 2.4, the antibodies bound to all 13 subtypes of influenza A viruses amplified in embryonated eggs with remarkable specificity without any cross-reactivity to the egg proteins. As expected, these antibodies were found to recognize the HA2 protein in both fully processed form or as part of the precursor HA (HA0). Furthermore, I tested the binding of Uni-1 antibodies in ELISA and found that they bind to recombinant HA proteins from both influenza A and B similarly (Figure 2.5 A). Importantly, they specifically recognized their respective peptides in the GST-Uni-1 fusion protein but not GST-PrP in ELISA as shown in Figure 2.5 B, confirming their specificity to the fusion peptide.

Although Uni-1 antibodies showed remarkable specificity in Western blot and ELISA, it was unclear whether Uni-1 antibodies could bind to native HA proteins from infected cell cultures. Thus, the ability of Uni-1 antibodies to bind native HA associated with other viral proteins in virus-infected cultures was determined. To this end, extracts from cells infected with Influenza A/Puerto Rico/8/34(H1N1) virus were immunoprecipitated using Uni-1, F10 as a positive control and normal IgG negative control. The immunoprecipitated proteins were then subjected to MS/MS analysis. As shown in Table 2.1, LTQ FT LC MS/MS analyses revealed that Uni-1 and the positive control (F10) antibodies were able to precipitate HA associated with other major viral proteins in virus-infected cell cultures while no viral protein was found to be precipitated with the IgG control, suggesting that Uni-1 antibodies were able to bind to HA protein from infected cultures.

Figure 2.4

Specificity and cross-reactivity of Uni-1 antibodies against diverse subtypes of influenza A viruses

Specificity and cross-reactivity of Uni-1 antibodies were tested by Western blot against 13 HA subtypes (1-13) of influenza A viruses propagated in embryonated eggs. Viruses in allantoic fluids were fractionated in SDS-PAGE, followed by detection of the HA proteins using the Uni-1 antibodies as shown in the upper gel panel. Rabbit polyclonal anti-NP antibodies were used as control as shown in the lower gel panel. The different mobilities of the HA proteins are due to the difference in size or processing stages of the various HA subtypes. The positive control (+) is a reference standard antigen H1N1 obtained from the National Institute for Biological Standards and Control (NIBSC), U.K. while the negative control (–) is allantoic fluid from uninfected embryonated eggs.

Figure 2.4

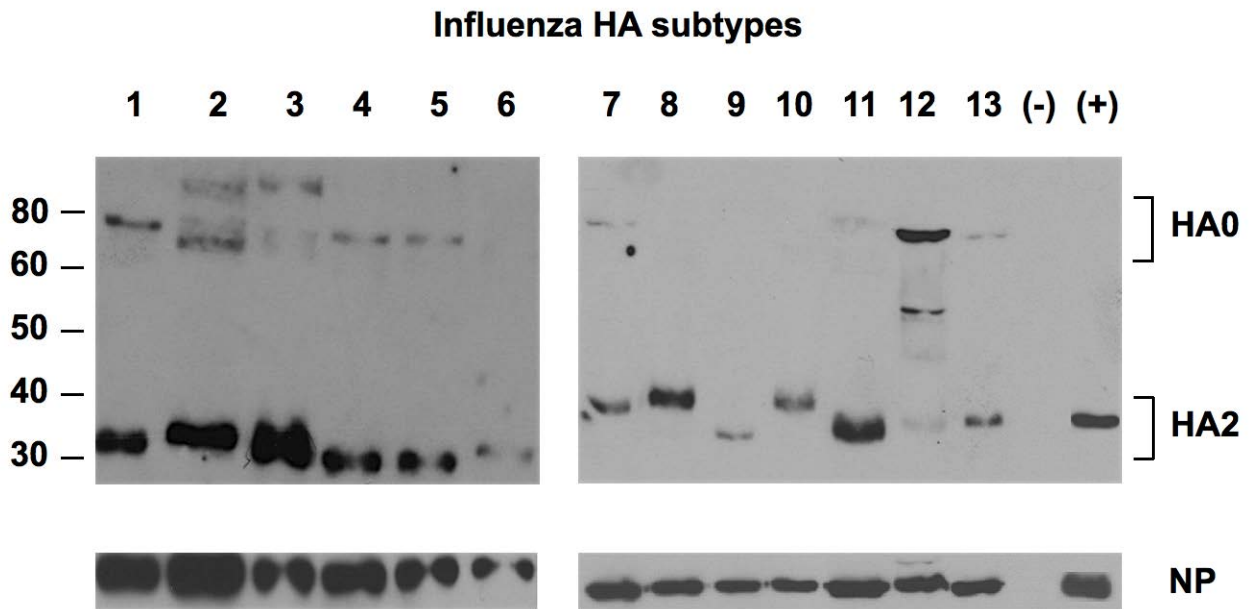


Figure 2.5

Binding of Uni-1 Abs to influenza fusion peptide in ELISA

(A) Recombinant HA proteins from different influenza A subtypes and one influenza B virus were detected with Uni-1 antibodies in ELISA. **(B)** Uni-1 antibodies bind to their respective peptide (GST-fusion peptide) and recombinant HA but not to GST-PrP control. Results represent mean \pm standard deviations from triplicates.

Figure 2.5

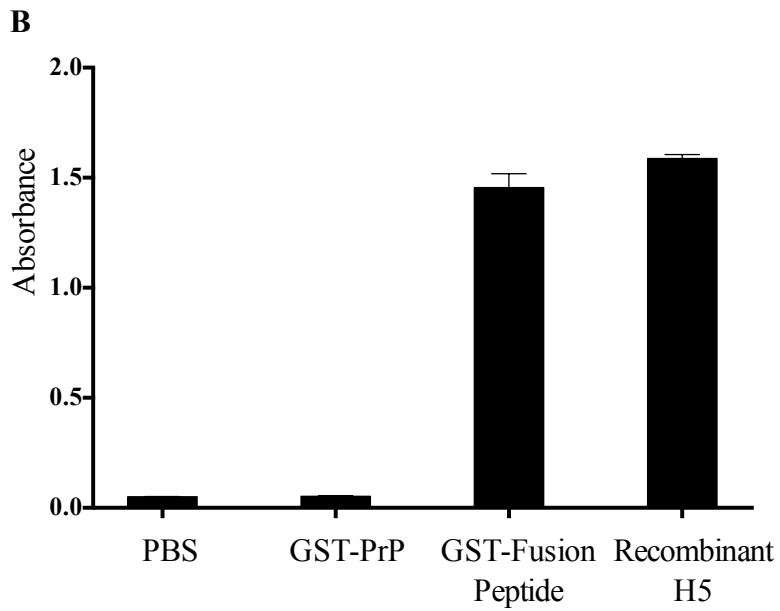
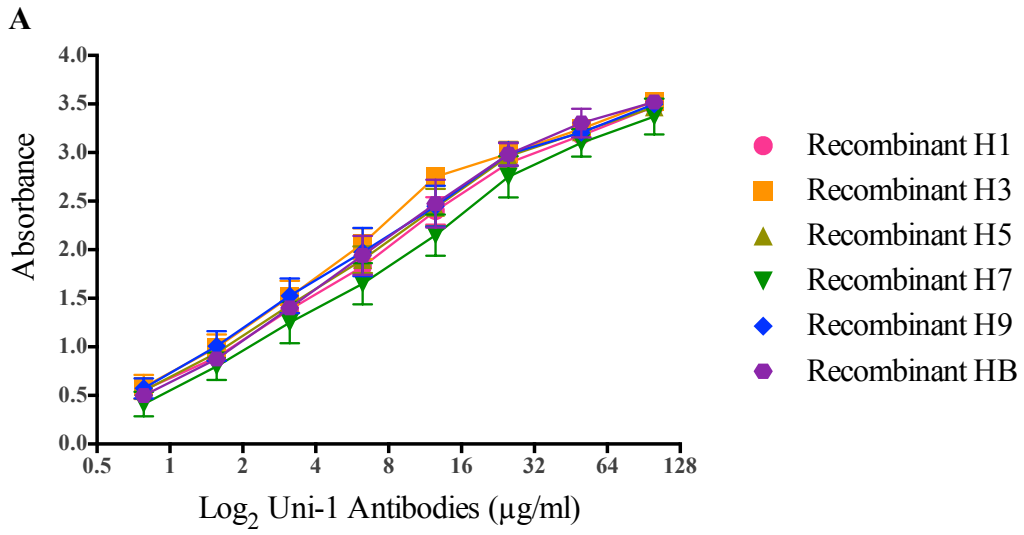


Table 2.1**Identification of the specific antibody-binding proteins by LC MS/MS analysis***

Antibody	MDCK cells	Detection of influenza H1N1 proteins			
		Number of peptides and protein sequence coverage (%)			
		HA	M1	NS1	NP
Uni-1	H1N1 infected	2 (3%)	6 (35%)	6 (43%)	2 (4%)
Uni-1	Uninfected	-	-	-	-
F10	H1N1 infected	2 (3%)	5 (29%)	4 (31%)	2 (5%)
F10	Uninfected	-	-	-	-
IgG	H1N1 infected	-	-	-	-

* Lysates from the uninfected or infected MDCK cells with influenza A A/Puerto Rico/8/34(H1N1) virus were immunoprecipitated by the specific antibody (Uni-1, F10 or IgG), and the proteins were identified by mass spectrometry. Reproducible results were achieved by parallel analyses from two independent experiments. Only the specific antibody-binding proteins are listed, whereas several non-specific binding proteins (albumin, ribosomal protein, tubulin, vimentin, elongation factor, high mobility group, Hnrp, etc) are not shown since those were also observed in the control samples. Dashes denote the undetected proteins in the pull-down extracts. The identified proteins are HA precursor (gi|66239966), M1 protein (gi|4996868), NS1 protein (gi|31096442), and NP (gi|187763982).

2.5.2 Uni-1 antibodies cross-neutralize multiple influenza A subtypes

Having observed that Uni-1 antibodies could not only cross-react with all HA subtypes but also bind to HA proteins from infected cell cultures, I next investigated whether they could inhibit virus replication. To this end, Uni-1 antibodies were first tested by plaque reduction assay against influenza A/Puerto Rico/8/34(H1N1) virus. As shown in Figure 2.6 A, pre-incubation of Uni-1 antibodies with the virus resulted in remarkable reduction in plaque size compared to IgG control. Furthermore, pre-incubation of Uni-1 antibodies with influenza A/Puerto Rico/8/34(H1N1), A/New Caledonia/20/99(H1N1), A/New York/55/01(H3N2) or H5N1 (TH04)-pseudotyped viruses resulted in a substantial reduction of viral replications in microneutralization assay (Figure 2.6 B-E). These results suggest that Uni-1 antibodies can inhibit replication of diverse strains of influenza A virus in a concentration-dependent fashion.

2.5.3 Uni-1 antibodies inhibit viral fusion rather than virus binding

To elucidate the mechanism underlying the inhibition of virus replication by Uni-1 antibodies, I determined whether Uni-1 inhibited the binding of virus to the cells or fusion step after the initial binding. While Uni-1 antibodies failed to prevent binding of the virus to the cells (Figure 2.7 A), significant inhibition of cell fusion was observed with Uni-1 antibodies (Figure 2.7 B). As expected, F10 antibodies prevented cell fusion compared to no antibody control which shows massive cell fusion. It is of note, however, that Uni-1 is less potent than F10 as Uni-1 at 10 $\mu\text{g/ml}$ (panel F) could not completely inhibit polykaryon formation in contrast to F10 at the same concentration. Consistent with initial design of the antibodies, these results confirmed that Uni-1 antibodies can cross-neutralize multiple influenza subtypes through impeding the fusion step required for viral uncoating.

Figure 2.6

Uni-1 antibodies cross-neutralize multiple influenza A subtypes.

(A) Uni-1 antibodies reduce the size of plaques. Influenza A/Puerto Rico/8/34(H1N1) virus (100 pfu) was pre-incubated alone or with 100 µg/ml of Uni-1 antibodies or control rabbit IgG for 30 minutes, then the virus-antibody mixture was transferred to confluent MDCK cells in a 6-well plate, incubated at 37 °C for 2 hours and subjected to plaque assay. Data represent one of two experiments. Uni-1 antibodies or rabbit IgG control were also tested for neutralization against the different viruses **(B) Uni-1 antibodies inhibit the replication of multiple influenza A viruses.** Influenza A/Puerto Rico/8/34(H1N1), A/New Caledonia/20/99(H1N1), A/New York/55/01(H3N2) viruses or H5N1-TH04–pseudotyped virus (virus-like particles with HIV-1 cores expressing H5 and N1 proteins) were tested in a microneutralization assay. Data are presented as percentage of neutralization ± standard deviations from three experiments.

Figure 2.6

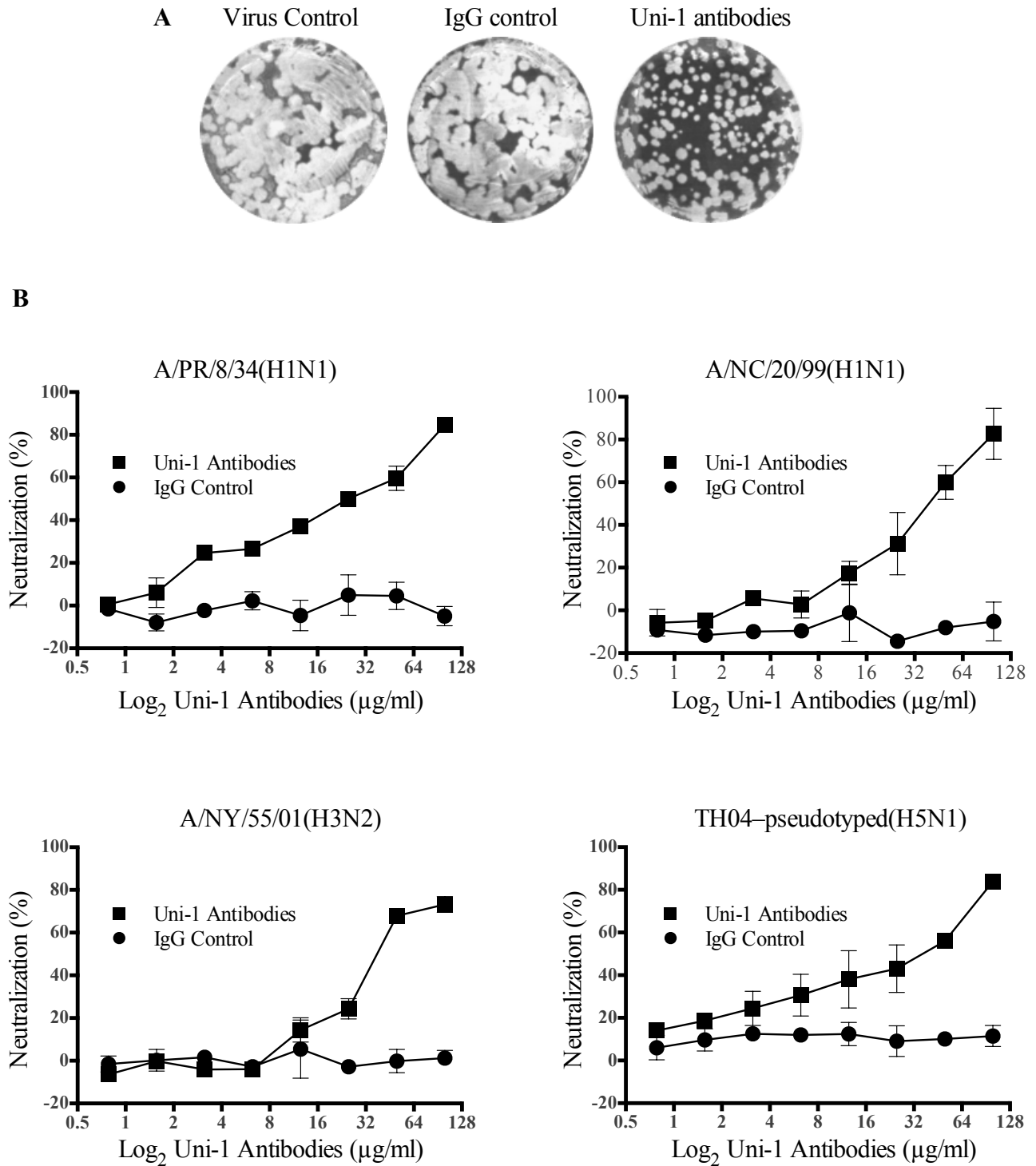


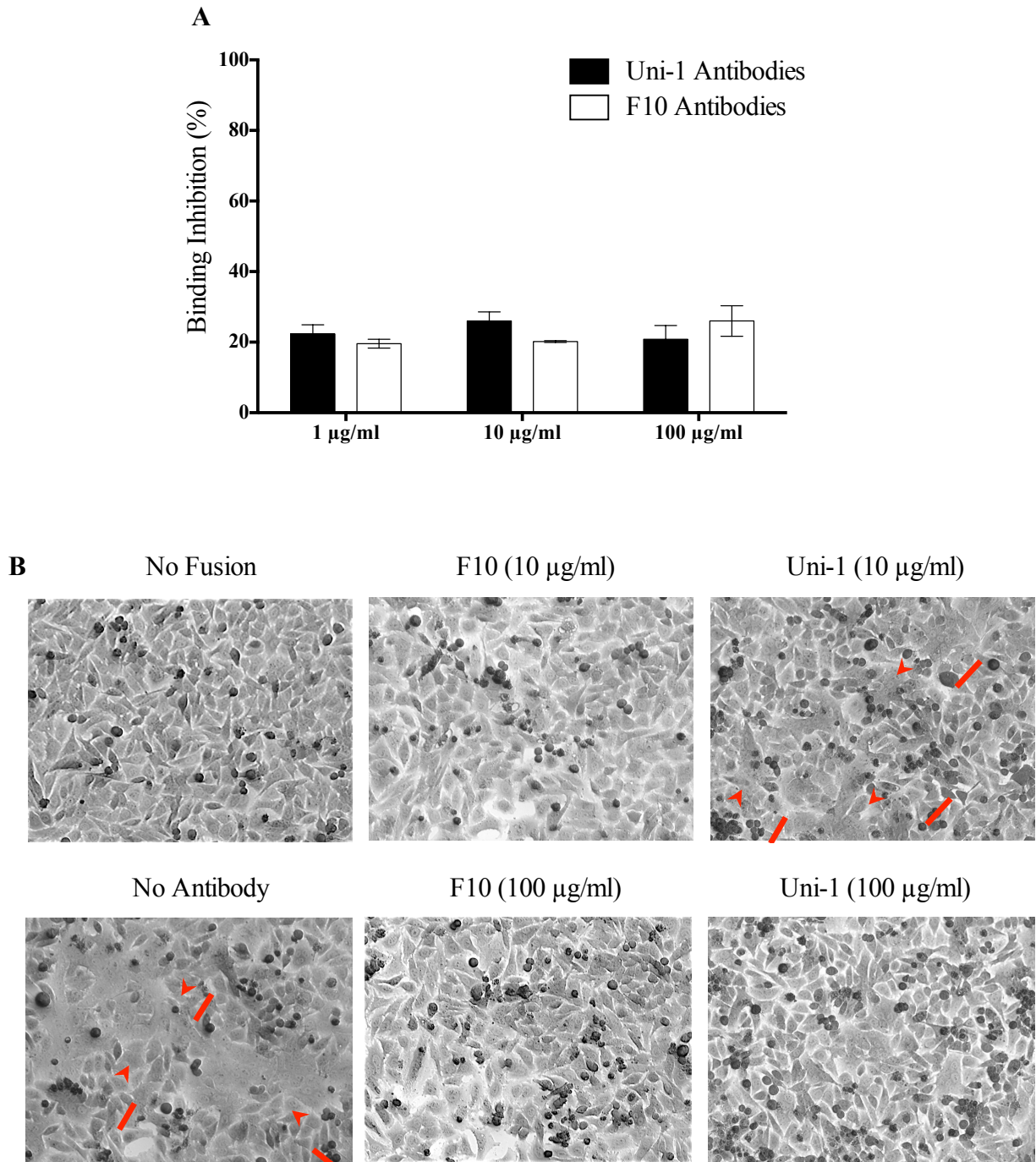
Figure 2.7

Uni-1 antibodies inhibit cell fusion rather than virus binding

(A) Uni-1 antibodies do not inhibit virus binding to cells. HEK-293T cells were incubated with H5-TH04–pseudotyped HIV viruses (500 ng of p24) in the presence of different concentrations of Uni-1 or F10 antibodies (known not to inhibit binding), or in the absence of antibodies, in PBS buffer at 4 °C. After 1 hour of incubation, supernatant and cell bound viruses were quantified by measuring p24 levels. Results are presented as percentage of binding inhibition compared to untreated virus \pm standard deviations from two experiments.

(B) Inhibition of cell fusion by Uni-1 antibodies. HeLa cells were transfected with H5-TH04–expressing plasmid and exposed to a pH 5.0 buffer for 5 minutes in the presence or absence of antibodies. H5-Transfected HeLa cells without exposure to pH 5.0 buffer show no fusion. While syncytia formation was observed when transfected cells were exposed to pH 5.0 buffer in the absence of antibodies. Syncytia formation was inhibited by F10 antibodies (positive control) at a concentration of 10 μ g/ml and 100 μ g/ml. Uni-1 antibodies inhibited fusion at 100 μ g/ml and only showed partially inhibition at 10 μ g/ml. Arrows indicate cell fusion. Pictures are representative of 10 fields. Experiment was repeated at least twice.

Figure 2.7



2.6 DISCUSSION

Current influenza vaccines provide substantial protection in humans, however they mainly induce strain-specific neutralizing antibodies (Epstein, 2006; Gerhard et al., 2006). Most of these antibodies target the highly variable immunodominant regions of the receptor-binding domain of HA; therefore they cannot induce heterosubtypic immunity (Gerhard et al., 2006; Sui et al., 2009). Although it has always been thought that heterosubtypic immunity is mainly mediated by cross-reactive CTLs (Epstein et al., 1998; Flynn et al., 1998), which target conserved epitopes in the viral internal proteins such as NP and PA proteins (Yewdell et al., 1985; Bennink et al., 1987; Epstein, 2006; Gerhard et al., 2006), cross-reactive antibodies against HA have been long known (Sanchez-Fauquier et al., 1987; 1991; Okuno et al., 1993; Tkáčová et al., 1997); however most of these antibodies lacked neutralization activity. With the recent technological advancement in antibody libraries, screening and isolation of such antibodies has been made easier. These antibodies may serve as a source for passive immunotherapy or as a guide for designing of new antiviral drugs. Most importantly, they may represent an important step towards the design of a universal vaccine against influenza virus. Recently, various groups have reported BnAbs with broad reactivity against group 1 (Throsby et al., 2008; Ekiert et al., 2009; Sui et al., 2009), group 2 (Ekiert et al., 2011) or both groups (Corti et al., 2011; Dreyfus et al., 2012) of influenza A viral HA. Although these antibodies target distinct conformational epitopes, they all share the fusion peptide at the N-terminus of HA2 subunit as part of these epitopes. Moreover, different reports have also shown that cross-protective antibodies against HA can also be conferred by immunization (Tumpey et al., 2001; Takada et al., 2003; Quan et al., 2008; Bommakanti et al., 2010; Steel et al., 2010; Wang et al., 2010; Wei et al., 2010) and natural infection (Kostolansky et al., 2002; Prabhu et al., 2009).

Following comprehensive bioinformatics analysis, we recently identified the N-terminal 14 amino acids of the fusion peptide in the HA2 subunit as the only universally conserved sequences among all viral HA proteins. More importantly, mono-specific antibodies (Uni-1 antibodies) against this linear epitope could detect virtually all viral HA proteins. Here, I demonstrated that Uni-1 antibodies bind to HA associated with other viral proteins and inhibit multiple strains of influenza virus in cell cultures. Moreover, mechanistic studies revealed that the inhibition of virus growth was due to Uni-1 impeding the fusion process. Clearly more studies are needed to determine whether antibodies targeting the universally conserved epitope in viral HAs could protect animals from influenza in a suitable animal model, an issue which is not addressed in this study, given that Uni-1 antibodies are of rabbit origin and because of limited quantities. Interestingly, Prabhu et al isolated a similar monoclonal antibody (mAb), designated 1C9, upon lethal infection of mice with HPAI H5N1 virus. The 1C9 antibody was found to bind to 9 amino acids within the Uni-1 peptide (GLFGAIAGF). This mAb inhibited cell fusion *in vitro* in the same fashion as Uni-1 antibodies. More importantly, it showed therapeutic and prophylactic protection in mice against HPAI H5N1 influenza A strains from two different clades (Prabhu et al., 2009).

In summary, the significance of my findings is clear: during the course of infection in tissue culture, the viral fusion peptide is exposed to an extent which is accessible to a neutralizing antibody, making it an attractive target for alternative antiviral intervention and vaccine development, particularly because these antibodies possess high specificity against influenza viral sequences and retain the potential to bind to all viral HAs. Thus, investigating the potential of the fusion peptide as an immunogen to induce cross-protection in animals would be of added values for future antiviral and vaccine strategies.

CHAPTER 3

CD40 Ligand Preferentially Modulates Influenza Nucleoprotein-specific Immune Response and Enhances Protection against Influenza Virus Infection

3.1 ABSTRACT

Influenza A NP-based immunization has the potential to induce heterosubtypic immunity. However, it is unclear whether only the different forms of vectors and vaccination regimens and/or the substantially weak NP protective immunity, compared to HA, could have contributed to the previously reported discrepancies in protection. Here, I evaluated different NP-based genetic immunization regimens and investigated the potential of CD40 ligand (CD40L), a key regulator of the immune system, as both a targeting ligand and a molecular adjuvant in NP-based host defense against influenza. I found that two subcutaneous doses of rAd vector expressing NP (rAd-NP) were sufficient to elicit an enhanced immune response in mice and complete protection against lethal challenge compared to plasmid DNA alone or plasmid DNA prime/rAd boost regimens. Furthermore, immunization with rAd carrying a fused gene encoding for secreted NP-CD40L fusion protein (rAd-SNP40L) substantially enhanced immunogenicity and protective efficacy; thus a single dose was protective against lethal challenge in mice. Mechanistically, rAd-SNP40L preferentially induced early and persistent B-cell germinal center formation, accelerated Ig isotype-switching and a T_H1 -skewed NP-specific immune response. Also, it significantly enhanced primary and memory NP-specific CTL activity and the frequency of polyfunctional $CD8^+$ T cells. Transfer of sera or $CD8^+$ T cells from rAd-SNP40L-immunized mice rendered the recipient naïve mice resistant to viral challenge, suggesting a role for both NP-specific antibodies and CTLs in protection. Interestingly, rAd-SNP40L afforded equally effective protection in $CD40L^{-/-}$ and $CD4^{-/-}$ mice, confirming that the enhanced protection against influenza is CD40L-mediated and $CD4^+$ T cell-independent. Single dose of rAd-SNP40L conferred complete protection against lethal challenge 4 months post-immunization, revealing the robust and long-lasting memory immune response against influenza.

3.2 INTRODUCTION

Current influenza vaccines largely induce strain-specific neutralizing antibodies against the two surface glycoproteins; HA and NA (Epstein et al., 2005; Gerhard et al., 2006). While these vaccines can provide substantial protection in a healthy population when vaccine strains match circulating ones (Gerhard et al., 2006), their high antigenic variability necessitates annual vaccine formulation and evaluation (Wood, 2002; Gerhard et al., 2006; Palese, 2006). Identification and characterization of broadly neutralizing or reactive antibodies against highly conserved epitopes in both HA (Throsby et al., 2008; Sui et al., 2009; Corti et al., 2011; Ekiert et al., 2011; Dreyfus et al., 2012) and NA (Gravel et al., 2010) may represent an important step towards a universal influenza vaccine. However, most of these antibodies either have limited neutralization potency or are restricted to certain subtypes. Most importantly, current vaccination and natural influenza infection mainly result in the recognition of the highly variable immunodominant head domains in HA and NA, which shield the more conserved regions in these two proteins (Nabel and Fauci, 2010; Steel et al., 2010).

On the other hand, internal proteins such as NP are highly conserved among all influenza subtypes and are under extensive research as candidate universal vaccine antigens using different immunization strategies. Several studies have shown that NP-based genetic immunization through plasmid DNA and/or recombinant viral vectors induces strong immune responses and protects against various influenza A subtypes in several animal species (Fu et al., 1997; Epstein et al., 2002; 2005; Breathnach et al., 2006; Laddy et al., 2009). However, more recent works suggest that NP based vaccines can only elicit limited immune responses and protection in animals (Patel et al., 2009; Rao et al., 2010). While the use of different vectors and/or immunization regimens in these studies may play a role in the

reported discrepancies, it is clear that NP induces substantially weaker protective immune response compared to HA as it required multiple doses in all previous reports.

Gene-based vaccination is an exciting means of inducing protective immune responses against viral infections. It offers many advantages compared to traditional vaccines including simplicity of production, high purity and the ability to induce humoral and cellular immunity (Oliveira et al., 1999; Wahren, 1999). However, DNA vaccines typically are weak immunogens, and innate and pre-existing immunity against recombinant viral vectors may lead to acute toxicity and limited efficacy (Thacker et al., 2009), especially after multiple doses. Furthermore, vectors such as rAd have been reported to inadvertently invade the brain via the olfactory bulb upon intranasal administration (Huang et al., 2008). While several strategies are currently under investigation to overcome pre-existing immunity and to reduce viral vectors antigenicity (Pereboev et al., 2004; Huang et al., 2008; Hangalapura et al., 2010; Kim et al., 2010) it might be useful to consider adjuvants to enhance potency of the highly conserved antigens such as NP so that one single injection can afford effective protection. One interesting approach is to modulate and enhance the host immune response by using molecular adjuvants such as cytokines, chemokines and costimulatory molecules.

Among the various costimulatory molecules that have been identified, CD40 and its ligand (CD40L) are ones of the most important factors that not only orchestrate humoral and cellular immunity but also regulate APCs (van Kooten and Banchereau, 2000; Bishop and Hostager, 2003; Quezada et al., 2004; Ma and Clark, 2009). CD40, a type I transmembrane protein and a member of the TNF receptor (TNFR) superfamily, is constitutively expressed on all APCs in addition to activated CD4⁺ T cells, CD8⁺ T cells and some non-hematopoietic cells such as fibroblasts, endothelial cells and epithelial cells (van Kooten and Banchereau, 2000; Bishop and Hostager, 2003; Quezada et al., 2004; Ma and Clark, 2009). CD40L, a type

II integral membrane protein and a member of the TNF superfamily, is mainly expressed by activated CD4⁺ T cells transiently as both multimer cell-surface or secreted protein (van Kooten and Banchereau, 2000). It is also expressed by other immune and non-immune cells including activated B cells, some DCs subsets, platelets as well as smooth muscle cells (Quezada et al., 2004; Ma and Clark, 2009).

Interaction between CD40 and CD40L promotes the expansion and survival of APCs, T cells and B cells to initiate and sustain immune responses (Bishop and Hostager, 2003; Quezada et al., 2004). Specifically, during DC-CD4⁺ T cell interaction, CD40 ligation on DCs enhances their survival, secretion of cytokines and upregulation of costimulatory receptors such as CD54, CD58, CD80 and CD86 as well as MHC class-I and II molecules, thus promoting their maturation into fully competent APCs (van Kooten and Banchereau, 2000). Concomitantly, it provides crucial bidirectional signals to stimulate CD4⁺ T cells themselves and/or to license DCs in order to initiate direct or indirect CTL priming (Bennett et al., 1998; Schoenberger et al., 1998). Moreover, CD40 signaling on B cells plays important roles not only in enhancing antigen presentation to T cells (Schultze et al., 1997; Bergwelt-Baildon et al., 2004) but also in their own activation, proliferation and differentiation. Specifically, it facilitates Ig production, Ig isotype-switching, germinal centers (GC) formation, and memory B cells maturation (van Kooten and Banchereau, 2000; Bishop and Hostager, 2003).

Several groups have investigated the potential of CD40L as a molecular adjuvant using different strategies including co-delivery of CD40L with antigens (Mendoza et al., 1997; Gurunathan et al., 1998; Tripp et al., 2000; Sin et al., 2001; Harcourt et al., 2003; Zheng et al., 2005; Liu et al., 2008; Gómez et al., 2009; Cao et al., 2010; Auten et al., 2012) or retargeting the antigen or the delivery vectors to CD40 on APCs (Manoj et al., 2004;

Pereboev et al., 2004; Huang et al., 2008; Lin et al., 2009; Hangalapura et al., 2010; Kim et al., 2010; Yao et al., 2010; Franco et al., 2011; Hangalapura et al., 2011; Sánchez Ramos et al., 2011). While these previous studies reported enhanced immune responses against various pathogens and tumors, little is known about *in vivo* B cell responses, functional roles of CD4⁺ and CD8⁺ T cells during induction phase as well as the contributions of antibodies and CD8⁺ T cells in protection. Moreover, few reports have employed CD40L to enhance the immune response against influenza virus (Yao et al., 2010; Sánchez Ramos et al., 2011). These have mainly focused on the highly variable HA protein and showed multiple dosing regimens and induction of partial protection.

Given that the previously published studies by various groups employed different vectors and/or immunization strategies, I investigated a combination of genetic immunization regimens in animal protection experiments and assessed the immune responses against influenza NP. Also, I determined the potential of CD40L as both an antigen-targeting ligand and molecular adjuvant to enhance NP-induced host defense against influenza.

3.3 OBJECTIVES

1. To compare different NP-based genetic immunization regimens including plasmid DNA alone, plasmid DNA prime/rAd boost and rAd only to determine their immunogenicity and protective efficacy.
2. To investigate CD40L as a molecular adjuvant and targeting molecule to enhance NP-based protection against influenza virus in mice.
 - a. To mechanistically examine the nature of the immune response in mice.
 - b. To functionally determine of the role of NP-specific antibodies and T cells in immune response induction and protection.

3.4 MATERIALS AND METHODS

3.4.1 Cell lines and virus

Mouse fibroblast NIH-3T3 (ATCC: CRL-1658), mouse mastocytoma P815 cells (ATCC: TIB-64) and MDCK cells were obtained from ATCC (Manassas, VA, USA), and QBI-HEK 293A cells were purchased from Qbiogene Inc. (Carlsbad, CA, USA). Cells were grown in complete DMEM with 10% FBS as described in section 2.4.1. Mouse adapted influenza A/Puerto Rico/8/34(H1N1) was used in this study for viral challenge. Virus stock was propagated and titrated as described in section 2.4.1.

3.4.2 PCR reagents, enzymes, genes and flow cytometry antibodies

PCR was done using High fidelity Iproof kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Primers used in cloning were synthesized by Bio S&T (Montreal, QC, Canada) and are listed in the Appendix. All restriction enzymes and T4 DNA ligase were purchased from New England BioLabs (Whitby, ON, Canada). The full-length coding sequences for influenza A NP gene from strain A/duck/Yokohama/aq10/2003(H5N1) (GeneBank Acc. No. AB212281) and mouse CD40L (GeneBank Acc. No. NM_011616) were synthesized by Bio S&T (Montreal, QC, Canada) in pUC19 vector (pUC19-NP and pUC19-CD40L, respectively).

All flow cytometry antibodies were obtained from eBioscience (San Diego, CA, USA) except for PerCP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and Pe-conjugated anti-mouse Syndecan-1/CD138 (BD Biosciences, San Jose, CA, USA).

3.4.3 Recombinant influenza A NP production

Recombinant NP (rNP) was produced in *E. coli* and used as an antigen in polyclonal anti-NP antibody production in NZW rabbits by Sigma-Genosys (Oakville, ON, Canada). Also, it was used to coat plates used in ELISA in order to measure anti-NP antibody titers in immunized mice.

3.4.3.1 Cloning of influenza NP into prokaryotic expression plasmid

Influenza NP was amplified from pUC19-NP by PCR using pQE2-NP sense and antisense primers to introduce *KpnI* and *HindIII* restriction sites, respectively. PCR product was then gel purified using QIAquick Gel Extraction Kit according to manufacturer's instructions (QIAGEN Inc., Toronto, ON, Canada), digested and inserted by directional cloning into pQE2 prokaryotic expression plasmid (QIAGEN Inc., Toronto, ON, Canada) generating pQE2-NP, in which the rNP is tagged at the N-terminus with six histidine residues (rNP-6xHis). Cloning was confirmed by sequencing and restriction digestion.

3.4.3.2 Expression and purification of recombinant influenza A NP

Expression of rNP was achieved by transforming *E. coli* BL21 (DE3) cells (Life Technologies Inc., Burlington, ON, Canada) with pQE2-NP plasmid. Transformed cultures were grown in Luria Broth (LB) containing 100 µg/ml ampicillin (Life Technologies Inc., Burlington, ON, Canada), and induced to express rNP-6xHis protein with 1 mM isopropyl-β-D-galactopyranoside (IPTG) (Life Technologies Inc., Burlington, ON, Canada) overnight at 37 °C. Bacterial cells were collected by centrifugation at 8,000 x g for 30 minutes at 4 °C. Cells were resuspended in lysis buffer (100 mM sodium phosphate, 10 mM Tris, 6 M guanidine HCL, pH 7.6) and sonicated 6 times for 45 seconds each on ice. Cellular debris

was pelleted by centrifugation at 10,000 x g for 30 minutes at 4 °C. Polyhistidine-tagged rNP protein was purified by Fast performance liquid chromatography (FPLC) by applying supernatant to a nickel-nitrilotriacetic acid (Ni-NTA) column according to the manufacturer's protocol (QIAGEN Inc., Toronto, ON, Canada). The rNP was eluted with elution buffer (100 mM sodium phosphate, 10 mM Tris, 250 mM imidazole, pH 7.6). Positive elution fractions as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3.1 A) were pooled, dialyzed and stored at -80 °C. As shown in Figure 3.1 B, protein was also detected using Histidine-Tag antibodies (Cell Signaling Technology Inc., Danvers, MA, USA) in Western blot.

3.4.4 Plasmid DNA

Plasmid DNA expressing influenza NP (pNP) was generated by subcloning NP gene from pQE2-NP plasmid into the *NotI* and *HindIII* sites in pcDNA3.1(-) mammalian expression vector (Life Technologies Inc., Burlington, ON, Canada). The construct was confirmed by sequencing and restriction enzyme digestion (Figure 3.2 A). Bulk endotoxin-free preparations of pNP as well as the empty control plasmid (pcDNA3.1) were prepared using endotoxin-free Plasmid DNA Giga purification kit (QIAGEN Inc., Toronto, ON, Canada).

To confirm protein expression, NIH-3T3 cells cultured in 6-well plates (3×10^5 /well) in complete DMEM with 10% FBS were transiently transfected with the plasmid constructs (pNP or pcDNA3.1) using FuGENE 6 Transfection Reagent (Roche Diagnostics, Laval, QC, Canada) as previously described (Flaman et al., 2011). Forty-eight hours later, cells were washed thrice with cold PBS and lysed using SDS-lysis buffer. Protein expression was confirmed by Western blot using rabbit polyclonal anti-NP antibodies (Figure 3.2 B).

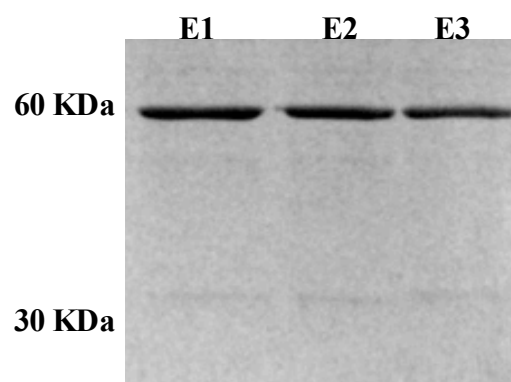
Figure 3.1

SDS-PAGE and Western blot analysis of recombinant influenza NP

Influenza NP was expressed in *E. coli* and purified by FPLC on a Ni-NTA column. **(A)** Purification fractions were separated on 12% polyacrylamide gel in SDS-PAGE and detected with Coomassie Blue staining. **(B)** Detection of recombinant NP by Western blotting using anti-His tag antibodies from these fractions. Analysis shows protein bands of about 57 KDa corresponding to the expected size of recombinant NP protein.

Figure 3.1

A Coomassie Blue staining on SDS-PAGE gel



B Western blotting using anti-His tag antibodies

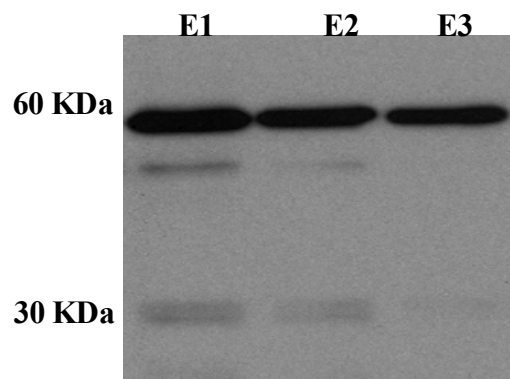


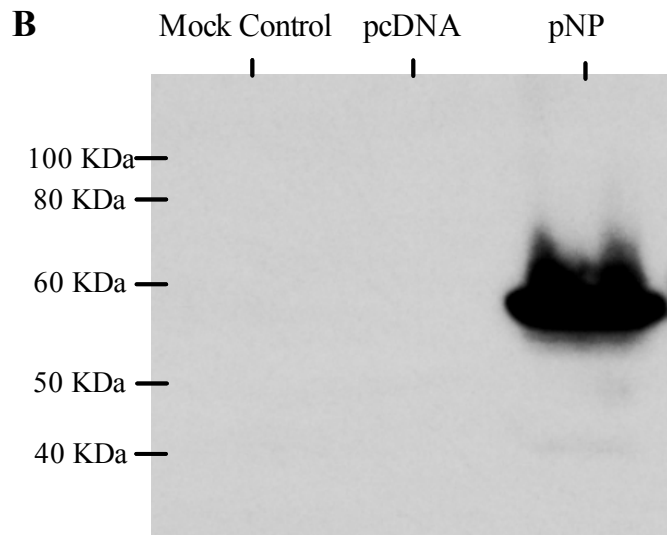
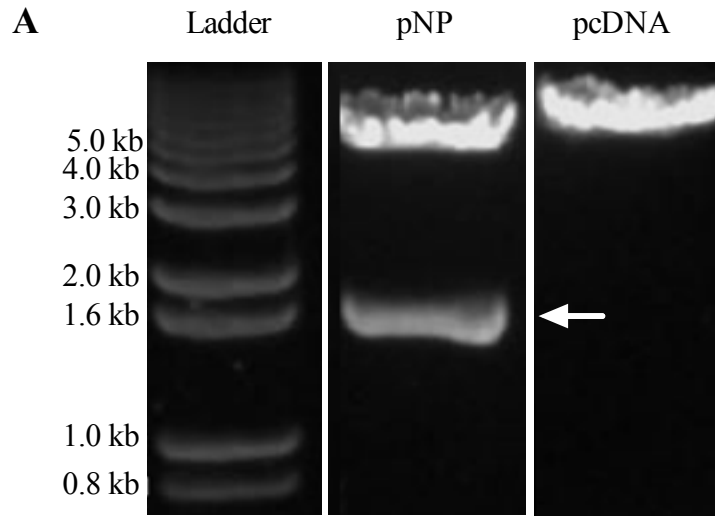
Figure 3.2

Confirmation of pNP cloning and protein expression

(A) Confirmation of pNP cloning by restriction enzymes. NP gene was cloned into the NotI and HindIII sites in pcDNA3.1 mammalian expression vector. The empty vector (pcDNA3.1) and plasmid containing influenza NP gene were digested with NotI and HindIII enzymes and resolved on a 1% agarose gel to confirm cloning. The size of influenza NP (1.5 kb) and the vector backbone (5.5 kb) were estimated by comparing them with a 1 kb ladder.

(B) In vitro protein expression in cell culture. NIH/3T3 cells were transiently transfected with pNP or empty plasmid (pcDNA3.1) and cell lysates were collected 48 hours later. Proteins expression was confirmed by Western blot on 12% SDS-PAGE under reducing conditions using polyclonal anti-NP antibodies.

Figure 3.2



3.4.5 Recombinant adenoviruses

Recombinant adenoviruses (rAds) were generated using AdenoVator™ Adenoviral Expression System with pAdenoVator-CMV5(Cuo)-IRES-GFP transfer vector (tAd) according to the manufacturer's instructions (Qbiogene Inc., Carlsbad, CA, USA). As shown in Figure 3.3 A, constructs were designed to either express: influenza NP (rAd-NP), a secreted form of NP (rAd-SNP) consisting of influenza NP and 23 amino acids from the human tyrosinase signal peptide (MLLAVLYCLLWSFQTSAGHFPR) at the N-terminus as previously described (Hauser et al., 2004), a fusion protein expressed by rAd-SNP40L consisting of SNP followed by a 27 amino acids from the bacteriophage T4 fibritin trimerization motif (GYIPEAPRDGQAYVRKDGEWVLLSTFL) connected to the ectodomain of mouse CD40L (amino acids 117-260) as previously described (Pereboev et al., 2004), unsecreted fusion protein (rAd-NP40L) or an empty control vector (rAd-Control).

3.4.5.1 Cloning of constructs into transfer vector

All constructs were firstly cloned into the *NotI* and *EcoRV* sites of the tAd. To generate tAd-NP, NP cDNA was PCR-amplified from pUC19-NP with NP-1 and NP-R1 primers to introduce *NotI* and *EcoRV* sites, respectively, and inserted into the tAd vector. To generate tAd-SNP, the NP gene was PCR-amplified from pUC19-NP introducing 43 base pairs (bp) of the secretion signal (S) at the 5' terminus and *EcoRV* site at the 3' end using NP-2 and NP-R1 primers. The PCR product was gel purified and re-amplified to complete the secretion sequence to 69 bps introducing *NotI* site at the 5' end using NP-3 and NP-R1 primers. The PCR product was gel purified, digested and ligated between *NotI* and *EcoRV* sites of the tAd vector. The tAd-SNP40L was assembled in three steps as shown in Figure 3.4. First, the SNP fusion gene was PCR-amplified from tAd-SNP using NP-3 and NP-R2




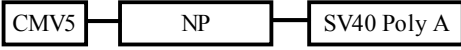

Figure 3.3

Recombinant Ad constructs and in vitro protein expression and secretion

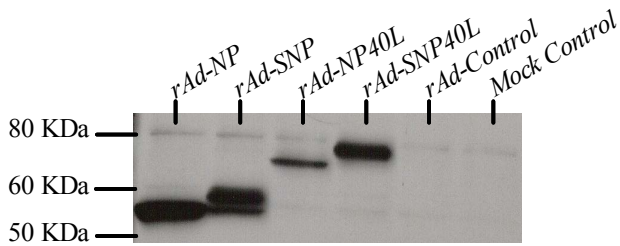
(A) Schematic representation of the rAd constructs. The rAd-SNP40L was generated to express secreted influenza A NP that is targeted to CD40 via trimerized CD40L as NP-F-CD40L fusion protein. The rAd-NP40L encodes the fusion protein missing the secretion signal. The rAd-SNP was designed to express secreted form of influenza A NP, while rAd-NP construct was generated to express influenza NP intracellularly. The rAd-Control is an “empty” control vector. S is an N-terminal leader sequence derived from human tyrosinase signal peptide. NP is influenza A/duck/Yokohama/aq10/03(H5N1) NP gene. F is a 27 amino acid fragment from the T4 bacteriophage fibrin trimerization motif fused with ectodomain (144 amino acids) of the mouse CD40L. Constructs were engineered in recombinant E1/E3 Adenovirus 5 vectors under the control of CMV5 promoter. Numbers indicate the expected molecular weight of the proteins from each construct. (B) Confluent NIH/3T3 cells were infected with rAds at MOI of 100 and expression of NP, SNP, NP40L and SNP40L was confirmed in cell lysates. (C) Secreted proteins were immunoprecipitated from supernatants using polyclonal anti-NP antibodies. Protein secretion was confirmed from both rAd-SNP (lane 2) and rAd-SNP40L (lane 6). Detection of NP from rAd-NP in supernatant (lane 1) is expected as it is well documented that small amounts of extracellular NP can be detected in cell culture supernatant (Prokudina et al., 2001). Protein expression and secretion were confirmed by Western blot on 8% SDS-PAGE under reducing conditions using polyclonal anti-NP antibodies.

Figure 3.3

A

Constructs	Products	Size of product
<i>rAd-SNP40L</i>		Secreted NP-CD40L ~ 78 KDa
<i>rAd-NP40L</i>		Unsecreted NP-CD40L ~ 75 KDa
<i>rAd-SNP</i>		Secreted NP ~ 59 KDa
<i>rAd-NP</i>		Unsecreted NP ~ 56 KDa
<i>rAd-Control</i>		No encoded protein

B Cell lysate



C Supernatant

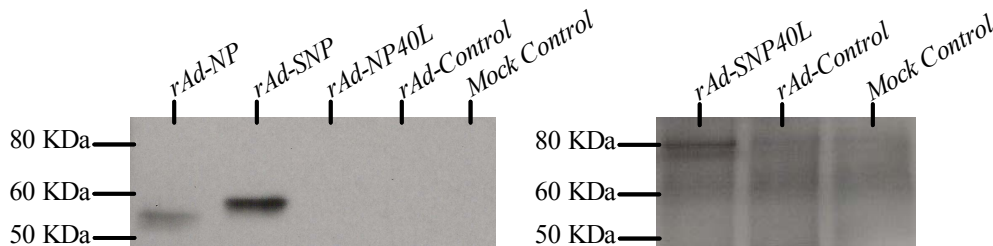


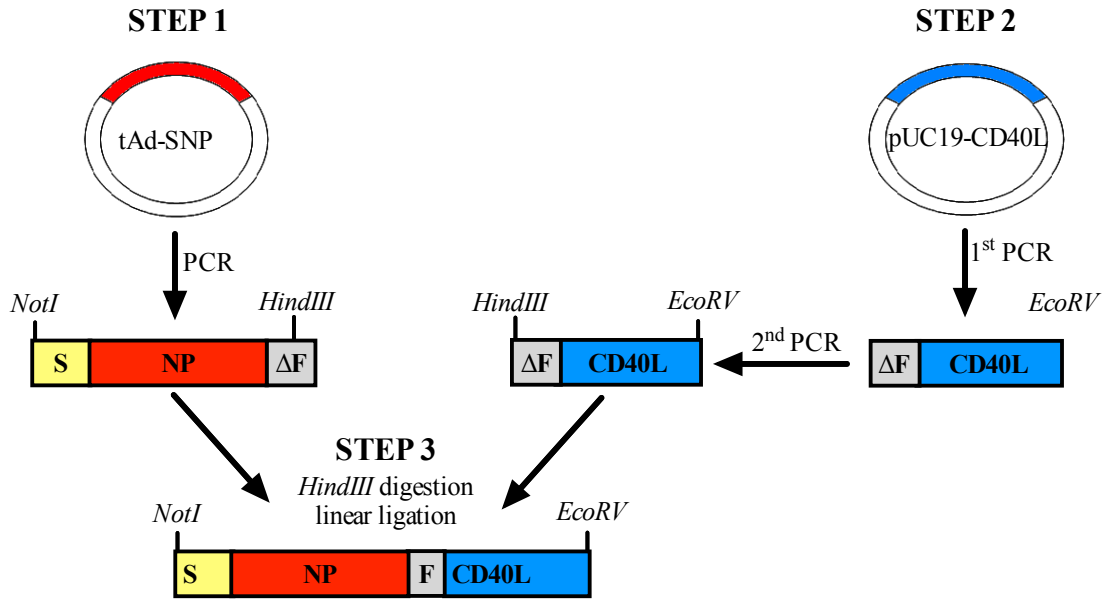
Figure 3.4

Generation of rAd-SNP40L

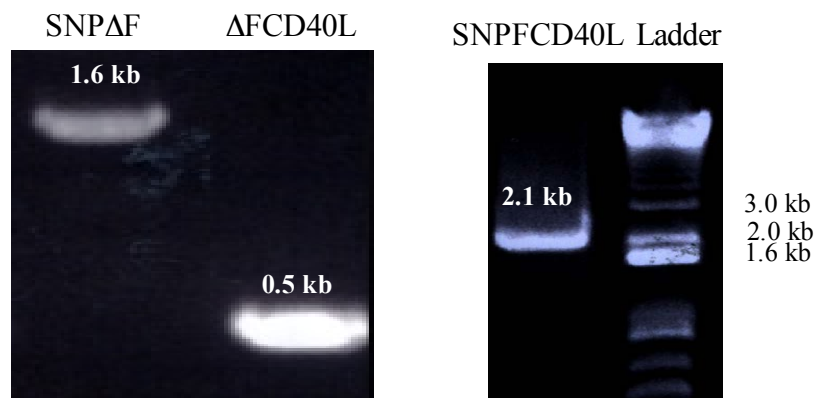
(A) Step 1. The SNP fusion gene was PCR-amplified from tAd-SNP to introduce 41 bps of the trimerization motif (F) at the 3' terminus, which contains an internal HindIII restriction site and to generate SNP Δ F. **Step 2.** The ectodomain of CD40L was PCR-amplified in a separate reaction to add 27 bps from the trimerization motif (F) at the 5' end and EcoRV site at the 3' end, respectively. The PCR product was re-amplified to add 29 bps of the trimerization motif, which contains the internal HindIII restriction site, and to generate Δ FC40L. **Step 3.** Both fragments, SNP Δ F and Δ FC40L, were digested with HindIII, ligated in a linear ligation, PCR amplified and inserted into the tAd vector. **(B)** PCR products from the three steps were resolved on a 1% agarose gel and purified for ligation and insertion into the transfer vector. The size of the SNP Δ F (1.6 kb), Δ FC40L (0.5 kb) and the SNPFC40L (2.1 kb) were estimated by comparing them with a 1 kb ladder.

Figure 3.4

A



B



to introduce 41 bps of the trimerization motif (F) at the 3' terminus, which contains an internal *HindIII* restriction site and to generate SNP Δ F. Second, the ectodomain of CD40L (nucleotides 361-795) was PCR-amplified in a separate reaction from pUC19-CD40L using CD40L-1 and CD40L-R primers to add 27 bps from the trimerization motif (F) at the 5' end and *EcoRV* site at the 3' end, respectively. This PCR product was re-amplified to add 29 bps of the trimerization motif, which contains the internal *HindIII* restriction site, using CD40L-2 and CD40L-R primers and to generate Δ FCD40L. Finally, both fragments, SNP Δ F and Δ FCD40L, were gel purified, digested with *HindIII*, ligated and PCR-amplified using NP-3 and CD40L-R primers. Full length SNPFC40L fragment was then gel purified, digested and inserted into the tAd vector. To generate tAd-NP40L, fusion gene was amplified from tAd-SNP40L using NP-1 and CD40L-R primers, and inserted into the tAd vector. Cloning was confirmed by DNA sequencing and restriction digestion.

3.4.5.2 Generation of rAd constructs

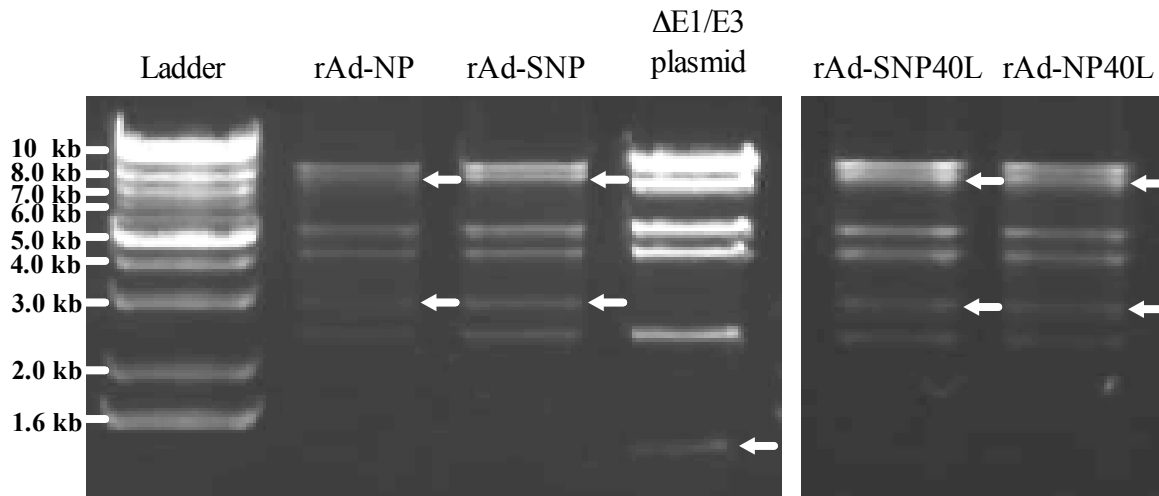
All transfer vectors containing the NP, SNP, NP40L or SNP40L or an empty transfer vector were *PmeI*-linearized and co-transformed with a Δ E1/E3 Ad5-backbone containing plasmid into *E. coli* cells (BJ5183) for homologous recombination. Recombinant plasmids were screened using *BstXI* restriction enzyme digestion according to the manufacturer's instructions (Figure 3.5). Positive plasmids were transfected into 70% confluent QBI-HEK 293A packaging cell line grown in complete DMEM with 5% FBS for generation of rAds using FuGENE 6 Transfection Reagent. Recombinant adenoviruses were isolated upon plaque formation 10–14 days after transfection and amplified to high titers in QBI-HEK 293A cells. Recombinant viruses were concentrated, titrated using Adeno-X Rapid Titer Kit (Clontech Laboratories Inc., Mountain View, CA, USA) and stored in PBS at -80 °C.

Figure 3.5

Confirmation of rAd generation by restriction digestion

The different potential recombinant plasmids after homologous recombination were digested with BstXI enzyme and resolved on a 1% agarose gel. Bands from potential clones were compared to the pattern observed in $\Delta E1/E3$ Ad5-backbone plasmid according to the manufacturer's instructions. Potential recombinant plasmids which show a shift in band size, appearance of new band or disappearance of a band (arrows) were selected and confirmed by sequencing. Positive clones were used to generate recombinant adenoviruses in QBI-HEK 293A packaging cell line. Clones shown are representative of the obtained positive recombinant adenoviruses plasmids.

Figure 3.5



3.4.6 Protein expression and secretion from rAds in cell culture

Generated rAds were used to infect confluent NIH-3T3 cells in 6-well plate at a MOI of 100. Forty-eight hours later, supernatants from rAd-infected cells were collected and immunoprecipitated with polyclonal anti-NP antibodies using Pierce Crosslink Magnetic IP/Co-IP Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Also, infected cells were washed thrice with cold PBS and lysed using SDS-lysis buffer. Protein expression and secretion from the transgenes were confirmed by Western blot using rabbit polyclonal anti-NP antibodies (Figure 3.3 B & C).

3.4.7 Western blotting

Samples were mixed with equal volume of 2X protein loading buffer and boiled for 10 minutes. Proteins were fractionated on 8 or 12% polyacrylamide gel in SDS-PAGE, followed by transferring the proteins to polyvinylidene difluoride (PVDF) membrane (Milipore Ltd., Etobicoke, ON, Canada). The membrane was washed in 1X tris buffered saline with 0.1% Tween-20 (TBS-T) for 10 minutes and blocked overnight with 5% skim milk in TBS-T. Membranes were washed for 1 hour at 10-minute intervals in TBS-T. Subsequently, the membrane was incubated with rabbit polyclonal anti-NP antibodies at (1:50,000) for 1 hour at RT. This was followed by incubation with HRP-conjugated goat anti-rabbit IgG (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada) at a dilution of 1/2000 for 1 hour at RT. The detection was carried out using the Immobilon Western chemiluminescent HRP substrate kit (Milipore Ltd., Etobicoke, ON, Canada). The membrane was exposed on Kodak film and developed using the Kodak XOMAT development machine.

3.4.8 Animal Experiments

3.4.8.1 Mice

Six to eight-week-old female BALB/c mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA). Eight to ten-week old female C57BL/6J, B6.129S2-Cd4tm1Mak/J (CD4 deficient; CD4^{-/-}), B6.129S2-Cd8atm1Mak/J (CD8 deficient; CD8^{-/-}) and B6.129S2-Cd40lgtm1Imx/J (CD40L deficient; CD40L^{-/-}) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). 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.

3.4.8.2 Immunization

Mice were subcutaneously immunized with plasmid DNA alone, plasmid DNA prime/rAd boost or rAd alone. For plasmid DNA immunization, mice were injected with 4 doses of 100 µg of either pNP or pcDNA dissolved in 100 µl PBS. Doses were given on days 0, 14, 28 and 42. For plasmid DNA prime/rAd boost, mice which received 3 doses of pNP or pcDNA were boosted with 1×10^9 pfu of either rAd-NP or rAd-Control on day 42, respectively. For rAd only regimen, mice were immunized with one or two doses of 1×10^9 pfu in 200 µl PBS of each rAd construct on days 0 and 28.

3.4.8.3 Virus challenge

On day 56 after DNA plasmid, DNA plasmid prime/rAd boost or two doses of rAd immunization, Balb/c were intranasally challenged with 25 μ l (10 x LD₅₀) of mouse adapted influenza A/Puerto Rico/8/34(H1N1) virus diluted in PBS. In some experiments, Balb/c mice were challenged 4 weeks or 4 months after a single dose of rAd immunization.

CD4^{-/-}, CD8^{-/-} and CD40L^{-/-} mice were intranasally challenged with 5 x LD₅₀ of the virus 4 weeks post-secondary immunization. After challenge, mice were weighed daily and clinically monitored for signs of illness for 14 days. Clinical scoring was performed according to the following grading scale: healthy (0); barely ruffled fur (1); ruffled fur but active (2); ruffled fur and inactive (3); ruffled fur, inactive and hunched (4); dead (5) as previously described (Walzl et al., 2000).

3.4.8.4 Samples

Mice were bled via retro-orbital plexus under diethyl ether anesthesia on days 0 (before immunization) and 14, 28, 42 and 56 after immunization. Serum was obtained by centrifugation of blood at 5,000 x g for 5 minutes and stored at -80 °C for antibody analysis by ELISA. For cellular immune response analysis, at indicated time points (post immunization), immunized mice were euthanized by cardiac puncture and cervical dislocation. Spleens and draining inguinal lymph nodes (LNs) were aseptically isolated and teased apart between the frosted ends of two sterile glass microscopic slides in complete RPMI 1640 medium with 10% FBS. Cell suspension was passed twice through a 70 μ m cell strainer (BD Falcon, Chelmsford, MA, USA) using the rubber end of a sterile plunger from a one cc tuberculin syringe in complete RPMI 1640 medium with 10% FBS to obtain a single cell suspension. For tissue viral titration, lungs were harvested from mice 6 days post-

challenge and stored at -80 °C until use. Frozen tissues were then thawed and homogenized in 0.5 ml of cold PBS and used for viral titration by plaque assay in MDCK cells as described in section 2.4.2.

3.4.8.5 CD8⁺ T cells adoptive and serum passive transfer

CD8⁺ T cells were purified from splenocytes obtained from rAd immunized Balb/c mice by negative selection using the Dynal ® Mouse CD8 Negative Isolation Kit (Life Technologies Inc., Burlington, ON, Canada). The purity of CD8⁺ T cells was determined using Pe-conjugated anti-mouse CD8 α (clone 53-6.7) and Pe-Cy7-conjugated anti-mouse CD3 (clone 145-2C11); they were found to be >95% pure. Naïve Balb/c mice were injected intravenously via the tail vein with 1×10^7 CD8⁺ T cells in 100 μ l PBS using a 27 gauge needle. Three days later, they were intranasally challenged with 10 x LD₅₀ of influenza A/Puerto Rico/8/34(H1N1) virus. In another experiment, Naïve Balb/c mice were injected intraperitoneally with 300 μ l of donor serum from Balb/c mice immunized with the different rAd constructs on days -3 to +1 relative to infection. On day 0, mice were intranasally challenged with 10 x LD₅₀ of influenza A/Puerto Rico/8/34(H1N1) virus. Lungs were harvested from mice 6 days post-challenge, homogenized and used for viral titration by plaque assay in MDCK cells as described in section 2.4.2.

3.4.9 Anti-NP antibodies measurement by ELISA

The end-point titers of anti-NP antibodies (total IgG, IgG1, IgG2a, IgG2b, IgG2c or IgM) in serum samples collected from immunized mice were determined by ELISA as described in section 2.4.5 with minor modifications. Briefly, 96-well plates were coated with 100 μ l/well of 4 μ g/ml of purified rNP. Plates were washed with PBS-T and blocked with

200 µl blocking buffer for 1 hour at 37 °C. After washing, 100 µl/well of each serum sample from immunized mice at the indicated time points were added in a two-fold serial dilution starting from 1:100 or 1:1000 dilution and incubated at 37 °C for 1 hour. After washing, appropriate HRP-conjugated goat anti-mouse antibodies (anti-total IgG, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG2c or anti-IgM) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) were added at 1:2000 dilution for 1 hour at 37 °C. IgG2a were detected in Balb/c mice, while IgG2c isotype was detected in C57BL/6J; I refer to IgG2c as IgG2a/c as previously described (Heer et al., 2007). After 6 washes, TMB substrate was used colorimetric development and the absorbance was read at 450 nm using a Synergy™ 2 Multi-Mode Microplate Reader. End-point antibody titers were expressed as the reciprocals of the final detectable dilution with a cut-off defined as the mean of pre-bleed samples plus three standard deviations.

3.4.10 Cell-mediated cytotoxicity assay

NP-specific cell-mediated cytotoxicity was conducted using lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, 3×10^7 splenocytes from immunized mice were re-stimulated *in vitro* with 5 µg/ml of synthetic NP MHC class-I restricted peptide (TYQRTRALV) (restricted to H-2Kd) in complete RPMI 1640 medium supplemented with 50 µM 2-β-mercaptoethanol and 1 µg/ml IL-2 to generate effector CTLs. After 5 days of culture at 37 °C, NP-specific cell-mediated cytotoxic activity was measured by LDH release in supernatant from effector and target cells co-cultured at different effector:target ratios for 4 hours at 37 °C. Target cells were P815 cells (H-2Kd) pulsed with 5 µg/ml of the synthetic

NP peptide in complete DMEM media with 10% FBS. The percentage of specific lysis was calculated as (experimental LDH release – effector spontaneous LDH release – target spontaneous LDH release) / (maximum LDH release – target spontaneous LDH release) x 100. Effector or target cells incubated in medium alone and with medium plus 5% Triton X-100 were used to determine spontaneous and target maximum release, respectively. Background lysis of unpulsed P815 cells incubated with effectors was subtracted from lysis from pulsed target cells.

3.4.11 CD8⁺ T cells intracellular cytokine staining

CD8⁺ T-cell IFN- γ , TNF- α and IL-2 responses were evaluated post-rAd immunizations as described (Hashem et al., 2012). Briefly, splenocytes (1×10^6) were cultured in complete RPMI 1640 medium with 10% FBS in the presence of 5 $\mu\text{g/ml}$ of synthetic NP MHC class-I restricted peptide (TYQRTRALV) (restricted to H-2Kd) for *ex vivo* restimulation and 1 $\mu\text{g/ml}$ Golgiplug (eBioscience, San Diego, CA, USA) to inhibit cytokine secretion for 6 hours. Stimulated cells were then washed twice with PBS with 5% FBS (FACS buffer) and stained with APC-eFluor 780-conjugated anti-mouse CD8a (clone 53-6.7) for 30 minutes at 4 °C. Cells were washed thrice with FACS buffer, fixed and permeabilized using ice cold BD Cytofix/Cytoperm for 20 minutes in the dark and washed with cold 1X BD perm/wash buffer. Cells were then stained for intracellular cytokines using the following antibodies: PerCP-Cy5.5-conjugated anti-mouse IFN- γ (clone XMG1.2); Pe-conjugated anti-mouse TNF- α (clone MP6-XT22) and Pe-Cy7-conjugated anti-mouse IL-2 (clone JES6-5H4) for 30 minutes at 4 °C in 1X BD perm/wash buffer. Cells were washed with cold 1X BD perm/wash buffer followed by fixation in 1% formaldehyde fixative. A BD LSRII flow cytometer was used for data acquisition, and analysis was completed with Flow

Jo, Version 8.8.4 (Tree Star Inc, Ashland, OR, USA). Unstained cells and single stained compensation beads (BD Biosciences, San Jose, CA, USA) were used as controls for background fluorescence and false positives due to fluorochrome bleeding. Results for IFN- γ , TNF- α and IL-2 were calculated as percent of CD8⁺ T cells. Polyfunctional CD8⁺ T cells were analyzed using Boolean gate analysis as previously described (Commandeur et al., 2011).

3.4.12 B cells flow-cytometry

Single-cell suspensions of LNs or spleens from immunized mice were washed twice with FACS buffer and stained with Pe-Cy7-conjugated anti-mouse CD19 (clone 1D3); APC-eFluor780-conjugated anti-mouse CD45R/B220 (clone RA3-6B2); PerCP-conjugated anti-mouse IgG; APC-conjugated anti-mouse IgD (clone 11-26); Alexa Flour 488-conjugated anti-mouse Ly77/GL7 (clone GL-7) and Pe-conjugated anti-mouse Syndecan-1/CD138 (clone 281-2) for 30 minutes at 4 °C. In another experiment, cells were stained with Pe-conjugated anti-mouse CD19 (clone 1D3); FITC-conjugated anti-mouse B7.1/CD80 (clone 16-10A1) and Pe-Cy5-conjugated anti-mouse CD40 (clone 1C10). Stained cell samples were washed twice and fixed with 1% paraformaldehyde. A BD LSRII flow cytometer was used for data acquisition and analysis was completed with Flow Jo, Version 8.8.4 as described above.

3.4.13 Data analysis

One-way or two-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 software (San Diego, CA).

3.5 RESULTS

3.5.1 Immunization with rAd-NP induces a strong antibody response in mice

To compare the different NP-based genetic immunization regimens shown in Figure 3.6, I first analyzed NP-specific total IgG titers in serum as a surrogate marker for immune response induction. Surprisingly and in contrast to previously reported findings (Pertmer et al., 1996), immunization with the two doses of pNP failed to induce any antibody response in mice (Figure 3.7, days 14 and 28). It was only after a third dose of pNP that mice started to show marginally detectable levels of antibodies in circulation (Figure 3.7, day 42). A fourth dose of pNP was able to elicit significant levels of antibodies compared to control groups, but boosting with rAd-NP generated at least a two-fold increase in antibodies on day 56 (Figure 3.7, day 56). On the other hand, mice immunized with rAd-NP showed significantly elevated levels of NP-specific antibodies even after a single dose (Figure 3.7, days 14 & 28). Immunization with rAd-NP induced significant antibodies response at all time points compared to all groups except for mice from pNP prime/rAd-NP boost group (Figure 3.7, day 56). These results demonstrate that immunization with rAd-NP alone is as effective as pNP prime/rAd-NP boost but more efficient than pNP in inducing immune response. Most importantly, two doses of rAd-NP were sufficient to elicit strong humoral response.

3.5.2 Immunization with rAd-NP confers complete protection against influenza

Next, I evaluated the different immunization regimens for their ability to induce protection against influenza A/Puerto Rico/8/34(H1N1) virus challenge. Mice immunized with either pNP prime/rAd-NP boost or two doses of rAd-NP were completely protected after viral challenge, whereas mice which received pNP or single-dose rAd-NP only showed

Figure 3.6

Schematic representation of immunization strategies

Balb/c mice were immunized with 4 doses of plasmid DNA alone (pNP or empty vector control), 3 doses of plasmid DNA (pNP or empty vector control) and boosted with rAd vectors (rAd-NP or rAd-Control, respectively) or with 2 doses of rAd vectors at the indicated time points. All mice were challenged on the indicated day.

Figure 3.6

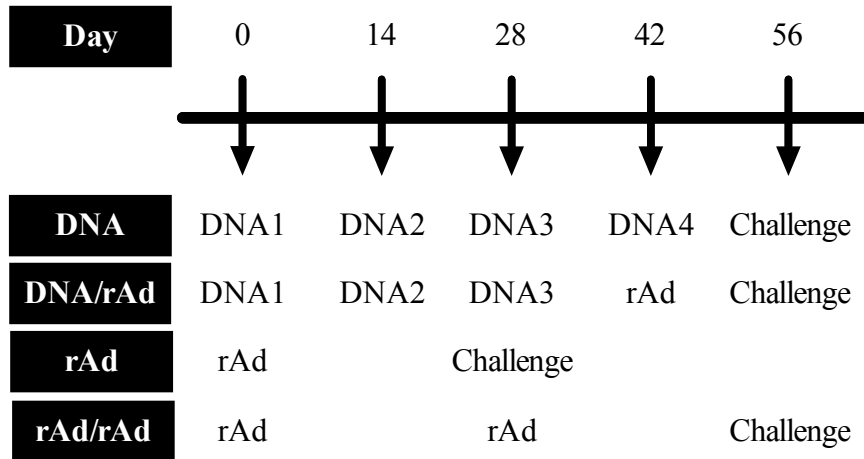
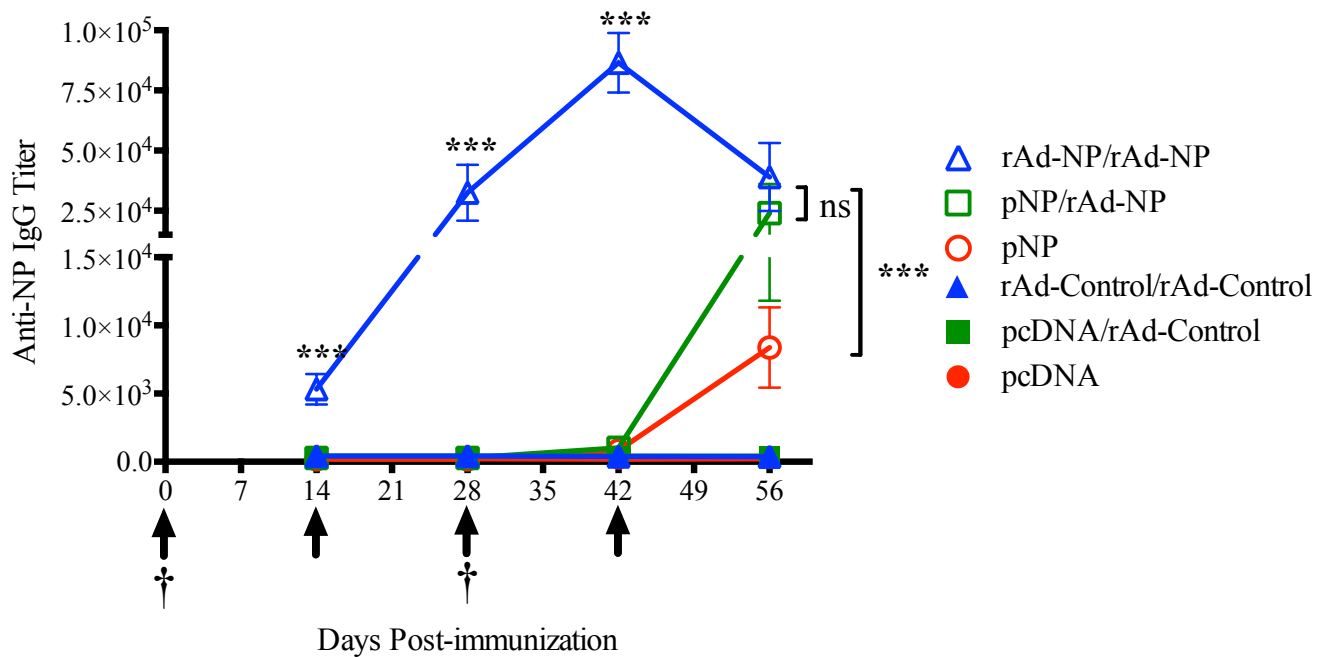


Figure 3.7

Immunization with rAd-NP induces strong antibody response in mice

End-point NP-specific antibody titers were determined from sera collected from immunized mice every two weeks by ELISA. Data are shown as mean end-point titer \pm s.e.m. of the total IgG from two experiments, with $n = 8$ mice in each experiment. \uparrow and \dagger indicate DNA and rAd immunization time points, respectively. *** represents $P < 0.001$ (one-way ANOVA with Bonferroni post-test).

Figure 3.7



survival rates of 50% and 40% respectively (Figure 3.8 A). Protected animals also significantly maintained their weight, in contrast to mice from pNP or single rAd-NP groups which lost up to 20% of their total body weight within 8 days after challenge (Figure 3.8 B). All animals in the control groups suffered from severe clinical symptoms and weight loss (Figure 3.8 B) and died between 5-9 days after viral challenge (Figure 3.8 A). As expected, mice immunized with either pNP prime/rAd-NP boost or two rAd-NP doses showed significantly lower virus titers in lungs on day 6 post-challenge compared to controls as well as to pNP and single rAd-NP groups (Figure 3.8 C). Specifically, they showed a 40-fold reduction in viral titers compared to control groups and a 10-fold reduction compared to pNP and single rAd-NP groups. On the other hand, immunization with pNP or one rAd-NP dose only reduced viral titers by 4 fold (Figure 3.8 C). Although pNP prime/rAd-NP boost strategy is as effective clinically as two rAd-NP doses (Figure 3.8), the former strategy requires 3 pNP injections, followed by another boost of rAd-NP. Thus, these results indicate that immunization with two doses of rAd-NP alone is the simpler procedure, and provides complete protection against influenza virus similar to pNP prime/rAd-NP boost.

3.5.3 Single dose of rAd-SNP40L provides durable protection against challenge

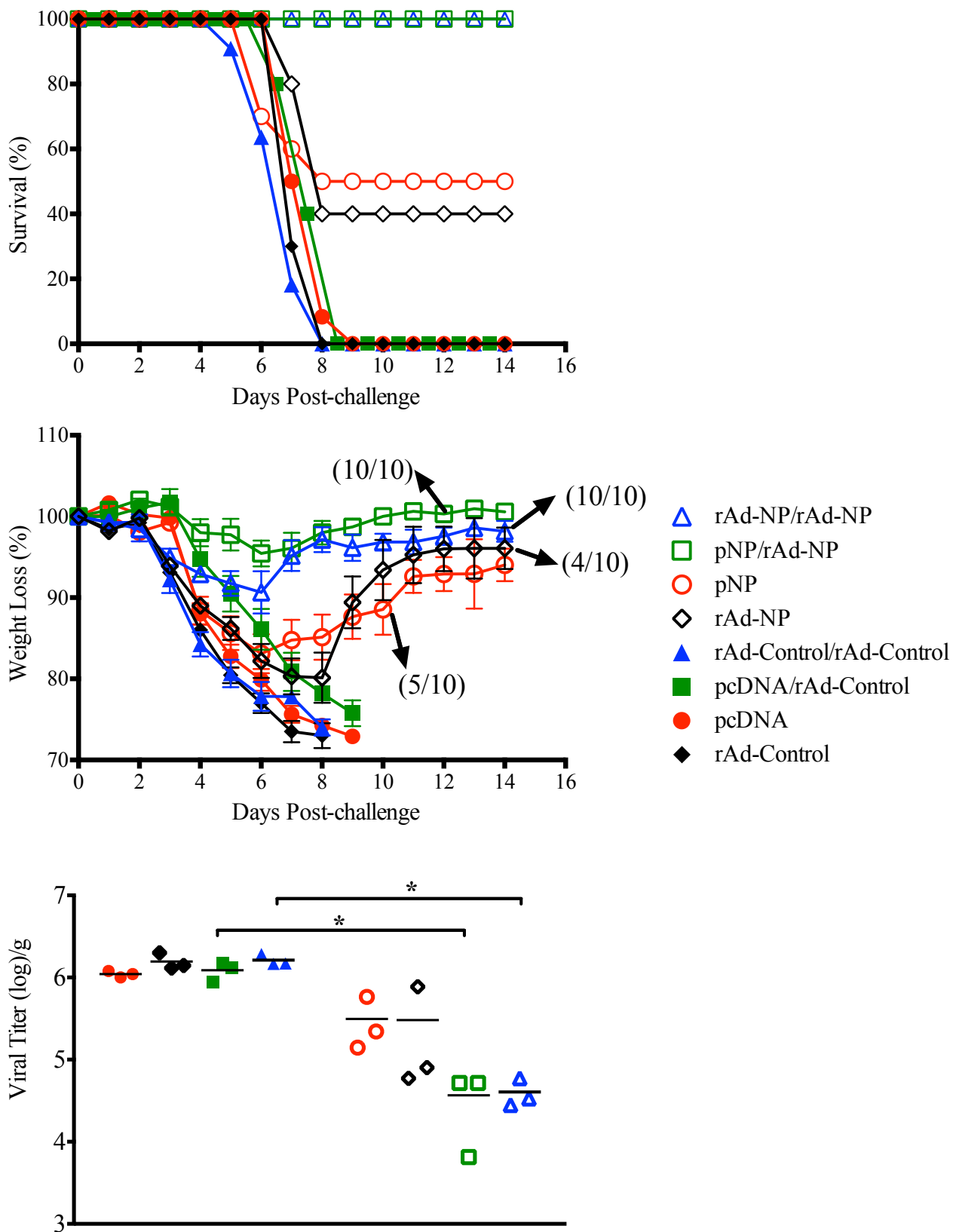
Having observed that two doses of rAd-NP are sufficient to provide complete protection against lethal influenza challenge, I determined whether using CD40L as an adjuvant and targeting molecule could enhance the protective efficacy of influenza NP. To this end, I generated rAd vector expressing influenza A NP fused to a secretion signal (S) at the N-terminus and trimerized form of murine CD40L ectodomain at the C-terminus (rAd-SNP40L) (Figure 3.3 A). Recombinant Ad-SNP40L can infect various cell types *in vivo*; thus the infected cells would secrete NP-CD40L fusion protein which will be targeted to CD40

Figure 3.8

Two doses of rAd-NP protect mice from viral challenge

(A) Survival of immunized mice after virus challenge. Data are presented as a percentage of surviving animals at each time point compared to the initial number of animals in each group. **(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. **(C) Lung viral titers.** Viral titer in lungs was measured on day 6 post-challenge as described in materials and methods. There is no statistical difference between groups received either pNP prime/rAd-NP boost or two doses of rAd-NP. Data are shown from one experiment with $n = 10$ mice per treatment group for A and B and $n = 3$ per group for C. * represents $P < 0.05$ (one-way ANOVA with Bonferroni post-test).

Figure 3.8



receptors on APCs. As controls, I generated rAd encoding a non-secreted form of NP-CD40L (rAd-NP40L) and rAd expressing non-targeting secreted-NP (rAd-SNP). Also, I included rAd-NP as well as an “empty” control vector (rAd-Control).

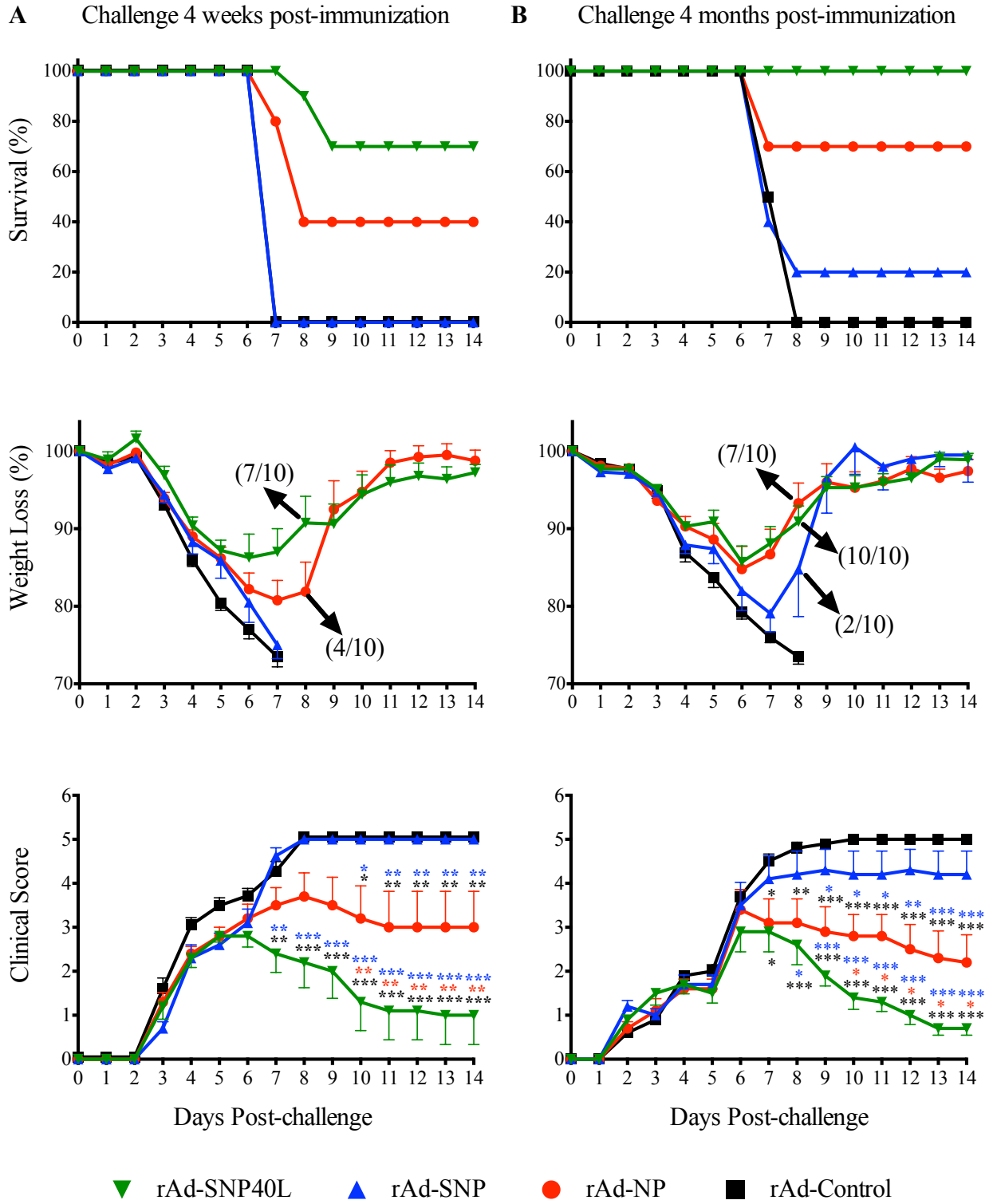
Initial pilot experiments showed that rAd-NP40L is unable to confer any protection nor to induce any detectable antibody response compared to other constructs, thus this construct was excluded from all the animal protection studies as well as from the subsequent investigation of immunological mechanisms (see Discussion). Noticeably however, when mice were challenged 4 weeks post-single immunization, rAd-SNP40L substantially enhanced survival rate by 30% and significantly decreased morbidity in challenged mice compared to rAd-NP which showed 40% survival rate only (Figure 3.9 A). Importantly, rAd-SNP40L immunized mice were completely protected against influenza when challenged 4 months post-immunization compared to rAd-NP which resulted in 70% protection; indicating an overall enhanced recall responses in these mice (Figure 3.9 B). Interestingly, none of the mice immunized with rAd-SNP survived when challenged 4 weeks post-immunization (Figure 3.9 A) and only 20% of the rAd-SNP-immunized mice were protected when challenged 4 months post-immunization (Figure 3.9 B). Furthermore, I found that rAd-SNP immunization only provided 50% protection even after two doses (Supplementary Figure S1, see Appendix), indicating that secreting influenza NP (without CD40L targeting) might not be an ideal strategy for vaccination. Overall, these results highlight the strength of NP-based recall responses and particularly confirm that targeting antigens via CD40L could provide enhanced rapid and long-lasting protective immune response in mice with a single dose.

Figure 3.9

Single dose of rAd-SNP40L confers enhanced protection against infection in mice

Survival curves, body weight loss and clinical score of Balb/c mice immunized with a single dose of the indicated rAd constructs and challenged with a lethal dose ($10 \times LD_{50}$) of the mouse adapted influenza A/Puerto Rico/8/34(H1N1) either 4 weeks **(A)** or 4 months **(B)** post-immunization. Data are presented as a percentage of surviving animals at each time point compared with the initial number of animals in each group. Weight loss is expressed as percentage of animal weight at each time point from their initial body weight. Clinical score was calculated as indicated in materials and methods. Data are shown from one experiment with $n = 10$ mice per treatment group in A and B. *** represents $P < 0.001$, ** represents $P < 0.01$ and * represents $P < 0.05$ (two-way ANOVA with Bonferroni post-test).

Figure 3.9



3.5.4 CD40L preferentially elicits T_H1-skewed NP-specific antibodies

I next investigated the effects of using CD40L as an adjuvant and targeting molecule on the induction of NP-specific antibody isotypes. To this end, mice were immunized with the generated rAd constructs and serum samples were collected every 2 weeks post-primary or secondary immunization to analyze the anti-NP antibody response. Immunization of mice with rAd-NP resulted in significant induction of NP-specific antibodies from all isotypes in both Balb/c (Figure 3.10 A & B) and C57BL/6J mice (Figure 3.11 A & B) 4 weeks post-primary or secondary immunization. However, rAd-SNP induced up to one fold higher NP-specific IgG1 antibodies and markedly reduced the titers of IgG2a and IgG2b isotypes by 3-6 and 4-10 folds, respectively, in both mouse models. It is of note that targeting the secreted NP to CD40 via CD40L (rAd-SNP40L) altered the isotypes of NP-specific antibodies by preferentially inducing IgG2a/c and IgG2b, and significantly reducing IgG1 levels by 10-20 fold and 5-8 fold compared to rAd-NP in Balb/c (Figure 3.10 A & B) and C57BL/6J mice (Figure 3.11 A & B), respectively. As expected, mice vaccinated with empty rAd-Control failed to mount any anti-NP antibody response. Similar results were also obtained from samples collected two weeks post-primary or secondary immunization (Supplementary Figures S2 and S3, see Appendix).

Regardless of the genetic background of Balb/c and C57BL/6J mice which could affect the phenotype of the induced immune response (Watanabe et al., 2004), targeting NP via CD40L always elicited higher levels of IgG2b and IgG2a, and very low levels of IgG1 indicating a T_H1-bias. Indeed, calculating the IgG1:IgG2a ratio clearly showed that secreted NP induces a T_H2-skewed response with a ratio of 6-6.3 and 3.5-4.6 in Balb/c and C57BL/6J mice, respectively (Figure 3.10 C and Figure 3.11 C).

Figure 3.10

CD40 targeting preferentially induces T_H1 antibody isotypes against influenza NP in Balb/c mice

Antigen specific antibody titers at 4 weeks after priming (**A**) or boosting (**B**) are shown for IgG1, IgG2a and IgG2b isotypes. (**C**) IgG1:IgG2a ratio was measured at 4 weeks after priming and boosting to determine the type of immune response induced (T_H2 vs. T_H1). Numbers on columns indicate the mean ratio. Mice were boosted at 4 weeks post-primary immunization. Data are shown as mean titer \pm s.e.m. from two independent experiments, with n = 8-10 mice per treatment group in each experiment. *** represents P <0.001, ** represents P <0.01 and * represents P <0.05 (one-way ANOVA with Bonferroni post-test).

Figure 3.10

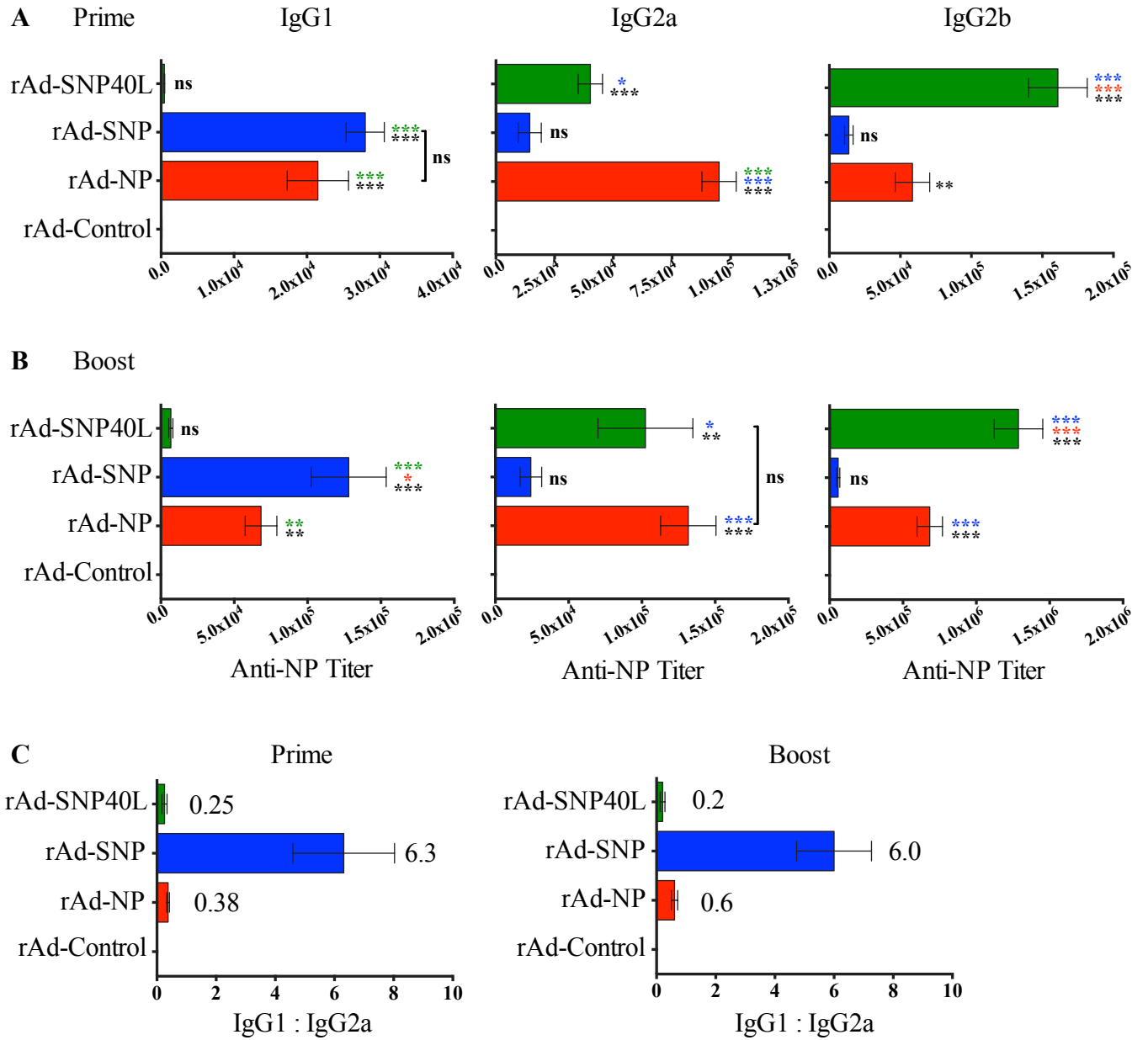
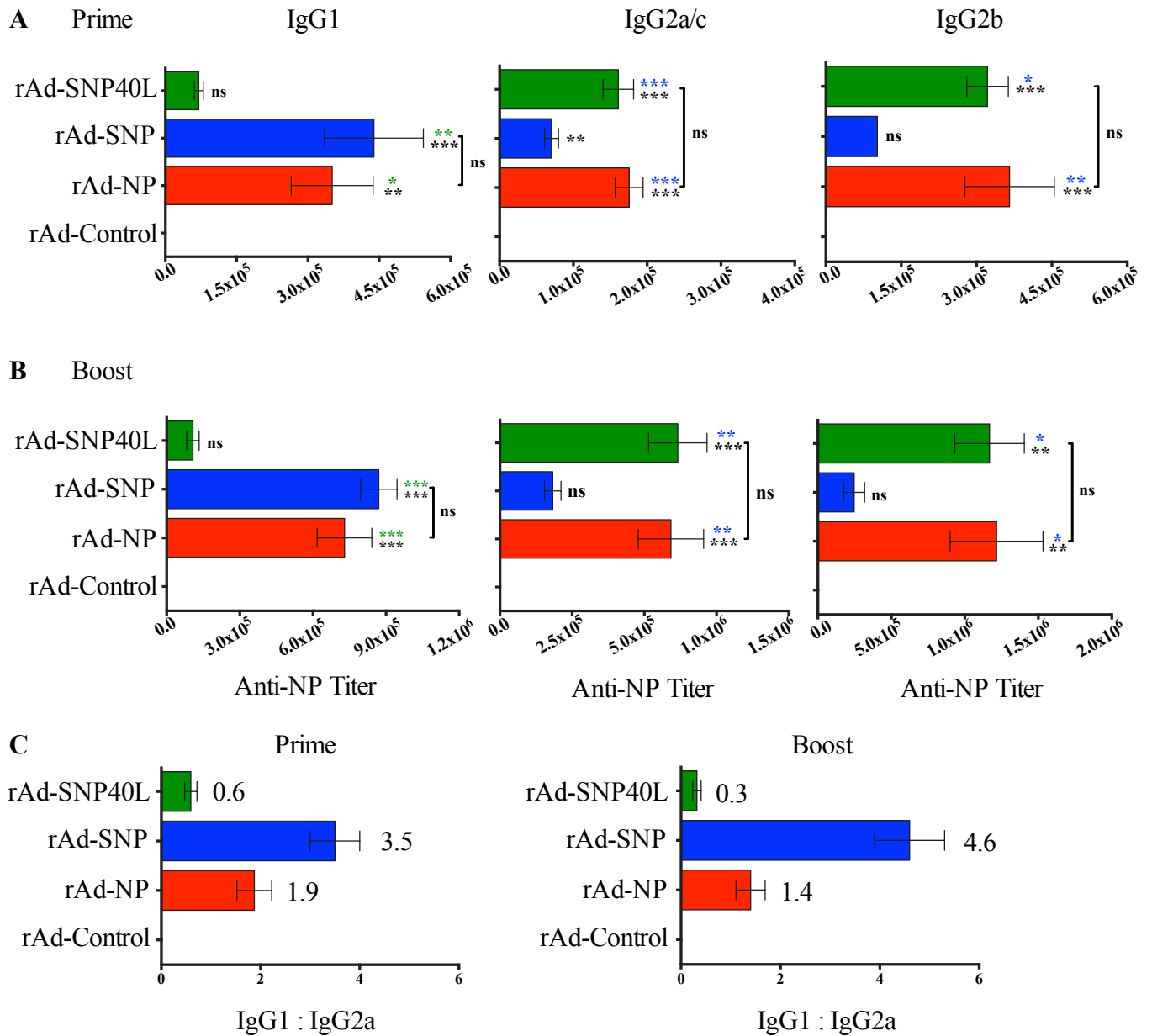


Figure 3.11

CD40 targeting preferentially induces T_H1 antibody isotypes against influenza NP in C57BL/6J mice

Influenza NP specific antibody titers at 4 weeks after priming (**A**) or boosting (**B**) are shown for IgG1, IgG2a/c and IgG2b isotypes. (**C**) IgG1:IgG2a ratio was measured at 4 weeks after priming and boosting to determine the type of immune response induced (T_H2 vs. T_H1). Numbers on columns indicate the mean ratio. C57BL/6J mice were boosted at 4 weeks post-primary immunization. Data are shown as mean titer \pm s.e.m. from one experiment, with n = 4-5 mice per treatment group. *** represents $P < 0.001$, ** represents $P < 0.01$ and * represents $P < 0.05$ (one-way ANOVA with Bonferroni post-test).

Figure 3.11



3.5.5 CD40L activates B cells and enhances plasma cell generation in draining LNs and spleen

CD40L activates B cells upon ligation and induces upregulation of costimulatory molecules. I examined the expression levels of CD40 and CD80 markers on B cells in draining LNs isolated from the different groups. As shown in Figure 3.12 A, mice received rAd-SNP40L had higher levels of these activation markers post-primary and secondary immunization, indicating that immunization with rAd-SNP40L resulted in antigen targeted to CD40 receptors on B cells, which promoted their activation. Furthermore, immunization with rAd-SNP40L upregulated the plasma cell marker CD138 on B cells in both LN and spleen (Figure 3.12 B) to higher levels compared to other constructs. These CD138⁺ cells were B220⁺ and CD19⁺ typical of plasmablasts, confirming activation and differentiation of B cells into antibody-secreting cells (ASC) in local lymphoid organs.

3.5.6 CD40L induces early and persistent B-cell GC formation and Ig isotype-switched memory B cells in draining LNs

Due to the T_H1-skewed NP-specific antibody isotypes in circulation, I next determined whether immunization with rAd-SNP40L regulates GC formation and isotype-switching in draining LNs differently from the other constructs. I first noted that targeting influenza NP to CD40⁺ cells substantially altered the magnitude as well as the kinetics of NP-specific IgM antibodies in serum. As shown in Figure 3.13, NP-specific IgM antibodies in rAd-SNP40L immunized mice peaked by day 28, albeit to low titers, and declined to low or undetectable levels by day 42 in contrast to other groups which showed significantly higher titers up to 4 weeks post-secondary immunization.

Figure 3.12

CD40L upregulates B cell activation markers and induces plasma cells differentiation

(A) Draining inguinal LNs were excised post-primary or secondary immunization from Balb/c mice immunized with the indicated rAd constructs. CD19⁺ cells were then analyzed for surface expression of B cell activation markers, CD80 and CD40. **(B)** Plasma cell marker (CD138) expression level on B220⁺CD19⁺ cells in draining LNs and spleens. Histograms are representative from one of two independent experiments with n = 2-3 mice per experiment (4 lymph nodes were pooled from each individual mouse).

Figure 3.12

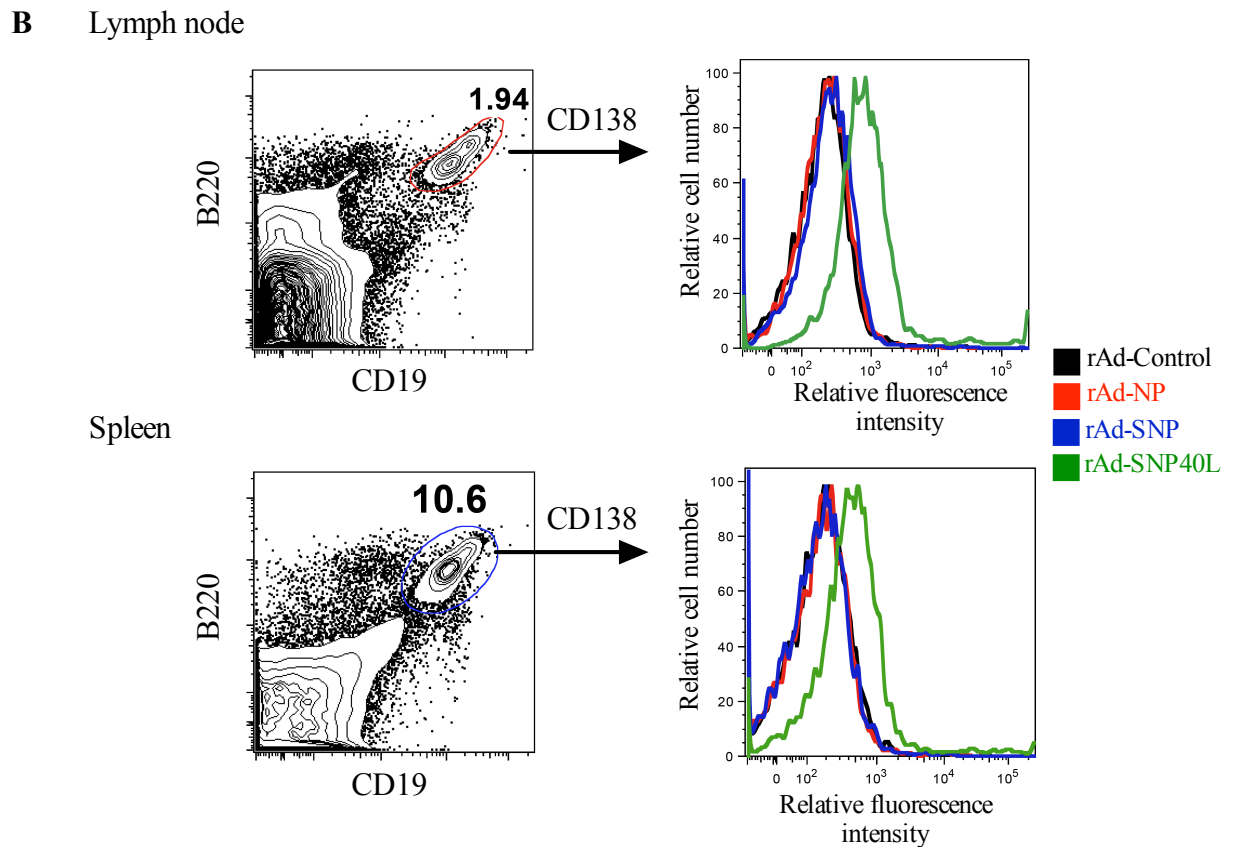
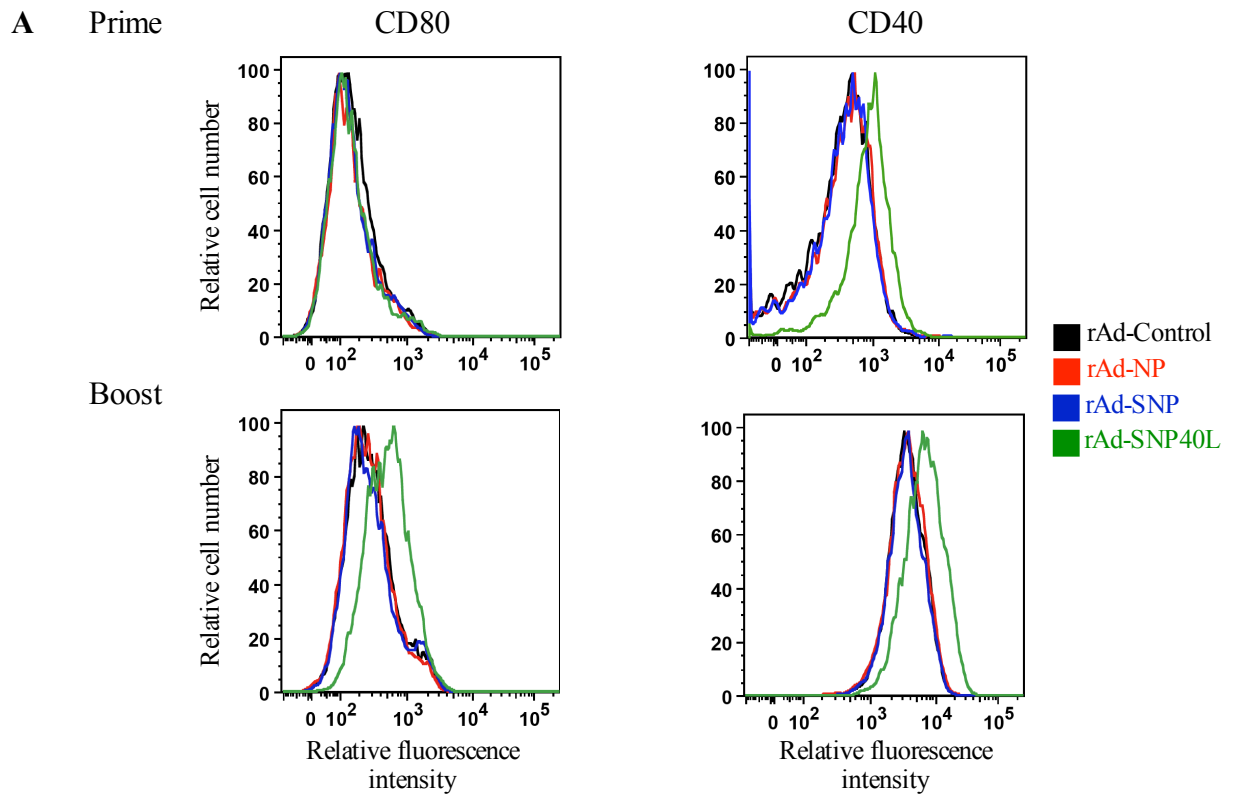
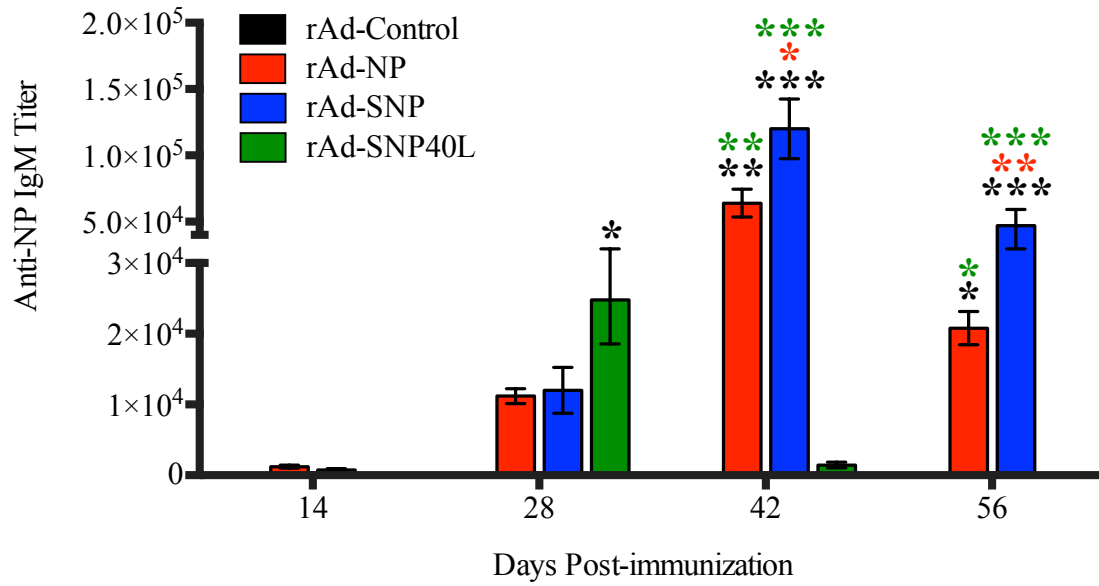


Figure 3.13

CD40L alters the kinetics and the magnitude of NP-specific IgM antibodies

Balb/c mice were immunized at day 0 and boosted 4 weeks later with the indicated rAd constructs. NP-specific IgM titers were determined every two weeks after priming and boosting. Data are shown as mean titer \pm s.e.m. from two independent experiments, with n = 8-10 mice per treatment group in each experiment. *** represents P <0.001, ** represents P <0.01 and * represents P <0.05 (one-way ANOVA with Bonferroni post-test).

Figure 3.13



These data suggested that immunization with rAd-SNP40L might have caused accelerated Ig isotype-switching and memory B cell induction.

Analysis of the GC marker GL7 on B cells in draining LNs collected from immunized mice revealed no differences in GL7⁺ B GC cells between the different groups until day 14 (Figure 3.14 A & B). By day 28, only rAd-SNP40L immunized mice started to show significantly higher frequencies of GL7⁺CD19⁺ GC B cells (25.9% of B cells) compared to other groups which showed low levels of total GL7⁺CD19⁺ GC cells (3.46-5.55% of B cells). Remarkably, it was not until day 56 that mice in remaining groups started to show an increase in GC formation, although at lower levels compared to rAd-SNP40L immunized mice (Figure 3.14 A & B). Further analysis also revealed a significantly enhanced numbers of isotype-switched (IgG⁺IgD⁻GL7⁺) memory B cells at day 28 (5.6% of B cells) and day 42 (7.8% of B cells) in the rAd-SNP40L immunized mice in contrast to other groups in which less than 1% of CD19⁺ cells showed isotype switching (Figure 3.14 C). By day 56, the number of isotype-switched memory B cells in rAd-SNP40L immunized mice was still higher than that in other groups (Figure 3.14 C). In fact, there was an evident overall upregulation of IgG and downregulation of IgD on B cells in LNs from mice immunized with CD40-targeted NP relative to other groups particularly post-secondary immunization (Figure 3.14 D). Collectively, these data strongly suggest that there was an early and persistent induction of GC formation as well as IgG⁺ memory B cells generation in mice immunized with rAd-SNP40L.

3.5.7 Enhancement of NP-specific cellular immune response by CD40L

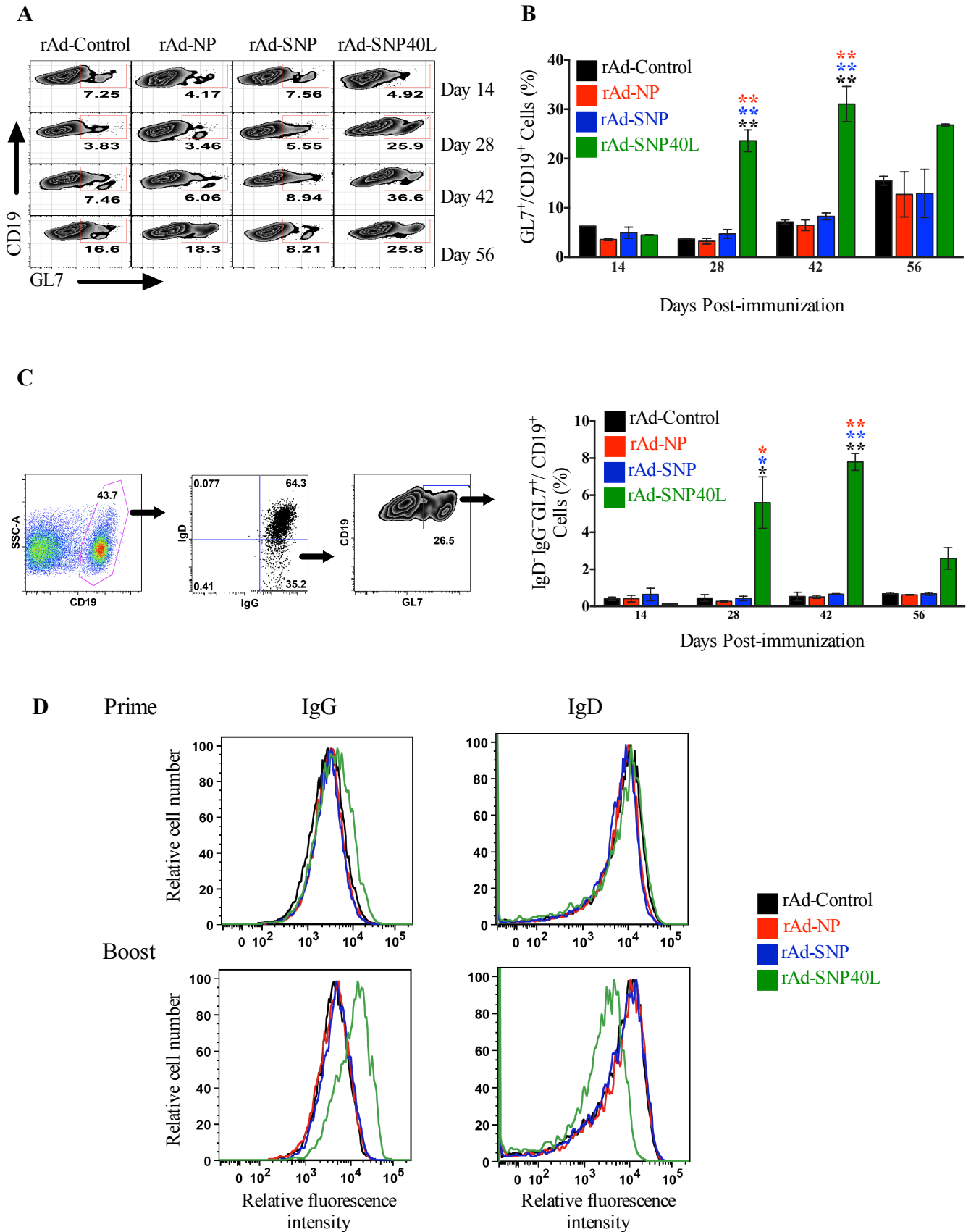
Polarization of the immune response towards a T_H1 phenotype using CD40L (Figure 3.10 and Figure 3.11) suggests an induction of CTL response. I next investigated the effects

Figure 3.14

CD40L induces persistent GC and isotype-switched B cells in draining LNs of immunized mice

Draining inguinal LNs were excised at indicated time points, and CD19⁺ cells were analyzed for expression of GL7. **(A)** Flow cytometry plots are representatives from one of two independent experiments. **(B)** Graphs represent frequencies of CD19⁺GL7⁺ B cells. **(C)** Following live lymphocytes gating, CD19⁺ cells were selected and gated on IgD⁻IgG⁺ isotype switched cells. CD19⁺IgD⁻IgG⁺ isotype switched B cells were analyzed for expression of GL7 to identify isotype-switched GC cells. Percentage of GL7⁺ cells and isotype-switched memory cells (IgD⁻IgG⁺GL7⁺) among CD19⁺ B cells were determined from 4 pooled draining LNs from each individual mouse per time point. Data are shown as mean ± s.e.m. from two independent experiments, with n = 2-3 mice per treatment group per time point in each experiment. *** represents P <0.001, ** represents P <0.01 and * represents P <0.05 (one-way ANOVA with Bonferroni post-test). **(D)** CD19⁺ cells were then analyzed for surface expression of IgG, IgD. Histograms are representatives from one out of two independent experiments with n = 2-3 mice per experiment (4 LNs were pooled from each individual mouse).

Figure 3.14



of CD40 targeting on NP-specific primary and secondary CD8⁺ T cell immune responses compared to other immunogens. Indeed, upon stimulation with the H-2Kd restricted immunodominant peptide of influenza NP, there was a significant enhancement of NP-specific IL-2, IFN- γ and TNF- α producing CD8⁺ T-cell frequencies in spleens from mice immunized with rAd-SNP40L at 4 weeks post-primary immunization compared to the rAd-Control immunized group (Figure 3.15). These cells were increased to significantly elevated levels upon secondary immunization in rAd-SNP40L immunized mice. As shown in Figure 3.16, the frequencies of CD8⁺ T-cell secreting IL-2, IFN- γ and TNF- α were higher by 3-5 fold compared to CD8⁺ T cells from other groups.

CD8⁺ T cells capable of producing multiple cytokines (polyfunctional CD8⁺ T cells) are a good indicator of a stronger cell-mediated immune response more than single cytokine-secreting CD8⁺ T cells (Darrah et al., 2007; Kannanganat et al., 2007; Almeida et al., 2007). To this end, I compared the levels of polyfunctional CD8⁺ T cells among the different groups. As shown in Figure 3.17 A & B, rAd-SNP40L induced significantly higher levels of NP-specific triple, double and single cytokine producing CD8⁺ T cells compared to other groups at 4 weeks post-primary and secondary immunization.

In order to measure effector functions of NP-specific CTLs, splenocytes isolated from vaccinated mice at different time points after immunization were tested for their ability to directly kill target cells *ex vivo* in a standard LDH release assay. As shown in Figure 3.17 C & D, targeting influenza NP via CD40L significantly enhanced NP-specific cell-mediated cytotoxicity of target cells (P815 cells pulsed with MHC-I restricted peptide) after primary and secondary immunization. Interestingly, enhancement of antigen-specific cell-mediated cytotoxicity was observed as early as two weeks post-primary immunization

Figure 3.15

CD40 targeted influenza NP enhances the frequencies of antigen-specific primary CD8⁺ T cell responses

Splenocytes were isolated from immunized Balb/c mice 4 weeks post-primary immunization. Cells from individual mice were stimulated *ex vivo* with synthetic NP peptide TYQRTRALV (restricted to H-2Kd) at 5 µg/ml for 6 hours in the presence of brefeldin-A (1 µg/ml). CD8⁺ T cells were then stained for intracellular IL-2, IFN-γ and TNF-α responses. (A, C, E) Flow cytometry plots are representative from one of two independent experiments. Graphs represent frequencies of (B) IL-2, (D) IFN-γ and (F) TNF-α producing CD8⁺ T cells 4 weeks post-primary immunization. Data are shown as mean ± s.e.m. from two independent experiments, with n = 2-3 mice per treatment group per time point in each experiment. *** represents P <0.001, ** represents P <0.01, and * represents P <0.05 (one-way ANOVA with Bonferroni post-test).

Figure 3.15

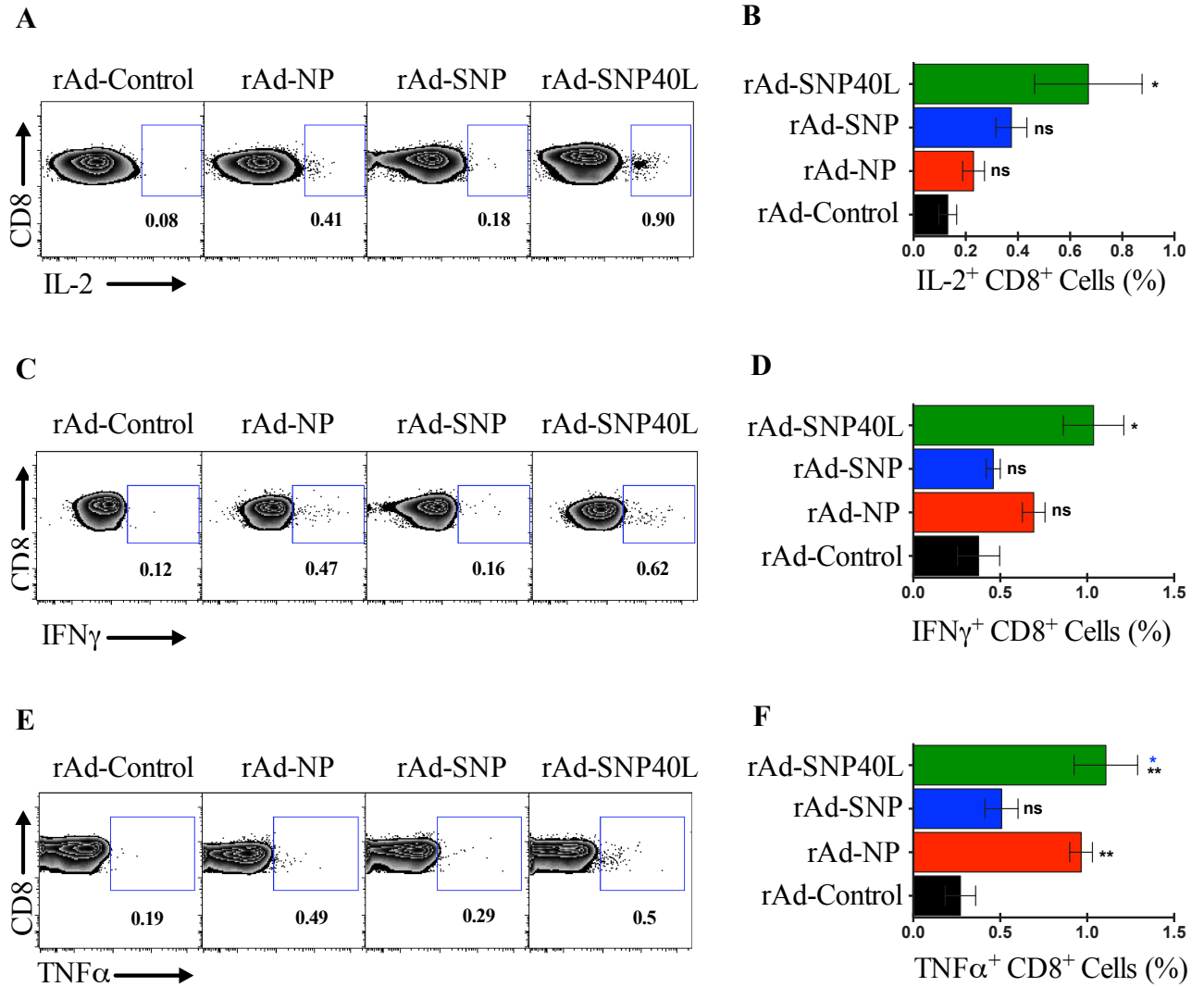


Figure 3.16

CD40 targeted influenza NP enhances the frequencies of antigen-specific memory CD8⁺ T cell responses

Balb/c mice were immunized on day 0 and boosted 4 weeks later with the indicated rAd constructs. Splenocytes were isolated from immunized mice 4 weeks post-secondary immunization. Cells from each individual mouse were restimulated *ex vivo* with synthetic NP peptide and stained for intracellular cytokines as described in Figure 3.15. (A, C, E) Flow cytometry plots are representative from one of two independent experiments. Graphs represent frequencies of (B) IL-2, (D) IFN- γ and (F) TNF- α producing CD8⁺ T cells 4 weeks post-boosting. Data are shown as mean \pm s.e.m. from two independent experiments, with n = 2-3 mice per treatment group per time point in each experiment. *** represents P <0.001, ** represents P <0.01, and * represents P <0.05 (one-way ANOVA with Bonferroni post-test).

Figure 3.16

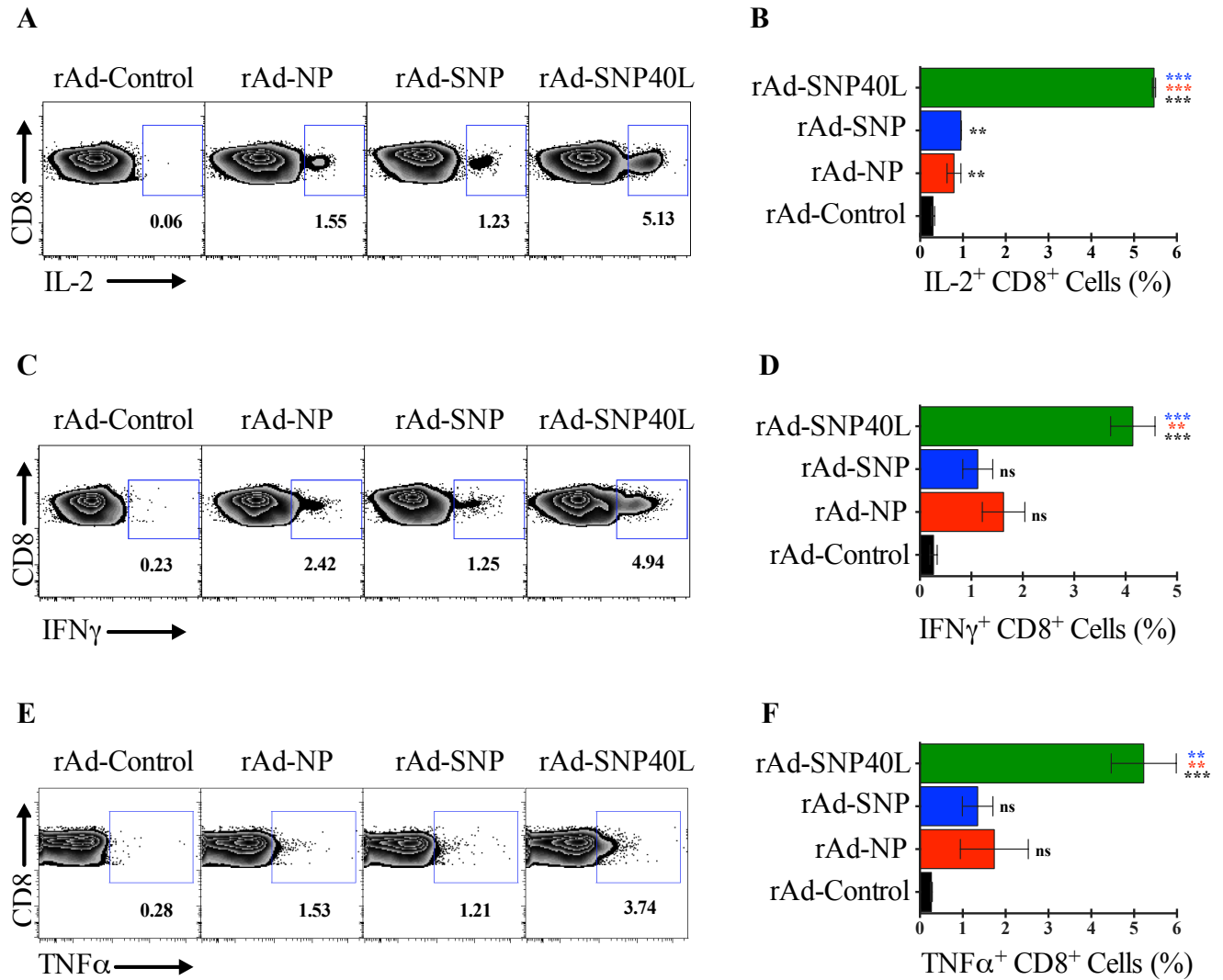
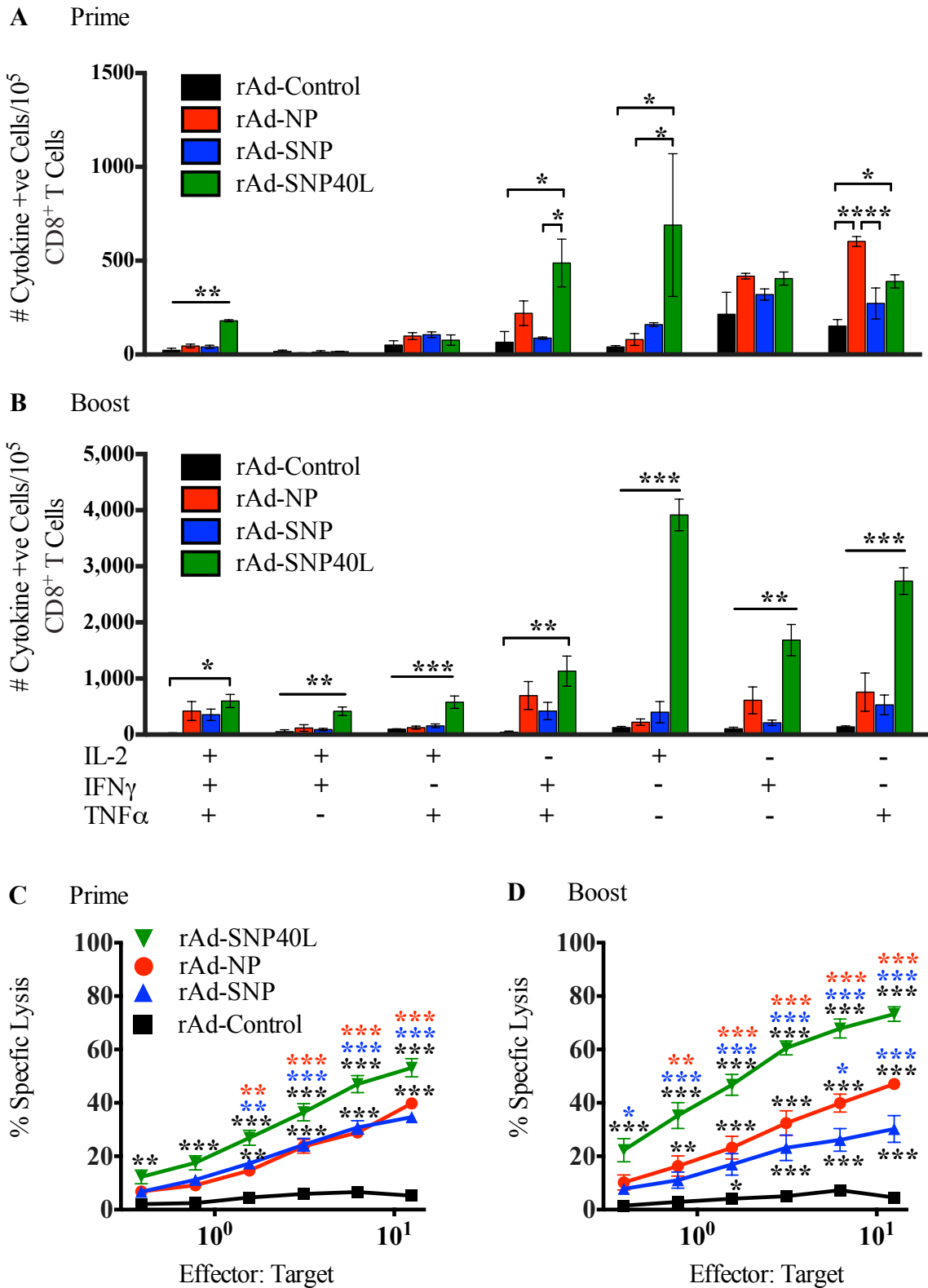


Figure 3.17

Immunization with rAd-SNP40L induces enhanced antigen-specific polyfunctional CD8⁺ T cell and cell-mediated cytotoxicity

(A & B) Splenocytes isolated from each individual immunized Balb/c mouse 4 weeks post-primary (A) or secondary (B) immunization were restimulated ex vivo with synthetic NP peptide and stained for intracellular cytokines as described in Figure 3.15. Data was analyzed on Flow Jo using Boolean gate analysis to determine the different single, double and triple-cytokine producing CD8⁺ T cells. Bar graphs represent frequencies of various combinations of cytokine producing cells normalized to represent cytokine producing cell numbers / 100,000 cells. (C & D) Approximately 3×10^7 splenocytes were isolated from immunized mice 4 weeks post-primary (C) or secondary (D) immunization. Cells from each individual mouse were cultured ex vivo with synthetic NP peptide TYQRTRALV (restricted to H-2Kd) at 5 µg/ml for 5 days to generate effector cells. NP specific cytotoxic activity was then measured using LDH release assay by co-culturing effector cells and P815 (H-2d) target cells pulsed with the same peptide at different Effector:Target ratios, and the percentage of specific lysis was calculated. Data are shown as mean ± s.e.m. from two independent experiments, with n = 2-3 mice per treatment group per time point in each experiment. *** represents P <0.001, ** represents P <0.01 and * represents P <0.05 (one-way ANOVA in A and B, and two-way ANOVA in C and D with Bonferroni post-test for both).

Figure 3.17



(Supplementary Figure S4, see Appendix). These results indicated that CD40L had not only improved CD8⁺ T cell responses qualitatively but also enhanced the quantity and functionality of CD8⁺ T cells as demonstrated by the significant increase in NP-specific polyfunctional CD8⁺ T cells and CTL responses. Taken together, these data confirm that targeting influenza NP to CD40 receptors skewed the immune response towards a T_H1 response and significantly boosted primary and memory cellular immune responses.

3.5.8 Both NP-specific CD8⁺ T cells and antibodies contribute to the enhanced protection

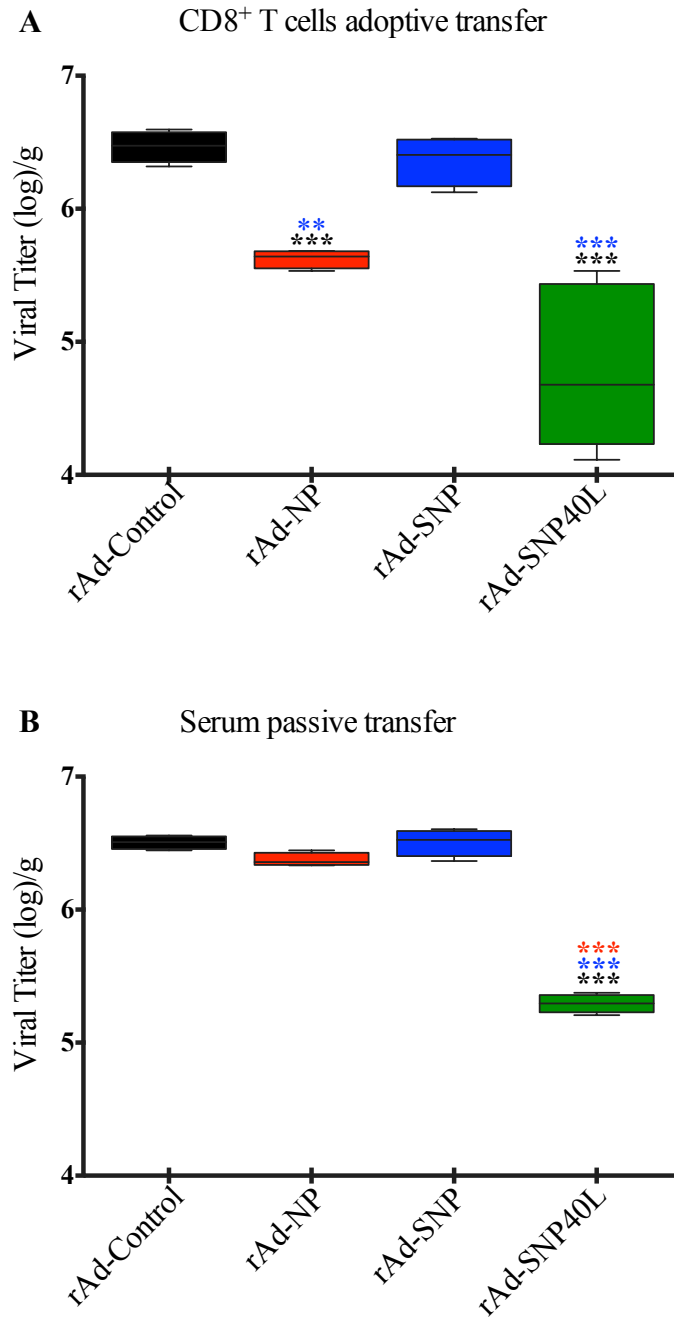
CD40L has not only altered and skewed the NP-specific antibody isotypes and B cell response but also enhanced the magnitude and quality of NP-specific memory CD8⁺ T cells. Therefore, I hypothesized that both the NP-specific CD8⁺ T cells and the T_H1 skewed anti-NP antibodies induced by CD40 targeting could contribute to the improved protection. Indeed, I found that adoptive transfer of CD8⁺ T cells from either rAd-NP or rAd-SNP40L immunized mice significantly reduced viral replication in lungs of challenged mice by one and two logs, respectively. Noticeably, mice received CD8⁺ T cells from rAd-SNP40L immunized group had more reduction in lung viral load compared to mice which received CD8⁺ T cells from rAd-NP immunized mice (Figure 3.18 A). Moreover, I passively transferred serum from vaccinated wild-type Balb/c mice into naïve mice followed by lethal influenza challenge. As shown in Figure 3.18 B, only mice that received pooled serum from rAd-SNP40L immunized mice showed significantly lower viral titers by more than one log in the lungs compared to other groups. These results indicate that rAd-SNP40L enhanced protection by augmenting the roles of both CD8⁺ T cells and NP-specific antibodies.

Figure 3.18

Targeting influenza NP to CD40⁺ cells improves the protection afforded by both CD8⁺ T cells and NP-specific antibodies.

(A) Naïve Balb/c mice (n = 4 per group) adoptively received 1×10^7 purified CD8⁺ T cells isolated from donor immunized mice. The recipient mice were challenged with a lethal dose (10 x LD₅₀) of the mouse adapted influenza A/Puerto Rico/8/34(H1N1) 3 days after adoptive CD8⁺ T cell transfer. (B) Serum from vaccinated donor mice (300 µl) was passively transferred into recipient naïve Balb/c mice for 5 days starting on day -3 to +1 relative to infection (n = 4 per group). Recipient mice were challenged on day 0 with a lethal dose (10 x LD₅₀) of the mouse adapted influenza A/Puerto Rico/8/34(H1N1) virus. Viral titer in lungs was measured on day 6 post-challenge as described in materials and methods. Data are shown as mean titer ± s.e.m. *** represents P <0.001 and ** represents P <0.01 (one-way ANOVA with Bonferroni post-test).

Figure 3.18



3.5.9 Enhanced protection is mediated through CD40-targeting

To confirm that the enhanced rAd-SNP40L protection observed in the animal studies is indeed mediated through CD40-CD40L ligation, I investigated the protective efficacy of rAd-SNP40L in a CD40L^{-/-} mouse model. As expected, all vaccinated mice, regardless of the type of rAd, generated NP-specific IgM antibodies but no IgG antibodies (Supplementary Figure S5, see Appendix), supporting the role of CD40L in promoting Ig isotype switching. However, when these mice were challenged with influenza virus, only mice immunized with rAd-SNP40L were protected from lethal viral challenge, which was accompanied by significantly less weight loss and better clinical scores (Figure 3.19). It is noteworthy that even though CD40L does not seem to reconstitute the immune system in this mouse model for Ig isotype-switching (see Discussion), these data suggest that targeting NP via CD40L improves the protective immunity largely through enhancing cellular immune responses in CD40L^{-/-} mice. More importantly, it clearly demonstrates that the induction of the improved protective immune response against influenza is CD40L-mediated.

3.5.10 CD40L induces CD4⁺ T cell-independent immune response and protection against influenza

Finally, I examined the role of as well as the need for CD4⁺ T cells during immune response induction in rAd-SNP40L immunization. As shown in Figure 3.20 A & B, CD4^{-/-} mice failed to mount a strong antibody response to rAd-NP or rAd-SNP vaccination, revealing the importance of CD4⁺ T cells in immune response induction. Specifically, IgG2a/c and IgG2b isotypes in rAd-NP immunized CD4^{-/-} mice were lower by 58-72 and 42-57 fold, respectively, after primary or secondary immunization compared to

Figure 3.19

rAd-SNP40L enhanced protection is mediated through CD40-CD40L ligation

Survival curves, body weight loss and clinical score in CD40L^{-/-} mice. Animals were immunized on day 0 and boosted 4 weeks later with the indicated rAd constructs and challenged with a lethal dose (5 x LD₅₀) of the mouse adapted influenza A/Puerto Rico/8/34(H1N1) virus 4 weeks post-secondary immunization. Data are presented as a percentage of surviving animals at each time point compared with the initial number of animals in each group. Weight loss is expressed as percentage of animal weight at each time point from their initial body weight. Clinical score was calculated as indicated in materials and methods. Data are shown from one experiment with n = 4-5 mice per treatment group. *** represents P <0.001, ** represents P <0.01 and * represents P <0.05 (two-way ANOVA with Bonferroni post-test).

Figure 3.19

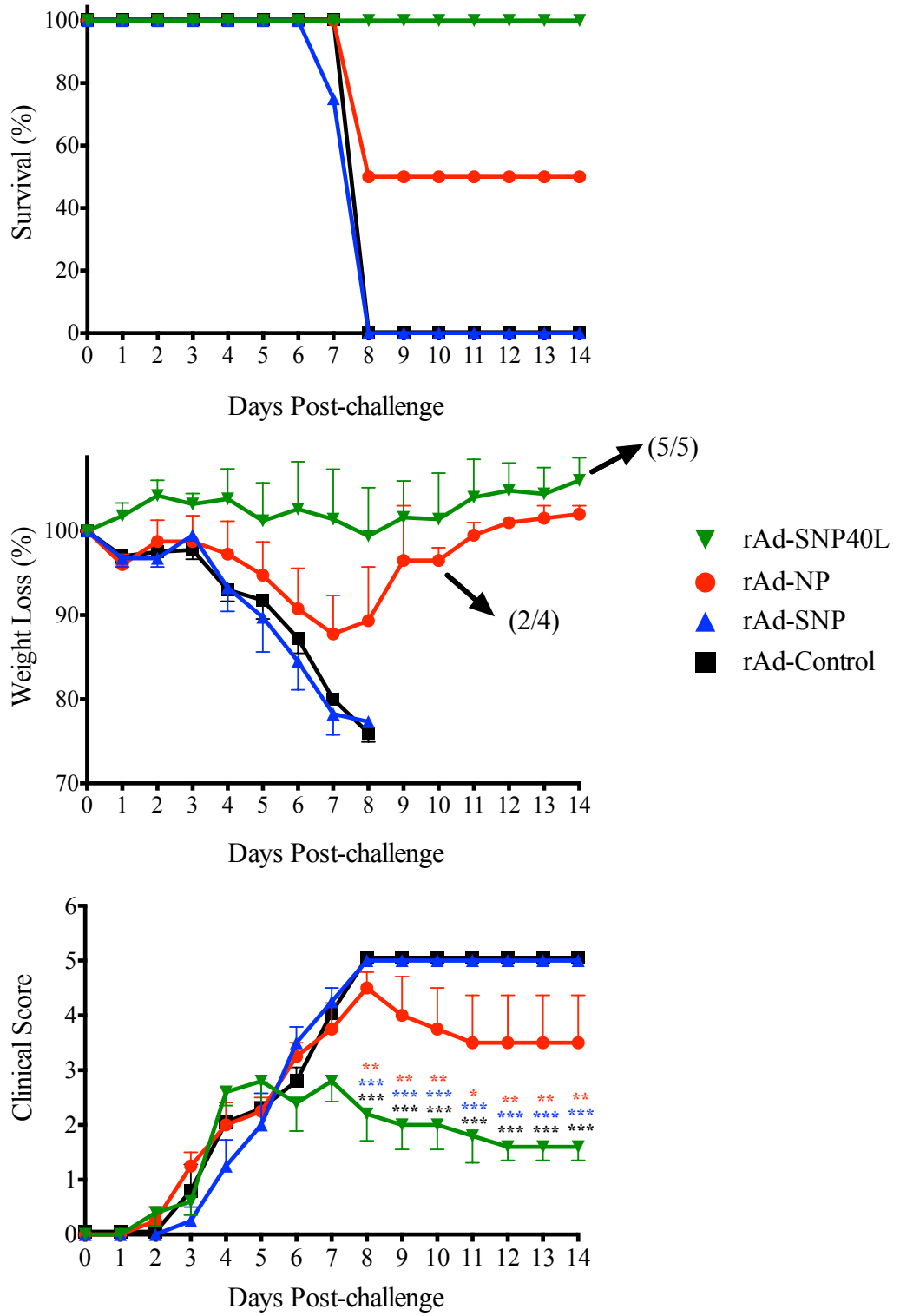
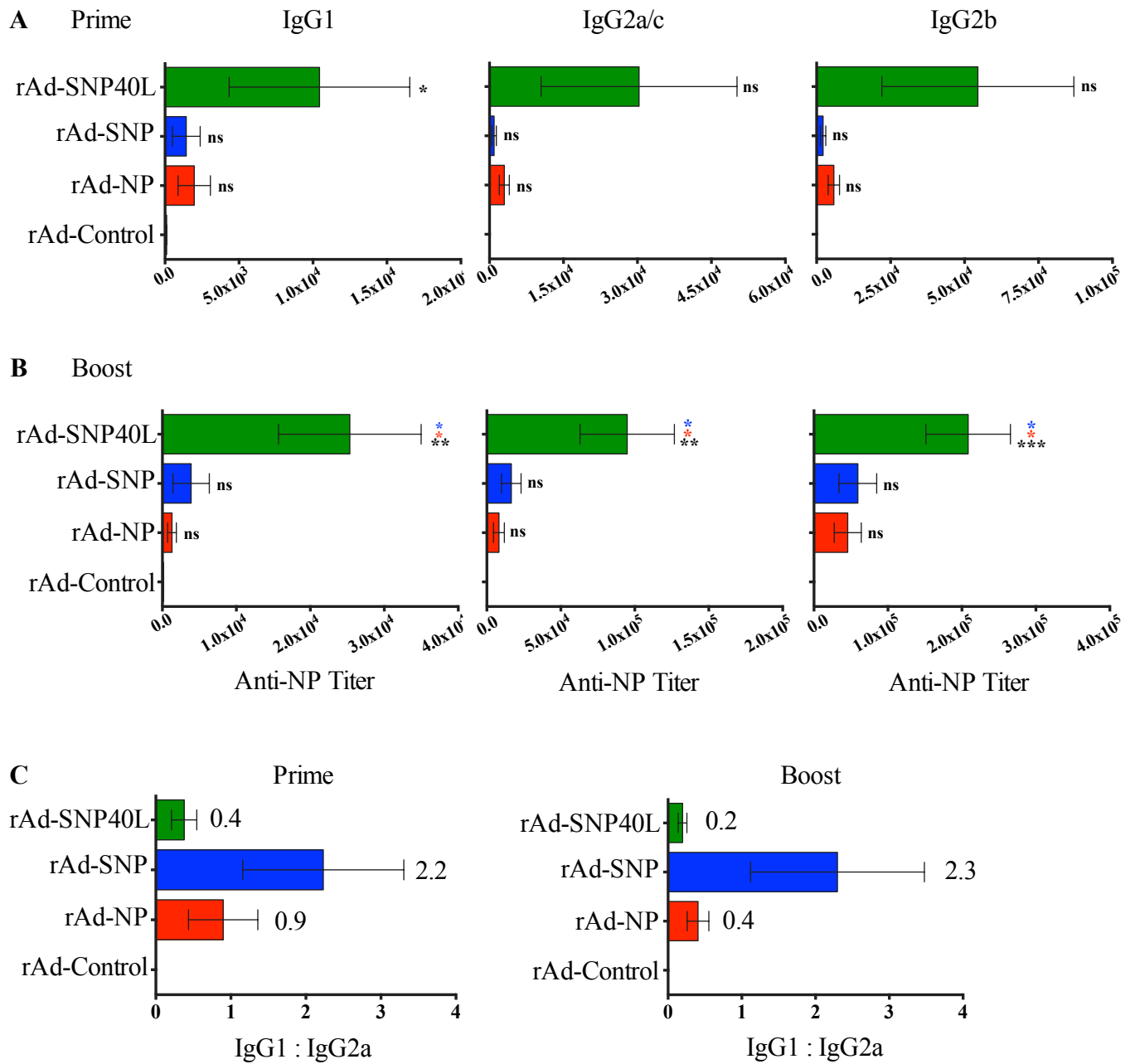


Figure 3.20

Immunization with rAd-SNP40L induces CD4-independent antibody responses against influenza NP

Influenza NP specific antibody titers at 4 weeks after (A) priming and (B) boosting are shown for IgG1, IgG2a/c and IgG2b isotypes. Here, IgG2c was detected in CD4^{-/-} mice. (C) IgG1:IgG2a ratio was measured at 4 weeks after primary and secondary immunization to determine the type of immune response induced (T_H2 vs. T_H1). Numbers on columns indicate the mean ratio. CD4^{-/-} mice were boosted at 4 weeks post-primary immunization. Data are shown as mean titer ± s.e.m. from one experiment, with n = 4-5 mice per treatment group. *** represents P <0.001, ** represents P <0.01, and * represents P <0.05 (one-way ANOVA with Bonferroni post-test).

Figure 3.20



C57BL/6J wild-type mice (Table 3.1). Reduction in antibody response in CD4^{-/-} mice was most prominent in IgG1 isotype upon immunization with either rAd-NP or rAd-SNP which elicited IgG1 titers up to 248 and 278 fold less than wild-type mice (Table 3.1), suggesting a critical role for CD4⁺ T cells in IgG1 production. As expected, the difference in IgG2a/c and IgG2b antibodies was less pronounced in rAd-SNP immunized mice since rAd-SNP vaccination induces mainly IgG1 and very low levels of these two isotypes in wild-type mice as shown in Figure 3.11.

In sharp contrast, immunization with rAd-SNP40L resulted in induction of high levels of anti-NP antibodies comparable to those observed in wild-type mice (Figure 3.20 A & B) with 4-7 fold difference only for all the isotypes (Table 3.1). Interestingly, the antibody isotypes in CD4^{-/-} mice were skewed towards a T_H1 response (IgG2a/c and IgG2b) in a similar manner as that in wild-type mice (Figure 3.20 C). Furthermore, when immunized mice were challenged with 5 x LD₅₀ of influenza virus, only 20-25% of rAd-NP or rAd-SNP vaccinated mice survived. On the other hand, all mice immunized with rAd-SNP40L survived the challenge and maintained their body weight with less severe clinical symptoms (Figure 3.21). Taken together, these data indicate that CD40-targeting via CD40L induced CD4⁺ T cell-independent immune response and afforded full protection not only in immunocompetent but also in CD4^{-/-} deficient animals against lethal influenza challenge.

Table 3.1

**Fold difference in antibody response between wild-type C57BL/6J and B6.129S2-
Cd4^{tm1Mak}/J (CD4^{-/-}) mice.**

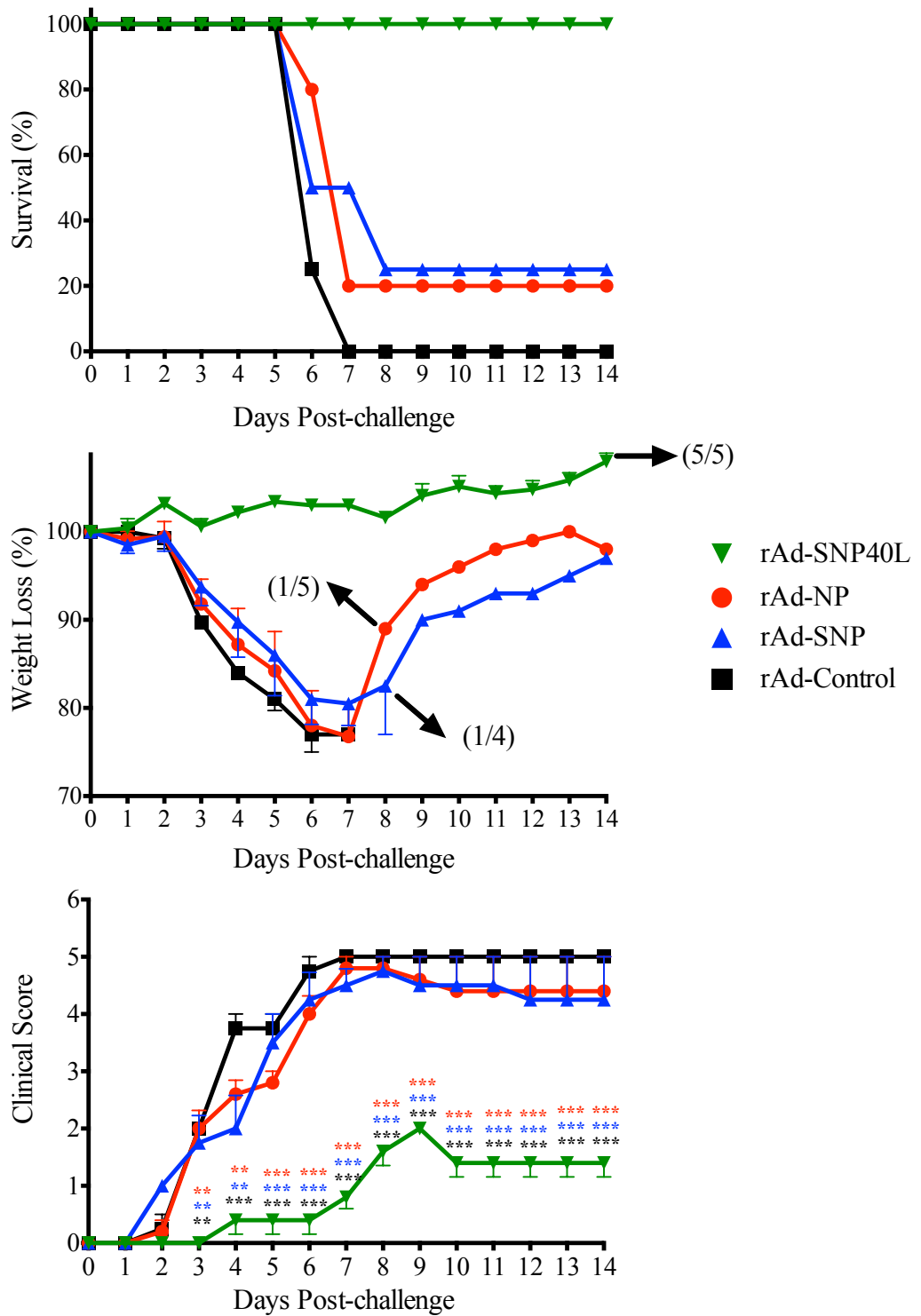
Antibody isotype	Primary immunization			Secondary immunization		
	rAd-NP	rAd-SNP	rAd-SNP40L	rAd-NP	rAd-SNP	rAd-SNP40L
IgG1	178	276	7	248	224	4
IgG2a/c	58	76	5	72	26	6
IgG2b	57	47	6	42	14	6

Figure 3.21

Immunization with rAd-SNP40L restores protection in CD4^{-/-} mice

Survival curves, body weight loss and clinical score in CD4^{-/-} mice. Animals were immunized on day 0 and boosted four weeks later with the indicated rAd constructs and challenged with a lethal dose (5 x LD₅₀) of the mouse adapted influenza A/Puerto Rico/8/34(H1N1) virus 4 weeks post-secondary immunization. Data are presented as a percentage of surviving animals at each time point compared with the initial number of animals in each group. Weight loss is expressed as percentage of animal weight at each time point from their initial body weight. Clinical score was calculated as indicated in materials and methods. Data are shown from one experiment with n = 4-5 mice per treatment group. *** represents P <0.001 and ** represents P <0.01 (two-way ANOVA with Bonferroni post-test).

Figure 3.21



3.6 DISCUSSION

CD40-CD40L ligation has a profound effect on APCs and subsequently on the induction of humoral and cellular immune responses. Although multiple studies have utilized CD40L as a molecular adjuvant co-delivered with antigens of interest using plasmid DNA or viral vectors (Mendoza et al., 1997; Gurunathan et al., 1998; Tripp et al., 2000; Sin et al., 2001; Harcourt et al., 2003; Zheng et al., 2005; Liu et al., 2008; Gómez et al., 2009; Cao et al., 2010; Auten et al., 2012) or as an adaptor protein to alter the tropism of delivery vectors (Manoj et al., 2004; Pereboev et al., 2004; Huang et al., 2008; Hangalapura et al., 2010; Kim et al., 2010; Franco et al., 2011; Hangalapura et al., 2011), several reasons have prompted me to investigate the effect of CD40L on influenza NP-induced immunity. Current influenza vaccines mainly elicit strain-specific neutralizing antibodies against the highly variable HA which necessitates annual vaccination of humans for better protection. Furthermore, the few studies reported the use of CD40L as adjuvant to enhance the humoral immune response against this highly variable protein only showed partial protection against influenza (Yao et al., 2010; Sánchez Ramos et al., 2011). While influenza vaccines based on the highly conserved viral genes such as NP have attracted great interest for the development of broadly protective influenza vaccines, previous reports with NP-based immunization have showed contradicting results. Although the use of different forms of gene-based vaccination and/or immunization regimens can cause such mixed outcomes, it appears that the substantially weaker protective immune response of NP compared to HA is involved. This is mostly because of the nature of the non-sterilizing immunity induced by NP-based vaccination which cannot prevent infection nor the occurrence of mild symptoms as NP-specific antibodies are not neutralizing antibodies. Thus, it would be of great interest to investigate the potential of CD40L as both a targeting and a stimulatory molecule to enhance NP-

specific immune response. I wished to determine the nature of the induced immune response and to dissect the functional roles of the non-neutralizing antibodies and T cell subsets in protection upon rAd-SNP40L immunization.

To achieve these goals, I first took note that the previously reported studies differ from each other in the use of vector, dose and immunization regimens. Thus, I investigated the protection induced by various combinations of these vectors and vaccination regimens especially upon finding that pNP, i.e. a DNA vaccine, was ineffective, with 4 injections needed to induce significant anti-NP antibody response, only affording partial protection. Here, I found that at least two subcutaneous doses of rAd-NP are more immunogenic than pNP prime/rAd-NP boost regimen in terms of immune response induction and afforded complete protection against viral challenge. Also, it is simpler to administer with fewer doses compared to 4 injections in the DNA prime/rAd boost regimen. Nevertheless, the ideal vaccine would require a single dose of rAd containing a highly conserved gene. Thus, it may be useful to consider adjuvants which preferentially boost humoral and cellular immune responses so that one single injection might afford effective protection.

To this end, the transgene was designed to encode a secreted-fusion-protein targeted to CD40 receptors on APCs. Initial experiments showed that the presence of the secretion signal was critical to ensure the release of the fusion protein from rAd-infected cells (Figure 3.3 C). Interestingly, the construct missing this secretion signal (S), designated as rAd-NP40L, failed to induce an immune response or afford protection against influenza challenge, underscoring the importance of secretion of the fusion protein.

It could be envisaged that as both proliferating and non-proliferating cells are susceptible to adenovirus infection (Huang et al., 2008; Kim et al., 2010), gene products with a secretion signal produced by adenovirus-infected cells would be secreted to increase the

antigen load *in vivo* (Figure 3.22). As the fusion protein is targeted to CD40 on APCs, the immune response against NP would be amplified due to CD40-CD40L ligation. Although APCs themselves are relatively refractory to adenovirus infection due to their lack of its main receptor, coxsackie-adenovirus receptor (CAR) (Huang et al., 2008; Kim et al., 2010), direct infection of these cells through different receptors or alternative mechanisms (Adams et al., 2009) can not only promote their own maturation but also result in secretion of the fusion protein into regional LNs which could provide direct activation of T and B cells. Thus, CD40L can act as an adjuvant and targeting molecule in both the peripheries (sites of injection) and/or the secondary lymphoid organs as illustrated in Figure 3.22.

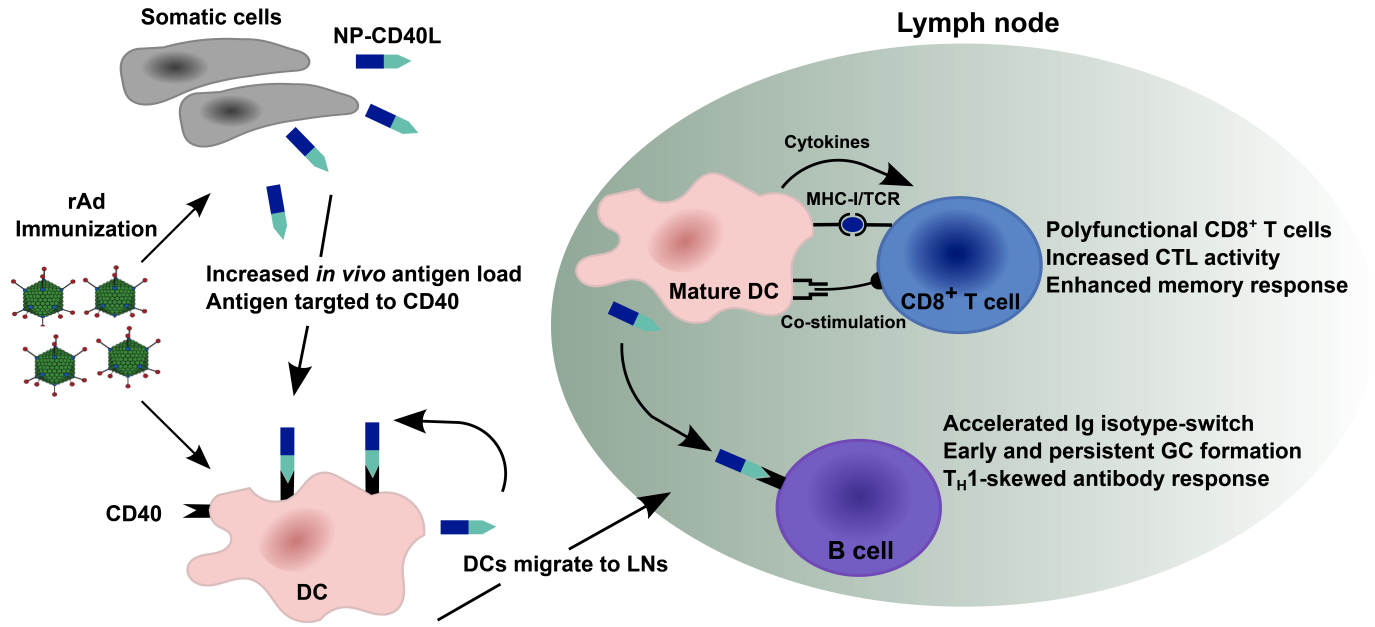
My observation that the three constructs (rAd-NP, rAd-SNP and rAd-SNP40L) elicited differently skewed immune responses in mice is noteworthy. Secretion of encoded NP using rAd-SNP resulted in T_H2 -skewed responses characterized by NP-specific IgG1 antibodies with very low levels of the other isotypes compared to rAd-NP which induced high levels of all IgG isotypes. Interestingly, targeting the secreted NP to CD40⁺ cells via CD40L significantly induced T_H1 -biased NP-specific antibody response. This dramatic shift from T_H2 to T_H1 was observed in both MHC haplotypes H-2d (Balb/c) and H-2b (C57BL/6J) and manifested by increased IgG2a/c and IgG2b and reduced IgG1 isotype levels. I also found that rAd-SNP40L preferentially altered the kinetics of B cell responses by significantly promoting early and long-lasting GC formation, plasma cell induction and isotype-switched memory B cells in draining LNs by day 28, consistent with its role on B cells. Targeting secreted NP via CD40L effects also resulted in a robust NP-specific CTL response as early as 14 days post-primary immunization. Moreover, it improved the quality of CD8⁺ T cells by significantly increasing the frequency of multiple-cytokine-producing CD8⁺ T cells which have been considered superior in mediating protection against infections

Figure 3.22

Schematic model of immune response induction using CD40-targeted antigen.

Subcutaneous immunization with rAd encoding secreted NP-CD40L fusion protein results in infection of proliferating and non-proliferating cells, thus secreted gene products increase the antigen loads *in vivo* at the site of injection. The fusion protein then binds to CD40 on APCs via CD40L to initiate immune response induction and amplification. DCs can also be directly infected with rAd leading to their maturation into professional APCs and subsequent secretion of the fusion protein into regional LNs to directly activate B cells. Thus, CD40L can act as an adjuvant and targeting molecule in both the periphery and/or the secondary lymphoid organs. In the LNs, the CD40L-activated DCs activate antigen-specific CD8⁺ T cells to induce enhanced CTL activity and multiple cytokines producing cytotoxic T cells. Meanwhile, secreted CD40-targeted antigens activate B cells and elicit accelerated Ig isotype-switch, early and persistent GC formation and T_H1-skewed antibody response.

Figure 3.22



and a surrogate marker for the quality of vaccine-induced T cells responses (Darrah et al., 2007; Kannanganat et al., 2007; Almeida et al., 2007). Thus, the immune response was markedly enhanced in rAd-SNP40L immunized mice at all levels. Indeed, a single dose of rAd-SNP40L was sufficient to induce superior and most importantly rapid and long-lasting protection compared to rAd-NP, confirming that the enhanced immune response led to improved protection.

The adoptive transfer study clearly demonstrates that CD8⁺ T cells are the major player in NP-based protection against influenza virus. Cells from both rAd-NP and rAd-SNP40L but not rAd-SNP immunized groups significantly reduced viral titers in lungs of recipient mice (Figure 3.18 A). Also, it shows that CD8⁺ T cells induced by rAd-SNP40L vaccination are more effective in inhibiting viral replication. On the other hand, passive transfer of anti-NP sera from rAd-NP or rAd-SNP immunized groups failed to convey protection to recipient mice. Importantly, sera from rAd-SNP40L immunized mice significantly reduced lung viral load (Fig. 4.18 B), suggesting a correlation between the shift in antibody isotypes and the inhibition of viral replication. Indeed, T_H1 related antibodies, IgG2a/c and IgG2b, are associated with effector mechanisms such as FcR binding, complement-fixation or complement-mediated lysis of infected cells (Yewdell et al., 1981; Germann et al., 1995; Lefeber et al., 2003). Collectively, these results demonstrated that targeting antigens via CD40L boosts CD8⁺ T cell responses and favors the induction of T_H1-biased antibodies which leads to stronger and longer-lasting protection. Also, they further extend the role of non-neutralizing anti-NP antibodies in protection against influenza by highlighting the importance of antibody-associated effector mechanisms.

Vaccination of CD40L^{-/-} mice with rAd-SNP40L elicited an NP-specific IgM response with very low levels or diminished IgG isotypes similar to rAd-NP and rAd-SNP

immunized groups, indicating that the levels of CD40L produced by the transient expression from rAd-SNP40L infected cells is insufficient to restore isotype-switching in these mice. Indeed, this is consistent with previously reported studies which showed that even constitutive expression of CD40L (Brown et al., 1998) or co-administration of high doses of recombinant CD40L (50 mg given 3 times/week) but not lower doses (5 mg given 3 times/week) (Jain et al., 2011) can only induce low to intermediate levels of antigen-specific isotype-switched antibodies in CD40L^{-/-} mice. Nonetheless, rAd-SNP40L immunization completely protected CD40L^{-/-} mice against influenza challenge. Most likely rAd-SNP40L protection is mediated through CTL response as my data suggest that CD8⁺ T cells play critical roles in protection (Figure 3.18 A). Furthermore, I found that none of the constructs generated in this study were able to confer any protection to CD8^{-/-} mice (data not shown).

The role of CD4⁺ T cells was apparent in rAd-NP and rAd-SNP immunized CD4^{-/-} mice as both groups failed to mount a strong antibody response or to show protection against influenza. On the other hand, rAd-SNP40L immunization enhanced all isotypes, including IgG1 antibodies, to levels very similar to those observed in wild-type mice with a 4-7 fold difference only (Table 3.1). Interestingly, it influenced the T_H1-T_H2 balance in a very similar fashion to wild-type mice by inducing a T_H1-polarized response (Figure 3.20 C). This observation is different from a previous study which showed T_H2-skewed response in CD4-depleted mice and balanced T_H1/T_H2 response in CD4^{-/-} mice (Zheng et al., 2005). Although the difference between the two studies cannot be fully explained, such a discrepancy could be due to the difference in immunization regimens. Specifically, while I administered two doses of rAd to target the encoded antigen as a fusion protein directly to CD40⁺ APCs via CD40L, Zheng and colleagues co-delivered CD40L with antigen through co-immunization/expression using DNA prime/rAd boost approach. Most importantly, my data

clearly show that targeting influenza NP using CD40L can be an effective approach to induce CD4⁺ T cell-independent protective immune response against influenza infection.

The significance of these results is paramount in regards to immunodeficiency. There is a growing need to develop new strategies to elicit CD4-independent immune responses or at least to partially substitute for CD4⁺ T cell loss in immunocompromised individuals with defective CD4⁺ T cells or CD40L deficiency. In HIV infection (McMichael and Phillips, 1997; Chougnet, 2003; Zhang et al., 2004; 2006) or aged individuals (Eaton et al., 2004) for example, the gradual loss of virus-specific CD4⁺ T cells is associated with significant reduction in CD40L expression. Such defects will not only result in impaired immune responses and increased risk of opportunistic infections but also in drastically reduced efficacy of vaccination (Malaspina et al., 2005). My data demonstrate that targeting antigens via CD40L can overcome such defects and induce protective immune response in CD4⁺ T cell and CD40L immunodeficiency.

In summary and as shown in Figure 3.22, data presented here show that using CD40L as adjuvant and targeting molecule enhanced the breadth, potency and durability of NP-specific immune responses as well as the protection in normal mice and in CD4^{-/-} and CD40L^{-/-} mice. Although I focused on vaccination using influenza NP, this work indicates an effective means of inducing durable immune response and protection. To my knowledge, this is the first report demonstrating that a single dose of NP-based immunization can elicit strong and long-lasting protective immune responses against influenza involving both CD8⁺ T cells and anti-NP antibodies.

CHAPTER 4

General Discussion and Conclusion

The influenza A virus is highly contagious and results in significant morbidity and mortality worldwide. While mass vaccination of a susceptible population is the best approach to prevent influenza infections, its propensity for mutation and gene reassortment can result in the occasional emergence of novel and unpredicted strains. This can give rise to a global influenza pandemic, such as the recent event caused by a triple reassortant swine-origin pH1N1 influenza virus. Since considerable time is required to develop and distribute vaccines, novel influenza strains can spread globally before a vaccine is available for mass immunization. Given the potential for widespread influenza infection, it is crucial to develop alternative approaches to better control infection.

Recent strategies for alternative therapy which explore the more conserved epitopes among the different subtypes of influenza virus as well as cross-neutralizing antibodies may offer protection against a broad range of strains. In my research, I have investigated the potential of broadly cross-reactive antibodies targeting the highly conserved fusion peptide in the HA protein, and a prototype vaccine based on the highly conserved NP.

4.1 Targeting a universally conserved epitope in the viral HA

The high susceptibility of the surface glycoproteins HA and NA to mutations due to the selective immune pressure in combination with both antigenic drift and shift, have resulted in significant structural variability between and within subtypes. Thus, the occurrence of antigenically conserved peptide sequences between and within subtypes of influenza NA and HA is uncommon. However, the quest for conserved regions in these mutating proteins has received increasing attention in recent years. The potential identification of such conserved regions may play an essential role in future vaccine development and avert the necessity of yearly vaccine updates.

Through comprehensive bioinformatics analysis of all available HA sequences from the NCBI influenza virus resource, we recently found that the HA2 N-terminal 14 amino acids (GLFGAIAGFIEGGW) of the fusion peptide are conserved among all known 16 subtypes of influenza A viruses as well as influenza B viruses with only minor substitutions (Figure 2.2). A key finding of my research was that antibodies targeting this universally conserved sequence (Uni-1 antibodies) cross-neutralized various influenza A virus strains from group 1 and group 2 HA subtypes. These antibodies inhibited viral replication via binding to their respective target “fusion peptide” in the stem region of the HA protein and blocked the fusion step required for viral uncoating.

Several groups have demonstrated the prophylactic and therapeutic potential of different BnAbs targeting conserved epitopes within the HA2 subunit in protection against multiple subtypes of influenza A virus including HPAI as well as pandemic influenza viruses. Interestingly, IC9 mAb, which targets a shorter form of the epitope targeted by Uni-1 antibodies, showed therapeutic and prophylactic protection in mice. Thus, passive immunotherapy with such antibodies could serve as an alternative or supplement to currently available agents at least for individuals at high risk, such as children, the elderly and the immunocompromised or during a pandemic situation. Furthermore, identification of highly conserved regions within HA and the isolation of BnAbs could aid in the development of small proteins and/or molecules that mimic these antibodies by targeting the conserved regions within the protein. However, the ultimate goal is to understand the mechanisms that would elicit the production of these antibodies and subsequently design a universal vaccine or develop strategies to confer long-lasting broader protection.

4.2 Targeting the highly conserved NP

Viral NP is a highly conserved structural antigen and a potential universal vaccine candidate. Several studies have shown that NP-based cross-protective or heterosubtypic immunity can be induced in humans and animals via natural infection or immunization. However, anti-NP antibodies are not protective on their own and while NP-specific CTLs could play an important role in the control of influenza virus infections by eliminating virus-infected cells and preventing viral spread, CTL mediated immunity is weak. Moreover, most of these studies have mainly focused on the use of different immunization regimens or delivery methods using viral vectors and plasmid DNA. Several other complementary strategies are under extensive investigation to adjuvant different vaccines. One such approach is to modulate and enhance the host immune response by using co-stimulatory molecules.

Here, I have utilized CD40L, which is a key linker between the innate and the adaptive immune responses, not only as an adjuvant but also as targeting molecule to efficiently stimulate APCs and enhance NP-specific immune responses. The data I presented here clearly demonstrate that this platform is highly efficient in preferentially modulating and augmenting the immune responses. Specifically, it skewed the immune response towards the T_H1 phenotype and enhanced humoral and cellular responses compared to untargeted NP. While the role of CTLs in protection is well documented, the induction of T_H1 -skewed anti-NP antibodies is noteworthy. In particular, passive transfer of these antibodies reduced viral replication in lungs of naïve mice demonstrating a novel role for the effector mechanisms associated with these non-neutralizing anti-NP antibodies. Importantly, a single immunization dose of CD40-targeted NP provided longer lasting immunity as demonstrated by the protection of animals even 4 months after immunization. Another key finding of this

study was the protection of immunodeficient ($CD40L^{-/-}$ and $CD4^{-/-}$) mice from lethal influenza challenge which is of great importance in regards to immunodeficiency. Furthermore, it clearly verifies that the enhancement of NP-protection is CD40L-mediated independent of $CD4^{+}$ T cells.

The overall significance of this work clearly points towards a platform capable of eliciting robust humoral and cellular immunity. Therefore, vaccination strategies based on highly conserved antigens such as HA2 subunit could be further improved in order to induce the unnatural cross-protective immunity.

4.3 Conclusion

One of the major inherent drawbacks of current influenza vaccines is the need for annual reformulation so as to antigenically match the circulating influenza strains. This is mostly due to the inability of these vaccines to induce cross-protective humoral and cellular immune responses against the different subtypes of influenza A virus. An ideal approach in controlling influenza would be a universal vaccine that protects against all influenza subtypes, including the emerging strains. Even a vaccine that could only protect against strains associated with human disease or against those within a particular subtype, would be an improvement over the current annual vaccination strategy. Such a vaccine should be able to elicit both cross-protective humoral and cellular immune responses for better control of this notorious virus.

The increase in published genome sequences and structural data about conserved epitopes in viral HA, as well as the ability to induce protection against multiple viral subtypes in animals using conserved internal antigens such as the NP, underscore the cross-

protective potential of these highly conserved epitopes and proteins and could bring us closer to the design of such a candidate vaccine. However, generation of an effective universal vaccine might necessitate an efficient induction of innate and adaptive immunity. Therefore, immunogenicity and protective efficacy of these promising candidates could be further improved by investigating different delivery regimens or by using molecular adjuvants such CD40L in order to induce the two arms of the immune system.

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APPENDIX

Figure S1.

Survival of Balb/c mice immunized with two doses of rAd constructs.

Balb/c mice were immunized with two doses of each rAd construct and challenged with 10 x LD50 of influenza A/Puerto Rico/8/34(H1N1) virus 4 weeks post-secondary immunization. Data are shown from one experiment with n = 8 mice per treatment group.

Figure S1

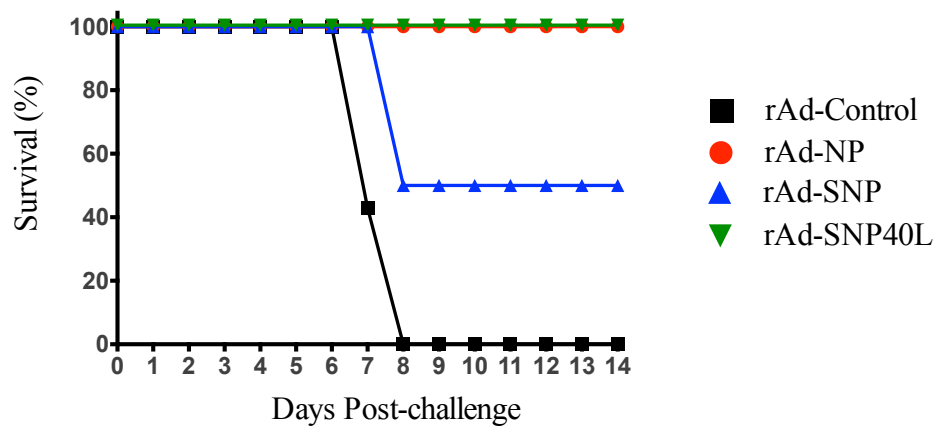


Figure S2

CD40 targeting preferentially induces T_H1 antibody isotypes against influenza NP in Balb/c mice.

Antigen specific antibody titers at 2 weeks after priming (**A**) or boosting (**B**) are shown for IgG1, IgG2a and IgG2b isotypes. Mice were boosted at 4 weeks post-primary immunization. Data are shown as mean titer \pm s.e.m. from two independent experiments, with n = 8-10 mice per treatment group in each experiment. *** represents p <0.001, ** represents p <0.01 and * represents p <0.05 (one-way ANOVA with Bonferroni post-test).

Figure S2

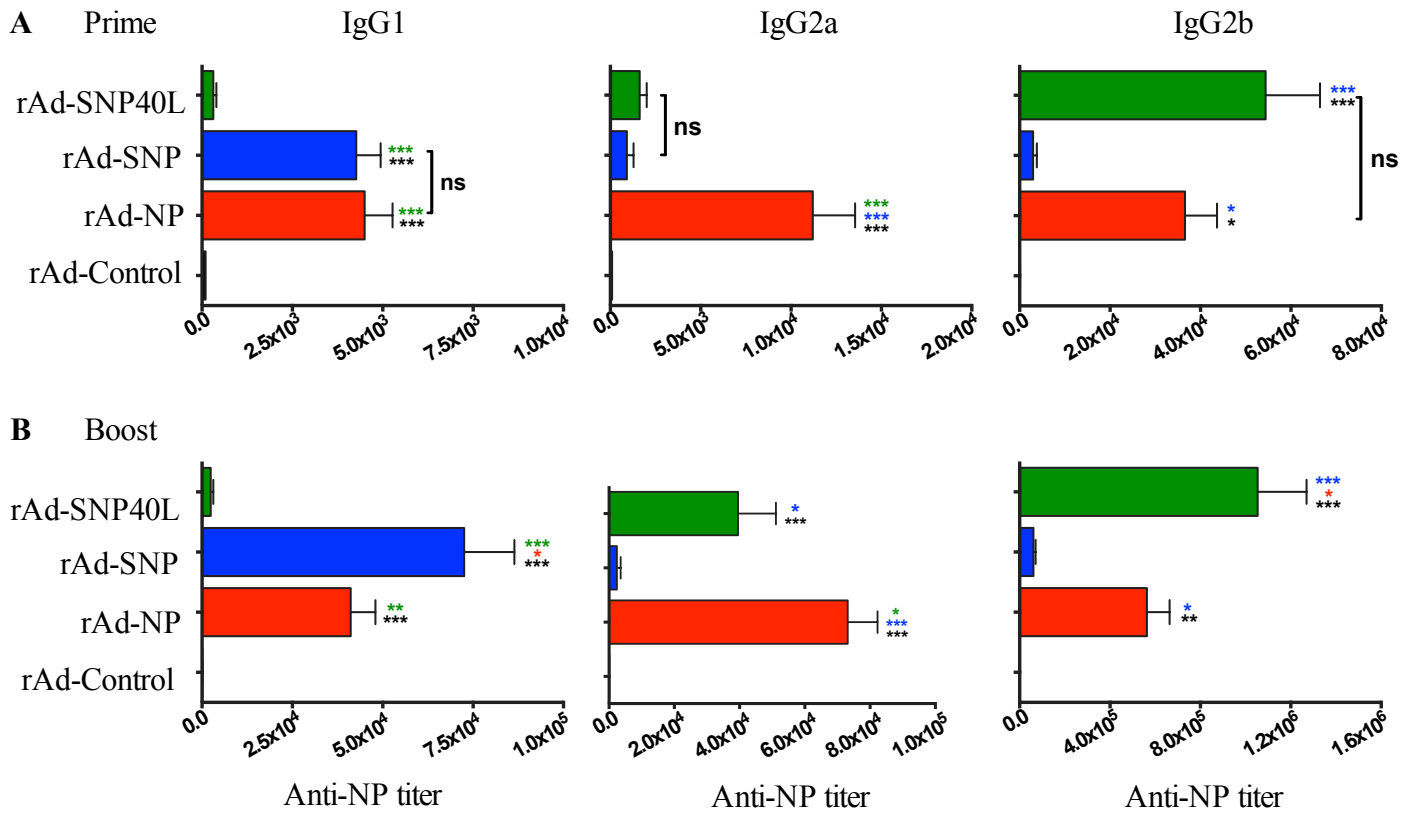


Figure S3

CD40 targeting preferentially induces T_H1 antibody isotypes against influenza NP in C57BL/6J mice.

Influenza NP specific antibody titers at 2 weeks after priming (**A**) or boosting (**B**) are shown for IgG1, IgG2a/c and IgG2b isotypes. Here, IgG2c was detected in C57BL/6J mice. C57BL/6J mice were boosted at 4 weeks post-primary immunization. Data are shown as mean titer \pm s.e.m. from one experiment, with n = 4-5 mice per treatment group. *** represents $p < 0.001$, ** represents $p < 0.01$ and * represents $p < 0.05$ (one-way ANOVA with Bonferroni post-test).

Figure S3

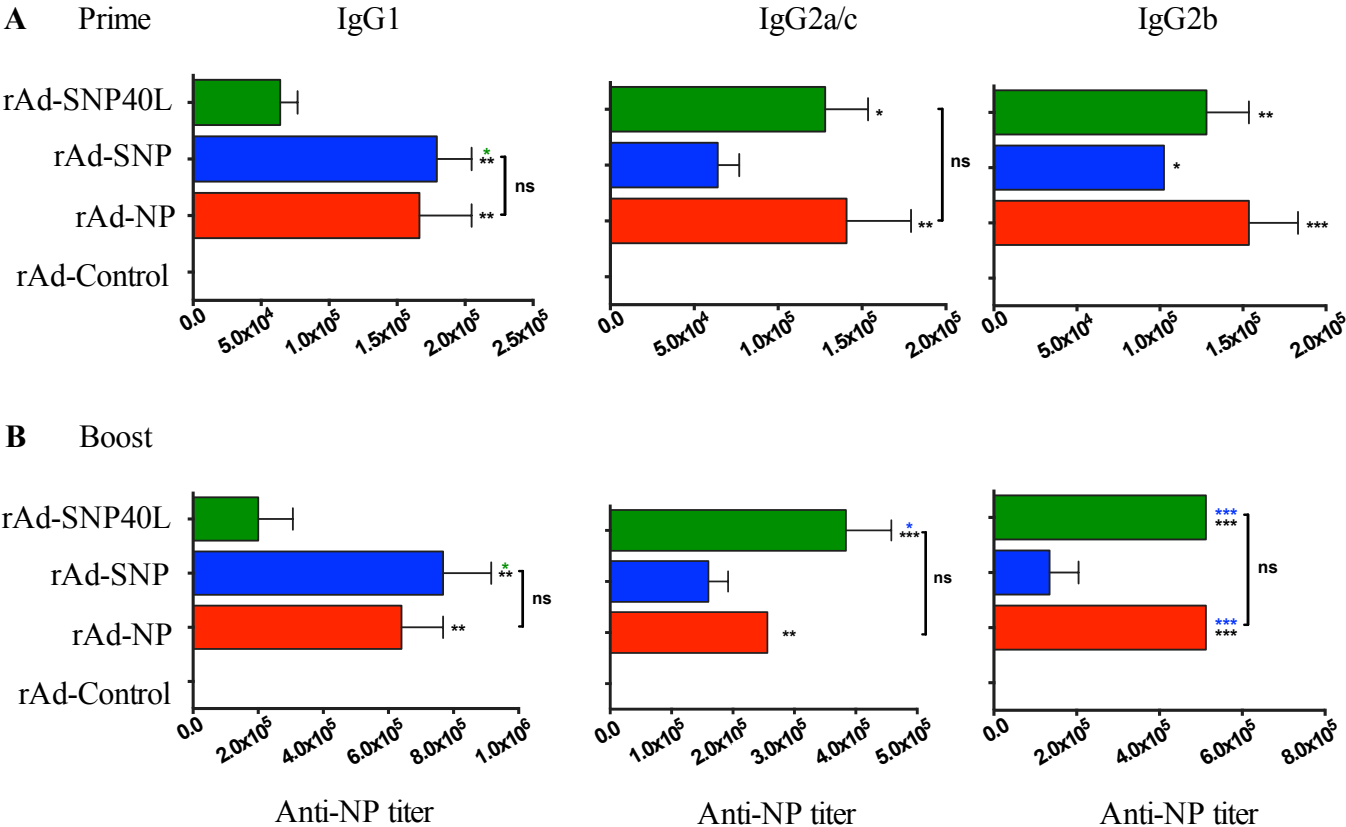


Figure S4

CD40 targeted influenza NP enhances antigen specific cell-mediated cytotoxicity 2 weeks after primary immunization.

Splenocytes isolated from immunized Balb/c mice 2 weeks post-vaccination were restimulated *ex vivo* with synthetic NP peptide to generate effector cells. NP specific cytotoxic activity was measured at different Effector:Target ratios, and the percentage of specific lysis was calculated. Data are shown as mean \pm s.e.m. from two independent experiments, with n = 2-3 mice per treatment group per time point in each experiment. *** represents p <0.001, ** represents p <0.01 and * represents p <0.05 (one-way ANOVA in **A-D**, and two-way ANOVA in **E** with Bonferroni post-test for both).

Figure S4

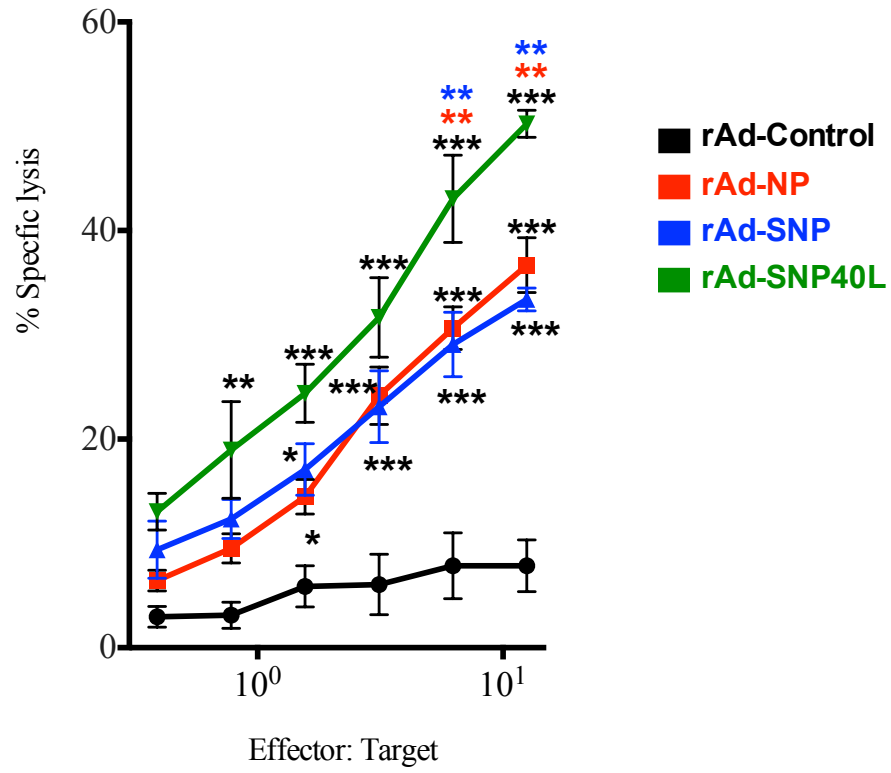
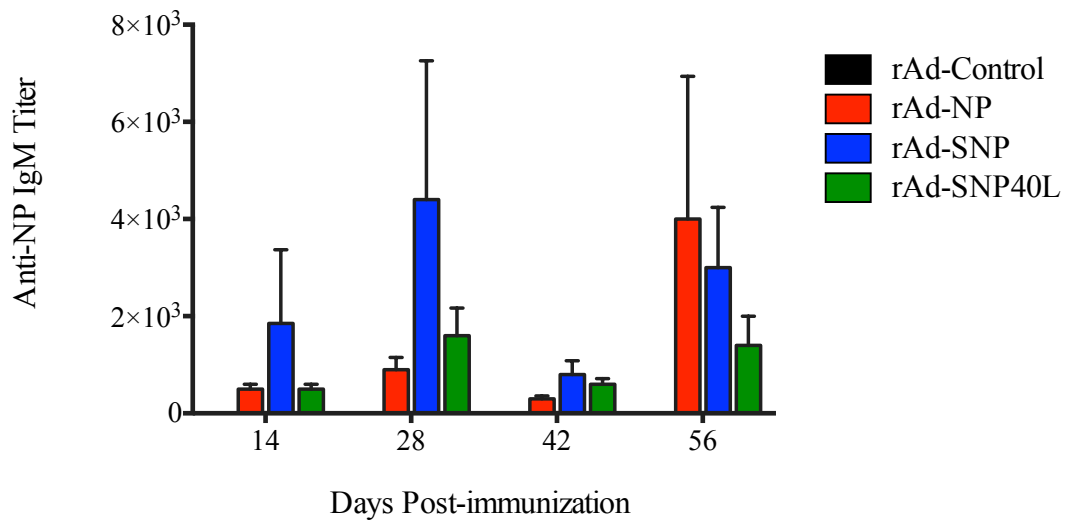


Figure S5

IgM antibody response in CD40L^{-/-} mice.

Influenza NP specific IgM titers were determined every two weeks after primary and secondary immunization in CD40L^{-/-} mice . Mice were boosted at 4 weeks post-primary immunization. Data are shown as mean titter \pm s.e.m. from one experiment, with n = 4-5 mice per treatment group.

Figure S5



GST-Uni1 fusion protein synthesis

Forward and reverse oligonucleotides (Uni-1 Fw and Uni-1 Rev) corresponding to the sequence of the mature Uni-1 peptide (GLFGAIAGFIEGGW) and the appropriate restriction sites were annealed and inserted into the pET19b-GST vector using the BamH1/Xho1 restriction sites generating pET19b-GST-Uni1 plasmid. Vector pET19b-GST is a modified version of pET19b (Novagen, San Diego, CA, USA) to which a GST fusion partner has been inserted between the poly-histidine tag and the multiple cloning site, along with a thrombin recognition cleavage site. The constructs were sequenced using an ABI 3130xl Genetic Analyzer from Applied Biosystems (Life Technologies Corp., Carlsbad, CA, USA). Plasmid pET19b-GST-Uni1 was then transformed in *E. coli* BL21(DE-3) competent cells (Stratagene, Kirkland, WA, USA). Expression was carried out as previously described (Gravel et al., 2010) by incubating a freshly transformed colony in LB media at 37 °C until a cell density measure at OD600 of 0.8 was obtained. Protein expression was induced with the addition of 1mM IPTG and allowed to proceed for 3 hours at 27 °C. Cells were harvested by centrifugation and purified using the Qiagen standard protocol for poly-histidine tagged proteins.

Mass spectrometric analysis

The tube gel samples were sliced, the proteins reduced with 10 mM dithiothreitol (DTT) at 56 °C for 1 hour, and alkylated with 55 mM iodoacetamide at RT for 1 hour, followed by in-gel tryptic digestion overnight. To remove the detergents, each sample was transferred to a tube-gel. The peptides were extracted with 0.1% trifluoroacetic acid (TFA) and 60% acetonitrile/0.1%TFA. After drying in a SpeedVac (Savant), the sample was reconstituted in 0.2% formic acid for LC MS/MS analysis. Nanoflow LC MS/MS analyses was performed using Nano-Acquity ultra-performance liquid chromatography system (UPLC) (Waters, Milford, MA) with a BEH130 C18 analytical column coupled to a 7-tesla hybrid linear ion trap Fourier transform ion cyclotron resonance (LTQ-FT Ultra ICR) mass spectrometer (Thermo Fisher, San Jose, CA). Two microliters of the sample were loaded and the peptides were trapped by a reversed phase Symmetry C18 nanoAcquity™ column (180 µm i.d. x 20 mm length, particle size 5 µm) at a flow rate of 5 µl/min for 3 min, and subsequently separated on a C18 analytical column (100 µm i.d. x 100 mm, particle size 1.7 µm, BEH130) at a flow rate of 500 nl/min. NanoUPLC separation was carried out through a 90-min linear gradient from 5% to 45%, and then with 85% of solvent B (97.9% acetonitrile, 0.1% formic acid and 2% water (v/v)) mixed with solvent A (0.1% formic acid and 99.9% water (v/v)). Mass spectral data was acquired in the data-dependent mode: ion-trap MS/MS survey scan over a mass range of m/z 300-2000. Survey scans were acquired in the ICR cell with a resolution of 100,000 at m/z 400 and at a normalized collision energy of 35% mode.

Primers list

Primer	Sequence (5'-3') ^a
Uni-1 Fw	GATCCGGCCTGTTTGGTGCGATTGCTGGTTTTATTGAAGGTGGCTGGTAAC
Uni-1 Rev	TCGAGTTACCAGCCACCTTCAATAAAAACCAGCAATCGCACCAAACAGGCCG
pQE2-NP ^b	GTAC <i>GGTACC</i> ATGGCGTCTCAAGGCAC
pQE2-NP ^c	GTAC <i>AAGCTT</i> TTAATTGTCATATTCCTCTG
NP-1 ^b	GTAC <i>CGGCCG</i> CATGGCGTCTCAAGGCACC
NP-2 ^b	GCTGTGGAGTTTCCAGACCTCCGCTGGCCATTTCCCTAGAGCGATGGCGTCT CAAGGCACCAAAC
NP-3 ^b	GTAC <i>CGGCCG</i> CATGCTCCTGGCTGTTTTGTAAGCCTGCTGTGGAGTTTCCA GAC
NP-R1 ^c	GTAC <i>GATATC</i> TTAATTGTCATATTCCTCTG
NP-R2 ^c	ACGT <i>AAGCTT</i> GCCCATCTCTTGGAGCTTCAGGAATATAACCATTGTCATATTC CTCTGCATTGTC
CD40L-1 ^b	GAATGGGTATTGCTTTCTACCTTCTTAGAGGATCCTCAAATTGC
CD40L-2 ^b	ATGGGC <i>AAGCTT</i> ACGTTTCGTAAGATGGCGAATGGGTATTGC
CD40L-R ^c	GTAC <i>GATATC</i> TCAGAGTTTGAGTAAGCCAAA

^a restriction sites are in bold and italics

^b sense primers

^c anti-sense primers

Collaborators Contributions

- Dr. Yi-Min She performed the LC MS/MS analysis and protein identification (Section 2.4.7 and Table 2.1) in the laboratory of Dr. Terry D. Cyr (CVE, BGTD, HPFB, Health Canada, Ottawa, ON, Canada).
- Genevieve Gingras generated the GST-Uni1 and GST-PrP fusion proteins (Section 2.4.4) in the laboratory of Dr. Yves Aubin (Centre for Vaccine Evaluation (CVE), Biologics and Genetic Therapies Directorate (BGTD), HPFB, Health Canada, Ottawa, ON, Canada).
- Bozena Jaentschke optimized rNP expression and purification and generated Figure 3.1.
- Michelle Lemieux performed all the sequencing of the plasmids generated in this study.
- Caroline Gravel, Monika Tocchi and Marsha Russell helped with the animal experiments.
- Dr. Jianhua Sui (Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA) provided the plasmids for the single-round HIV luciferase reporter virus pseudotyped with surface antigens of influenza A/Thailand/2-SP-33/2004(H5N1) (H5-TH04).
- Dr. Earl Brown, University of Ottawa provided the Mouse adapted influenza A/Puerto Rico/8/34(H1N1).
- Polyclonal anti-NP antibodies were generated by Sigma-Genosys (Oakville, ON, Canada).
- Primers as well as the pUC19-NP and pUC19-CD40L plasmids were synthesized by Bio S&T (Montreal, QC, Canada).

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