uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Amine Saad

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Microbiology and Immunology)

GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Induction of Adaptive Immunity by a Novel Influenza Vaccine: Immunization by mRNA Administration

TITRE DE LA THÈSE / TITLE OF THESIS

Francisco Diaz-Mitoma

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

Ashok Kumar

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Paul MacPherson

Jonathan Angel

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Induction of adaptive immunity by a novel influenza vaccine: immunization by mRNA administration

Amine Saad

Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirement for the degree of

Masters of Science in Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

University of Ottawa

Your file Votre référence
ISBN: 978-0-494-73810-8
Our file Notre référence
ISBN: 978-0-494-73810-8

Abstract:

Vaccination is one of the major strategies available for combating viral infections in humans. The seasonal inactivated influenza virus vaccine elicits type-specific, protective neutralizing antibodies that are detectable in the serum of a vaccinated person; however, the very nature of the virus requires a reformulation of the vaccine to match the currently circulating strains in any given year. In the present study, a novel vaccination approach was developed to induce protective immunity against Influenza A. The approach consisted of delivering naked or protected mRNA encoding for Hemagglutinin as a vaccine. The vaccine was administrated to B6C3F1 mice and the overall immune response raised by an mRNA-based vaccine was assessed through the evaluation of the cellular and humoral responses induced by this type of vaccination and the *in vitro* protection it confers against Influenza A virus. Results from these studies suggest that mRNA vaccination in the context of infectious diseases, specifically influenza, is feasible and that mRNA vaccines are capable of inducing a balanced immune response as characterized by the production of protective neutralizing antibodies and the induction of cellular immunity against influenza.

Acknowledgments:

I would like to thank my supervisor, Dr. Francisco Diaz-Mitoma for his continuous support and guidance. I would also like to thank Dr. Dave Anderson for his patience and willingness to discuss issues regarding my thesis.

Special thanks to my thesis advisory committee, Dr. Ashok Kumar, Dr. Earl Brown, Dr. Ken Dimock, Dr. Marko Kryworuchko and Dr.Ali Azizi for their tremendous help and input over the last two years. Dr. Earl Brown, your valuable help and constant encouragement were a great source of motivation. Dr. Jennifer Arnold, I wouldn't even know where to start, I am grateful to have you as a friend and a mentor.

Last but not least, I would also like to dedicate this thesis to my parents and sisters for their support over the years. You always had faith in my ability for accomplishing greater things.

Table of Contents

List of Abbreviations:

CD: cluster of differentiation

CHO: Chinese hamster ovary

Cpm: counts per minute

cRNA: complementary RNA

DC: Dendritic cells

DEPC: Diethyl pyrocarbonate

DMSO: dimethyl sulphoxide

ELISA : Enzyme-linked immunosorbent assay

Flu AV :Influenza A virus

FCS : Fetal calf serum

GMP : Good manufacturing practice

HA : Hemagglutinin

HRP : Horseradish peroxidase

I.P : Intra peritoneal

IRES: Internal ribosomal entry site

M: Matrix

MFI: Median fluorescence intensity

MHC: major histocompatibility complex

MOPS : 3-(N-morpholino)propanesulfonic acid

MPL: monophosphoryl lipid A

mRNA: Messenger RNA

mV: Milli volts

NA: Neuraminidase

nAbs: Neutralizing antibodies

NISV : Non-Ionic Surfactant Vesicle

NP: Nucleoprotein

O.D Optical density

PBS: Phosphate buffered saline

PEG: Polyethylene Glycol

Pfu: Plaque forming units

PHA: phytohemagglutinin

PRR: Pattern recognition receptor

RBC: Red blood cell

RDRP: RNA-dependent RNA polymerase

RNP: ribonucleoprotein

RT: Room temperature

SEM: Standard error of the mean

ssRNA: single strand RNA

TLR: Toll like receptor

WHO: World health organization

List of Figures:

A. Introduction

1. Context

The interaction between mankind and pathogens has been, is and will always be a major force in shaping everyday life. A Pathogen is an agent (viral, bacterial, fungal, protozoal) which, in essence, generates ("gennan") suffering ("pathos"). Pathogens resulting in infectious diseases are a particularly serious threat to both humans and animals with the most common infectious disease being seasonal influenza [hereinafter referred to as influenza]. Due to the ease by which seasonal influenza infects and spreads throughout a population, influenza results in a heavy burden on the health care system characterized by increased hospitalization costs, loss of human life and a heavy economic burden due to the loss of work productivity. The World Health Organization (WHO) estimates that annual cases of seasonal influenza-induced severe illness amount to approximately 5 million people around the world and that up to 500,000 deaths arise annually as a direct consequence of influenza epidemics (WHO, 2003). The influenza-mediated burden on the health care system and on the economy will only continue to increase as worldwide rapid travel continues with countless travelers acting as potential mobile reservoirs for this family of viruses.

One of the most efficient weapons developed in the battle against infectious diseases is vaccination. In simple terms, a vaccine is a biological product that is for direct administration to a human or animal for the purpose of triggering an immune response against an infectious agent. The biological product can range from whole pathogens, to proteins or to nucleic acids. The actual history leading to the development of the first vaccine remains slightly shrouded in mystery with many considering that the use of vaccines started in 1796 with the British physician, Edward Jenner, who used pox fluid derived from cows infected with cowpox to

vaccinate humans against smallpox. In 1734, however, the French philosopher Voltaire suggested that the Chinese had been, at least for the previous two centuries, inoculating their children against smallpox by having the children inhale the dried scabs collected from infected patients (Voltaire, 1743). A worldwide vaccination campaign was started in 1967 by the WHO, based on the findings of these earlier experiments, to diminish naturally occurring smallpox. Within ten years, the naturally occurring smallpox virus was successfully eradicated with the last known case being registered in Somalia in 1977 (Behbehani, 1983). Another striking example of vaccine efficacy was the containing of the spread of viruses by the Mumps vaccine. The use of the trivalent mumps, measles and rubella (MMR) vaccine in the U.S.A yielded a reported 99% reduction in cases of mumps between 1968 and 1993 (Van Loon *et al*, 1995). These are just a few examples of the many vaccines available to the public which have steadily and reliably proven the robustness of a vaccination program as a successful approach to eliminate or diminish the threat that many pathogens pose.

Unlike the infectious diseases discussed above, influenza has continued to evolve and always managed to stay slightly ahead of developments in vaccine technology. Influenza (commonly referred to as "the flu") is an enveloped RNA virus that belongs to the *Orthomyxoviridae family*. The first acknowledged report of an influenza case was made in 412 B.C. by Hippocrates who described an ailment whose symptoms greatly resemble those associated with our modern day "flu" (Hollenbeck, 2005). This example demonstrates for just how long humans have been living in close proximity to this virus and yet there is still no definitive vaccine. As mentioned above, influenza outbreaks, specifically those caused by the Influenza A virus (Flu AV), can lead to a heavy health and economic burden. Unlike the other members of the *Orthomyxoviridae family*, Flu AV has a propensity to mutate its genome (Nobusawa and Sato, 2006). Moreover, as aquatic birds constitute the natural reservoir of Flu

AV, this specificity allows genetic material to exchange in the case of co-infections giving rise to strains never before seen by the human immune system.

Currently, pandemics are what the global heath community is mostly concerned about and ever-vigilant for. The WHO defines pandemics as "a global disease outbreak" caused by the surfacing of a mutated virus, in this instance influenza, for which the population has a modest to non-existent immunity. The pandemic-inducing disease would spread readily from person-to-person, leading to a serious, potentially deadly, illness that could sweep across a country and around the world within a very short period of time (Koh *et al*, 2008).

Historically, epidemiologists reference three major Influenza pandemics within the 20th century. The first one occurred in 1918 and is commonly referred to as the "Spanish flu". This pandemic is considered to be one of the deadliest epidemics ever known to mankind. The absence of viable treatments combined with poor health practices, particularly in developing countries, led to 50 million casualties worldwide over a period of nine months characterized by three successive waves of infection (Morens and Fauci, 2007). The causative agent was a Flu AV of the H1N1 subtype of avian origin (Simonsen L, 1999). The next notable pandemic was the 1957 "Asian flu" caused by an H2N2 Flu AV. The virus originated in China and rapidly spread around the globe. The morbidity and mortality rates associated with Asian flu were relatively mild when compared to the Spanish flu pandemic. Nonetheless, 250,000 people were rapidly infected in Hong Kong while serological data in the U.S.A showed that the virus had disseminated in more than half the American population (Simonsen L, 1999; Kilbourne ED, 2006). The 1968 Hong Kong Influenza (caused by an H3N2 subtype) which arose in Southeast Asia was the last of the noted influenza-mediated pandemics and was the least severe of the three (Rambaut *et al*, 2008). The Hong Kong Influenza spread swiftly

around the globe causing very little mortality, except in the U.S.A, with the preexisting H2N2 immunity from the Asian flu believed to have been responsible for mitigating the impact of the Hong Kong Influenza on the world population (Eickhoff TC and Meiklejohn G, 1969; Viboud C *et al,* 2005). Interestingly, the H1N1 and H3N2 subtypes have been the predominant circulating strains behind seasonal flu outbreaks whereas the H2N2 subtype hasn't re-emerged since the Hong Kong pandemic (Beigel, 2008).

In an attempt to better prevent and/or minimize the probability of another influenza pandemic, some researchers have tried to study whether the appearance of the virus followed a cyclical pattern with regard to seasonality (Hilleman MR. 2002). Applying such an approach, however, to influenza has not proven to be beneficial in furthering the understanding of the influenza virus given the often unforeseen and sporadic flu crises that arise in discrete global locations. As such, the majority of the research community continues to work towards developing effective treatments against influenza such as vaccines and a wide array of antiviral drugs.

Currently there are two major anti-influenza drug treatments available on the market:

1) molecules which block the Matrix 2 (M2) ion channel (such as amantadine) have a mechanism of action which prevents the M2-mediated acidification of the virion which thwarts the uncoating of the virus; and

2) neuraminidase (NA) inhibitors (such as Oseltamivir commonly known as Tamiflu) which block the release of viral particles from the surface of the host cell.

M2 channel blockers have been largely dismissed as an effective treatment for influenza because of the high incidence of resistance (92% in the U.S.A) rendering this class of drugs almost useless (Bright *et al,* 2006). An important point to note is that the majority of

the resistant strains examined displayed the same single point mutation in the M2 gene which exemplifies just how adaptable the target virus can be (Simonsen *et al,* 2007). By contrast, NA inhibitors have proven to be efficient in mitigating the infection outcome when administered within two days of the onset of flu symptoms (Hayden and Pavia, 2006; Treanor *et al,* 2000). The main drawback to this approach is that patients may not accurately self-diagnose the first symptoms of influenza and, if the critical time for administration of NA inhibitors passes, it is then too late to administer the drug and the patient develops a full blown influenza infection. In addition, recent reports from clinicians in Europe suggesting the emergence of resistant isolates combined with recent publications reporting the emergence of variants from the highly virulent H5N1 strain which were unaffected by NA inhibitors, indicates that anti-influenza drugs have not solved the problem (Le,Q.M. *et al,* 2005).

An alternative and complementary approach to preventing influenza infection involves the use of vaccines. Two types of vaccine formulation are currently available on the market. The first class of formulation consists of inactivated products which may be a whole virion, a split virion or a subunit vaccine. For whole virion vaccines the entire virus is propagated in eggs before being collected and killed with formaldehyde. Split-virion vaccines are derived from virus particles which have been treated with detergents or organic solvents such as ether (Kilbourne and Arden, 1999). Subunit vaccines are extremely pure formulations comprising mainly the Hemagglutinin (HA) and NA proteins since the blockade of these two proteins, which are exposed at the surface of the virion, prevents viral entry and release from the host cell (Tamura *et al,* 2005). In direct comparisons, the killed whole-virion vaccine has proven to be the most immunogenic hence the most efficacious preparation due to the presence of viral single-stranded RNA which possesses intrinsic adjuvant capabilities mainly through TLR 7 activation (Hilleman, 2002).

The second class of vaccine formulation is represented by a live attenuated virus which is derived each season from a cold-attenuated virus (Influenza A and B) (Maassab HF, 1967). The cold-attenuated virus is prepared from the wild type virus by serial passage in eggs at 25°C. This yields a mutant virus which is restricted to the human upper respiratory tract. The predicted HA and NA are introduced into the attenuated virus through co-infection with the wild type virus (carrying the proper genes) while gene re-assortment takes care of the exchange of the genes. This approach yields great protection, not only to the homologous strains, but also cross-protection against heterologous influenza A viruses, which stands in contrast to the inactivated vaccines (Tamura *et al,* 2005). This vaccine is federally regulated and approved for use in the U.S.A for 5 to 49 year old patients only because of fears of respiratory infections in the higher risk groups.

Given the highly mutagenic ability of the influenza virus, the vaccines must be seasonally formulated. The WHO, through its influenza surveillance centres, recommends the variants to be included in the yearly vaccine preparation. These variants represent the strains which are predominantly circulating during a given season between February and April. With these selection procedures in mind, and in view of the fact that inactivated or live attenuated vaccines are based on predictions of possible future circulating strains, it is easy to imagine how a mistake in the forecast would lead to little or no protection against some of the strains that are behind an outbreak in any given year. For example, during the 2007-2008 season, the WHO misjudged the predominant circulating strains which led to only 44% of those vaccinated being less likely to become infected with influenza compared to the unvaccinated (CDC, 2008). In light of these facts, new ways of inducing protection against influenza are of ever growing importance as the traditional approaches are failing to provide highly effective prevention.

The protection against viral challenge elicited by live and attenuated vaccines will now be discussed in more detail. Live and whole particle vaccines were found to be better than the other alternatives such as subunit or split virion vaccines because they 1) trigger a broad immune response due to the variety of antigens they consist of, and 2) comprise viral single-strand RNA. The latter's importance results from its natural ability to activate Toll-Like Receptor 7 (TLR7). TLRs are germline-encoded receptors which recognize a wide range of pathogen-associated molecular patterns (PAMPS). These PAMPs vary from nucleic acids (DNA or RNA) to lipid moieties derived from pathogens (Romagne, 2007). They are part of a family of pattern recognition receptors (PRRs) which are present on innate immunity cells and are responsible for the recognition of non-self by the innate immune system leading to the activation of an inflammatory response. TLRs and PRRs have been mostly regarded as a mechanism for the host to rapidly sense and contain pathogens through actions mediated directly by the innate immune system.

Recently, however, TLRs have also been examined for the active role they play in mounting and steering an effective adaptive immune response. First, the induction of TLRs creates a pro-inflammatory environment which causes a further recruitment of leukocytes. Second, TLRs are differentially expressed on innate immune cells and can lead to specific responses depending on the activated cell type. For example, dendritic cells which are essential to the triggering a Th1 versus Th2 response, express diverse but distinct groups of TLRs depending on the cellular subset (see Table 1 below for a summary). This selective activation leads to differential cytokine secretion, which combined with antigen presentation, will determine the nature of the raised adaptive immune response (see Figure 1 below).

Supporting the crucial role of TLR agonists in the context of vaccines, a recent clinical trial demonstrated a significantly enhanced immunogenicity of a commercial inactivated Flu

AV vaccine combined with a TLR agonist (Cooper *et al,* 2004). The same study showed that this approach allowed a significant decrease in the antigen dose, lowering it to a tenth of the prescribed dose. Hence, a coordinated stimulation of both innate and adaptive arms of the immune system appears to be crucial to attaining, if not improving, protection while lowering costs.

In the light of such findings, a less traditional approach to influenza vaccine development would be to use messenger RNA (mRNA) as a vaccine against influenza. This approach would take full advantage of the intrinsic nature of this nucleic acid as a template for protein synthesis (antigen source) and as an adjuvant (TLR7 agonist). A more detailed description of the variables involved in this study follows below. In brief, the main variables of interest are:

1) influenza A virus (Flu AV) selected due to the heavy economical and sociological impact factors cited earlier
2) Hemagluttinin since it represents the logical target of choice for vaccine development against Flu AV
3) The use of mRNA as a novel therapeutic
4) *In vivo* models which will best illustrate the potential efficacy of an mRNA-based vaccine

	Freshly Isolated DCs			
	CD4+	CD8+	DN	pDC
TLR1	++	++	++	++
TLR2	++	++	++	++
TLR3	-	++	++	-
TLR4	++/-	++/-	++/-	++/-
TLR5	++	-	++	+
TLR6	++	++	++	+
TLR7	++	-	++	++
TLR8	++	++	++	++
TLR9	++	++	++	++

Table 1. TLR expression profile by mouse dendritic cells. (Iwasaki and Medzhitov, 2004)

+, ++ and – indicate the relative mRNA expression of each TLR by the dendritic cell (DC) subsets. + / – indicate contradictory data: some groups noting expression (+), while others did not find any (–).pDC: plasmacytoid dendritic cell. DN: double negative

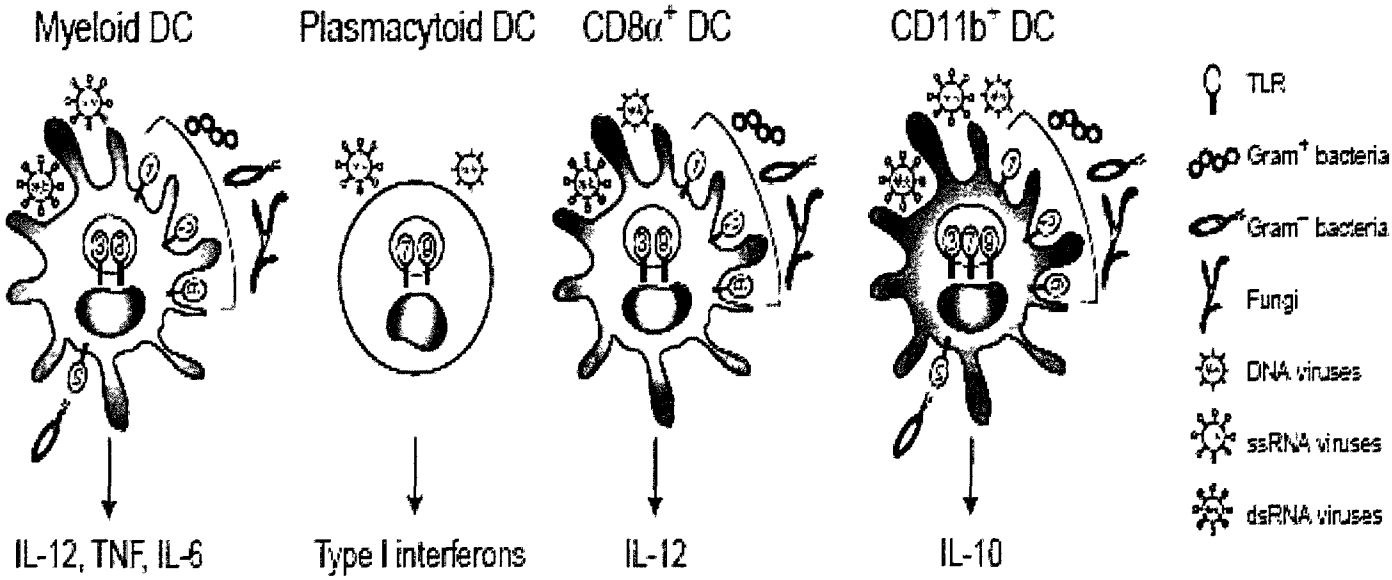


Figure 1. Sets of TLRs expressed by dendritic cells (Iwazaki and Medzhitov, 2004)

2. The Influenza A Virus:

Each influenza strain is classified according to the genus, the host (if isolated from a non-human), geographical site of isolation, isolate number, year of isolation and finally the HA and NA subtype if it is a Flu AV.

a. Structure and Genetics

The Flu AV belongs to the *Orthomyxoviridae family* along with four other members which are Influenza virus B, Influenza virus C, Thogtovirus and Isavrius. These studies will focus exclusively on Flu AV. Influenza A viruses are further classified into subtypes according to variations in the surface glycoproteins from which they are made. Thus, two surface glycoproteins, HA and NA, define each Flu AV. To date, there are 16 different HA subtypes and 9 NA (Bouvier and Palese, 2008). Historically, only four combinations of these subtypes have been known to lead to an epidemic: H1N1, H1N2, H2N2 and H3N2 (Palese and Shaw 2006). H5N1, H7N7 and H9N2 subtypes have also been isolated from humans but without any associated epidemic (Cheung and Poon, 2007). Research in the field has yet to provide a conclusive explanation as to why these subtypes have been the only ones to be isolated from humans. Nonetheless, the one aspect that remains confirmed is that Flu AV is host restricted.

Structurally, InfAV has a lipidic envelope which is derived from the host cell. HA and NA extend beyond the surface of the virus membrane (Bouvier NM, Palese P, 2008). M2, a third protruding surface protein, forms homotetramers which act as an ion channel (Zebedee and Lamb, 1988). Underneath the viral membrane, lies a layer formed by the M1 protein which encapsulates the virion core, which consists of eight single-strand RNA segments coated with nucleoprotein (NP) and polymerase complex (see Figure 2 below) (Palese and

Shaw 2006). As noted, the virion contains Flu AV's genome which consists of eight single-strand RNA segments encoding 11 proteins (Table 2).

The extra three proteins are derived from two distinct molecular processes wherein alternative splicing produces the M2 and NEP/NS2 proteins from the M1 and NS genes respectively (Lamb and Choppin, 1979; Lamb and Choppin, 1981). The third protein, PB1-F2, is generated from a second open reading frame in the PB1 gene (Chen *et al,* 2001). Each segment of the genome is complexed within a ribonucleoprotein (RNP) formed by the NP and the trimeric viral RNA-dependent RNA polymerase (RDRP: made of PB1, PB2 and PA) and the viral RNA segment itself.

Segment	Segment length in nucleotides	Encoded protein(s)	Protein length in amino acids	Protein function
1	2341	PB2	759	Polymerase subunit: mRNA cap recognition
2	2341	PB1	757	Polymerase subunit: RNA elongation, endonuclease activity
		PB1-F2	87	Pro-apoptotic activity
3	2233	PA	716	Polymerase subunit: protease activity
4	1778	HA	550	Surface glycoprotein: major antigen, receptor binding and fusion activities
5	1565	NP	498	RNA binding protein: nuclear import regulation
6	1413	NA	454	Surface glycoprotein: sialidase activity, virus release
7	1027	M1	252	Matrix protein: vRNP interaction, RNA nuclear export regulation, viral budding
		M2	97	Ion channel: virus uncoating and assembly
8	890	NS1	230	Interferon antagonist protein: regulation of host gene expression
		NEP/NS2	121	Nuclear export of RNA

Table 2: Genomic segments of Influenza A (A/PR/8/1934) virus and corresponding encoded proteins (Palese and Shaw, 2006)

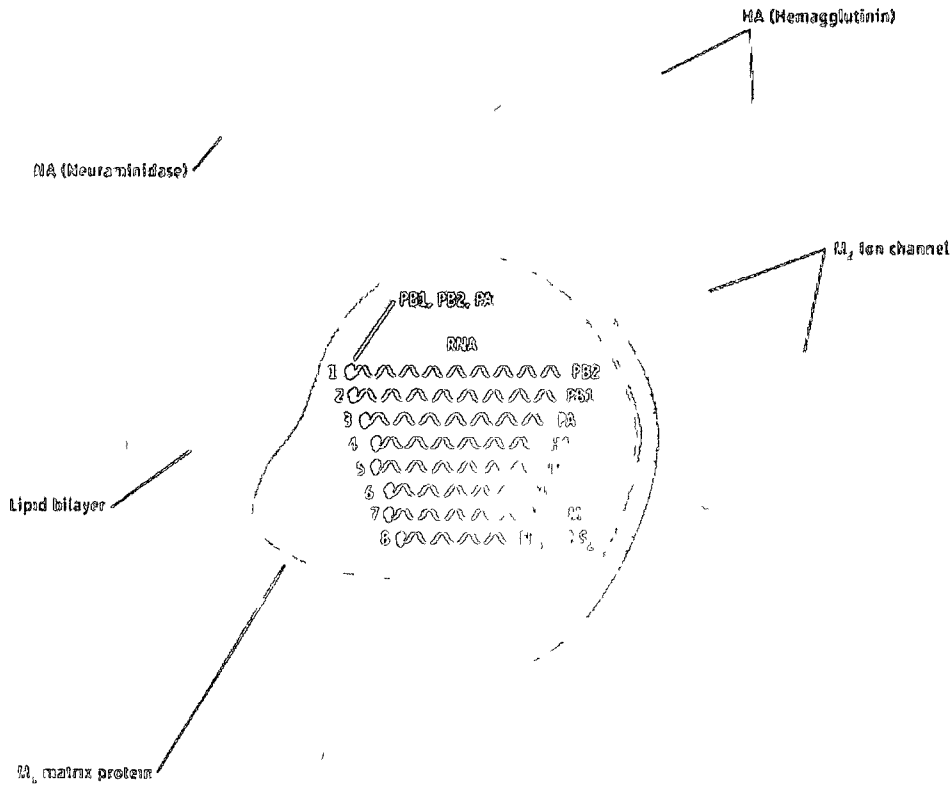


Figure 2: Influenza A virion structure and organization. (J. Kaiser, 2006)

b. Viral Life Cycle

The infectious cycle can be briefly summarized as:

1. Attachment;
2. Entry;
3. Synthesis of viral RNA/proteins;
4. Packaging of RNA and assembly of the virus and
5. Budding and release

First, the viral cycle is initiated upon HA-mediated recognition of sialic acid residues on the surface of the host cell. Sialic acids are sugars commonly found on many cell types across numerous species (e.g. fowl, swine). The chemistry of this residue determines the bias towards a specific host since sialic acid can form a link with the sixth (α-2,6) or third (α-2,3) carbon of galactose. The tropism of human and avian Flu AV is in total agreement with the chemistry since the latter linkage characterizes duck gut respiratory epithelial cells whereas the former is mainly found in human respiratory epithelium (Couceiro *et al,* 1993; Ito *et al,* 1998).

The attachment triggers clatherin-mediated endocytosis of the virion followed by a drop in the pH of the endosome which induces a change in the conformation of HA exposing a fusion peptide (Matlin *et al,* 1981). This peptide then inserts into the cellular membrane of the host bringing the virion in close proximity to the cellular membrane allowing the fusion of the virion membrane and the endosomal membrane (Stegmann *et al,* 1987). As a direct consequence of this fusion episode, many pores are opened between the endosomal and viral membrane causing the release of RNP into the host cell cytoplasm (Stegmann *et al,* 1987).

This release of RNP into the host cell is made possible by the acidification of the endosome wherein the hydrogen ions are transported inside the virion, through the M2 ion channel, which in turn weakens protein-protein interactions between the M1 matrix and RNPs (Martin and Helenius, 1991). This sequence of events allows the RNPs to flow freely into the cytoplasm and then translocate to the nucleus, through interactions between viral nuclear localization signals and cellular proteins (Cros and Palese, 2003).

Once in the nucleus, transcription of the viral genome starts. In summary, the viral RDRP uses the negative sense RNA to synthesize two positive-sense RNA species: one for translation (mRNA) and the other to be used as a template for more viral genomic RNA synthesis (cRNA for complementary RNA). The RDRP, owing to its intrinsic properties mediated by PB1 and PB2, will subsequently steal 5' capped primers from host mRNAs transcripts through a mechanism termed cap snatching (Krug, 1981). The viral genomic RNA encodes a sequence of five to seven uracils which are transcribed by RDRP into a stretch of adenosines forming the Poly(A) tail of each viral mRNA (Li and Palese, 1994). The combination of these two sequential events produces fully mature viral transcripts which are readily exported to the host cytoplasm and translated (Palese and Shaw 2006). NEP/NS2, along with M1, is responsible for the export of viral RNA (cRNA and mRNA) (Cros and Palese, 2003). The free RNPs are then directed to plasma membrane of the host cell where HA, NA and M2 islets have already assembled through specific sorting signals. M1 complexed RNPs will then gather underneath these islets and push the virus buds out onto the host cell membrane surface (Bouvier and Palese, 2008). The last step of the process is the NA-mediated release of the virus that is attached to the cell surface through HA-sialic acid interactions (Luo *et al*, 1999).

3. Hemagglutinin

a. Characteristics:

As already highlighted above, Flu AV HA is critical to the virus: without HA's receptor binding and fusion capabilities the virus wouldn't be able to initiate an infectious cycle. HA is a rod-shaped trimer located on the surface of the virus. Wilson *et al.* were the first group to discover the crystallographic structure of HA (see Figure 3 below) (Wilson *et al*, 1981).

The cycle of HA from synthesis to degradation has been fully characterized. HA is initially formed as a precursor termed HA0 which is then translocated to the endoplasmic reticulum where it undergoes trimerization and post translational modifications such as glycosylation (Klenk *et al,* 1975; Lazarowitz and Choppin, 1975). HA is then transferred to the cell surface via the Golgi apparatus. Once at the cell surface, HA is cleaved by serine protease yielding three main structures: HA1, HA2 and the fusion peptide (Lazarowitz and Choppin, 1975). This final step, of serine cleaving, results in fully active HA molecules. Three coiled HA2 molecules form the long stem of the HA which allows for insertion into the viral membrane. The globular head, responsible for binding sialic acid residues, is formed by three HA1 molecules and is fundamental in the recognition of the virus by the immune system.

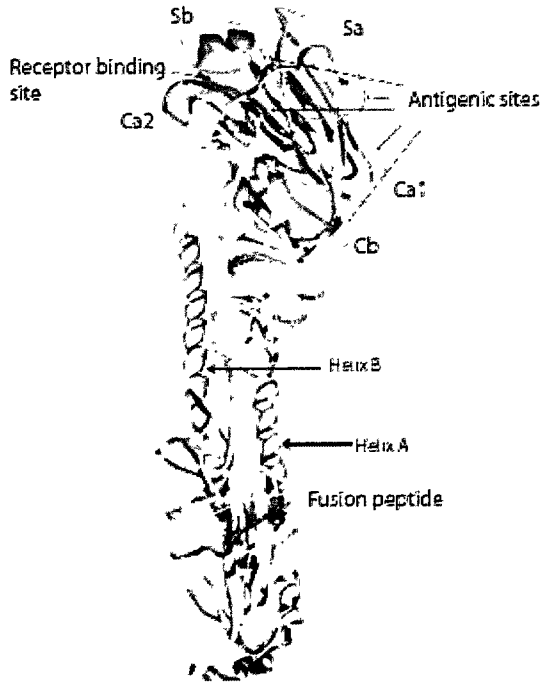


Figure 3: Representation of an uncleaved HA molecule. (Stevens *et al,* 2004)

b.HA and Immunity:

The surface antigens present on the Flu AV virion, primarily HA and NA, are the major determinants with regard to the immune response in the infected host. They are the first proteins the defenses of the host encounter. Given that HA is the molecule that the virus uses for entry into the host cell, obstructing the receptor binding regions on its globular head via the use of HA-specific antibodies should, theoretically, prevent attachment and entry of the virus into a host cell. Indeed, this type of antibody, termed a neutralizing antibody (nAb), is usually highly induced in the host upon infection. Underlining the importance of nAbs, humoral responses against the virus have been reported wherein anti-HA antibody responses have been found to account for more than half of the directed response (Wrammert *et al,* 2008).

An important fact to note is that these nAbs create an evolutionary pressure on the virus and drive the selection of escape mutants. These mutations occur essentially in the exposed HA1 part of HA and are subsequently transmitted to the progeny (Hedestam *et al,* 2008). These point mutations define what is commonly referred to as antigenic drift where the HA molecule acquires mutations, under selective pressure, in its globular head which would allow escape from recognition by nAbs. Another mechanism for escape from the effect of the nAb is antigenic shift. This has a more serious impact than antigenic drift since with antigenic shift the virus acquires a new HA segment through gene re-assortment with a second influenza A virus (human or non-human) which the host may never have encountered. This can happen, for example, in the case of a co-infection in a duck which is already asymptomatically infected with an avian Flu AV virus.

4. Messenger RNA as a Therapeutic Agent:

Messenger RNA is a made of nucleic acids which, when translated, generate a protein and can be obtained by *in vitro* transcription of plasmid DNA. Immune responses can also be mounted against the protein it encodes rendering its use possible in vaccine therapy. Through the years, the use of mRNA as a therapeutic has presented many challenges due to its highly sensitive nature. As is widely known, it is readily degraded by ubiquitous ribonucleases which are extremely difficult to exclude from lab equipment. Moreover, even when this hurdle is cleared, RNAses are present everywhere within the human body. Hence, mRNA therapy seems, a priori, to be almost destined for failure. Yet, in early 1990, Wolff *et al*, demonstrated that mRNA injected *in vivo* into murine muscle can readily be taken up and expressed by somatic cells. This study laid the foundation for subsequent mRNA research and proved that the concept of mRNA therapy was no longer lacking in possibility (Wolff *et al*, 1990). Conry *et al*, took it a step further by demonstrating that mRNA delivered intra muscularly to mice was expressed and capable of inducing a specific immune response (Conry *et al,* 1995).

Once over the feasibility hurdle, mRNA vaccine therapy showed numerous advantages when compared to more traditional approaches. In direct comparisons of mRNA to DNA, the former has been shown to produce more protein at its peak of expression (Probst *et al,* 2007). Secondly, its susceptibility to endogenous RNAses can also be seen as a positive feature since the mRNA will only be expressed for a defined period of time. In contrast, DNA and other types of vaccines can persist longer in the body generating an unwanted and uncontrolled source of antigen. These concerns over the half-life are not only a serious issue from the physiological point of view, but the prolonged presence of an antigen can be problematic in terms of tolerization of the immune response. Finally, mRNA is not capable of integrating

within the host genome which has been a matter of great concern when it comes to gene therapy with DNA.

Another aspect to consider regarding mRNA therapy is the mechanism of delivery. Four different methods have already been characterized using mice:

1) direct injection of naked mRNA into the site of interest (Conry *et al,* 1995);
2) encapsulation into cationic liposomes followed by injection (Martinon *et al,* 1993); Cationic polymers (e.g. protamine) have also been used as mRNA carriers (Hoerr *et al,* 2000);
3) transfection of *in vitro* matured antigen presenting cells which are subsequently re-injected into patients (Boczkowski *et al,* 1996); and
4) gene gun delivery using mRNA coated gold particles (Steitz *et al,* 2006)

Of these four delivery methods, only the naked (# 1) and transfection (# 3) approaches have been assessed in humans with mixed results (Weide *et al,* 2008; Palucka *et al,* 2006). Recently, Weide *et al,* found that direct injection of protamine-condensed mRNA was feasible and safe in the treatment of metastic melanoma (Weide *et al,* 2009). A brief review of the ongoing clinical trials for mRNA vaccines for use in cancer treatment listed on the National Institute of Health website suggests that this domain of research is still in its infancy with most clinical trials still recruiting or ongoing. As such, it is difficult to fully evaluate the potential uses of mRNA therapy based on clinical studies wherein the patient's immune system is already deregulated. To date, mRNA vaccines have never been applied to infectious diseases such as caused by Flu AV.

5. In Vivo Model:

Developing effective anti-influenza therapeutic and anti Flu AV vaccines requires animal models which are readily available, easy to house and which yield reproducible results. Given these factors, the mouse model is the one most typically used. Moreover, the mouse immune system is also extensively characterized which makes it an ideal model. Another aspect of importance is the easy accessibility to immunological reagents to study humoral and cellular responses in the mouse. Most importantly, mice express TLRs, specifically TLR7 (Zhong *et al,* 2006). Other models are known in the field; however, they are not as well characterized as mice thus making them less attractive as a choice for testing an entirely novel vaccine. Given these factors, the mouse system was the logical and best possible choice for these studies.

B. Materials and Methods:

Source of the HA mRNA

HA mRNA was commercially purchased from Mitoprod (Bordeaux, France) based on the Solomon Islands HA sequence that was provided to them. Mitoprod's mRNA was extracted from yeast which had been blasted with gold particles coated with DNA encoding for HA mRNA (For more information: www.mitoprod.com). All mRNA used in the experiments was from a single batch order.

Transient Transfection to Verify Protein Expression

CHO-K1, ovarian hamster epithelial like cells, were transiently transfected with HA mRNA to verify the expression of the HA protein. Briefly, CHO-K1 cells were plated on coverslips at $2x10^5$ cells/ml in a 12-well plate with complete Ham's F-12 (Wisent Bioproducts, Canada; media containing 10% heat-inactivated FCS, 100U/ml penicillin and 100 µg/ml streptomycin). Following a 48 hour incubation period at 37°C and 5% CO_2 cells were 80% confluent and ready for transfection. RNA transfection of CHO-K1 cells was carried out using a non-lipid cationic reagent (Transmessenger Transfection reagent, Qiagen). Transfection buffer, 94 µl, was mixed with 4 µl of enhancer reagent and 4 µg of HA mRNA in a 2 µl volume for a total final volume of 100µl. After incubation for 5 min at room temperature (RT), 8 µl of Transmessenger was added and incubation was continued for an additional 10 min at RT. Cells were then washed with PBS and resuspended in serum free Ham's F-12 containing the RNA-Transfection reagent mixture for three hours at 37 °C 5% CO_2. The cells were then washed twice with PBS followed by incubation for 18 hours at 37°and 5% CO_2 with fresh complete Ham's F-12.

For flowcytometric analysis, the cells were washed three times in PBS then trypsinized and fixed for 30 min with 500ul of 2% paraformaldehyde in PBS containing 0.01% triton X-100 in 15ml tubes. The cells were then blocked in 1% BSA in PBS for 1 hour at RT at which time a 1:100 dilution of mouse anti-HA primary antibody (Affinity Bioreagents, Cat #MA1-83250) was added directly to the blocking solution for one hour at RT followed by three washes in PBS. Blocking solution containing 1µl of anti-mouse FITC-conjugated secondary antibody (Sigma, Cat # F-4143) was then added and the tubes were incubated for 1 hour at 37°C in the dark followed by three washes in PBS. The cells were then analyzed on a flowcytometer based on 50,000 gated events.

For microscopic analysis, the cells were fixed for 30 min with 500ul of 2% paraformaldehyde in PBS containing 0.01% triton X-100 onto coverslips. The cells were then blocked in 1% BSA in PBS for 1 hour at RT at which time a 1:100 dilution of mouse anti-HA primary antibody (Affinity Bioreagents, Cat #MA1-83250) was added directly to the blocking solution for one hour at RT followed by three washes in PBS. Blocking solution containing 1µl of anti-mouse FITC-conjugated secondary antibody (Sigma, Cat # F-4143) was then added and the plates were incubated for 1 hour at 37°C in the dark followed by three washes in PBS. The slides were then mounted with mounting media and analyzed by fluorescent microscopy.

Analysis of mRNA Attachment to Cationic Liposomes:

Twenty µl of cationic liposomal preparation, as summarized in Figure 4 below, was diluted in 980 µl of distilled water and placed in a cuvette. The cuvette was then placed in a zetasizer (Zetasizer, Malvern instruments) and the solution was analyzed for its overall charge and

particle size. Cationic liposomes are positively charged and mRNA is negatively charged, therefore an indication of attachment is provided by neutralization of the overall charge.

Vaccine Preparation:

1) Cationic Liposomes

Prior to use, all glassware used for the preparation of the vaccine formulations was washed with RNAaseZAP (Sigma cat # R-2020) and rinsed with RNase free distilled water. Commercially available cationic liposomes were purchased from NOF Corporation (Coatsome EL-01-C, Japan). Antigen and adjuvant which were incorporated in cationic liposomes were prepared as described in Figure 4. The components of each vaccine formulation were mixed in the amounts indicated in table 3.

Monophosphoryl lipid A (MPL) was used as an adjuvant. It is an ADP-ribosylating enterotoxin, which is a derivative of lipid A from *Salmonella minnesota* lipopolysaccharide. It is one of the few licensed vaccine adjuvants used in humans (e.g adjuvant used in HPV vaccine). Fluviral is one of the licensed split virion vaccine against seasonal influenza which is used as a positive control in the mouse experiments. Fluviral is an ideal positive control because it is known to trigger robust systemic immune responses. It also serves as a point of comparison since it is a vaccine against influenza that is accepted as a standard of care in clinical medicine. The Fluviral used is the trivalent seasonal influenza vaccine used during the 2007-2008 season and contains two subtypes of influenza A; the H1N1 Solomon Islands/3/2006 and the H3N3 Wisconsin/67/2005; as well as the Influenza B Malaysia/2506/2004 subtype.

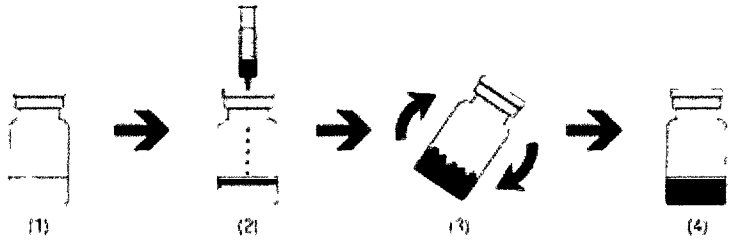


Figure 4: Basic cationic liposome–containing vaccine preparation steps

(1) liposomes are brought to RT;

(2) an aqueous solution containing mRNA-MPL or MPL alone is added to the liposome film at RT;

(3) the vial gently is shaken five times by hand; and

(4) the liposomal preparations are then ready for use

2) Non-Ionic Surfactant Vesicle (NISV)

NISV were prepared using a molar ratio of lipids of **5:4:1** or 22.5 mg of monopalmitoyl glycerol (Larodan AG,Sweden, Cat # 31-1600), 21.5 mg of synthetic cholesterol (Sigma-Aldrich Cat # C9913) and 7.5 mg of dicetyl phosphate (Sigma-Aldrich, Cat # D2631), respectively. The lipid mixture was placed in a flat bottom 10 ml glass beaker, while ensuring none of the powder sticks to the side of the glass beaker. The lipid mixture was then melted in a heated oil bath at 120°C for 10 min, with occasional swirling of the beaker.

While maintaining the melted lipid solution at 120°C, an emulsion was formed by the simultaneous addition of 2 ml of 25mM bicarbonate buffer, pH7.6 (preheated to 50°C). The emulsion was then homogenized for 2 min at 50°C at 8,000 rpm. Hemagglutinin mRNA with or without KLK, and 400 units of RNase inhibitors (Promega), were added while continuing the constant homogenization at 50°C for 8 additional minutes. The vaccines were then cooled to 30°C and incubated for 2 hours on a shaker at 220 rpm.

KLK is an abbreviation for the positively charged KLKL5KLK decapeptide. It is derived from a protein found in the hemolymph of the flesh fly (*Sarcophaga peregrina*) and is known for its antimicrobial effects. Recently, KLK has been shown to be a potent stimulator of Th-2 adaptive immune responses when co-administered with antigens. For the purpose of this study KLK was synthesized via solid phase synthesis on a Symphony Multiple Peptide Synthesizer (Protein Sciences). KLK was purified on a C-18 column (Gilson) and fractions verified via mass spectrometry. The dose used in this study was 10 μg per dose per animal which is noticeably lower compared to several studies which used up to 130 μg of KLK as an adjuvant (Agger *et al*, 2006). A lower dose of KLK was used since an excess amount might impede the translation of HA mRNA by preventing the ribosomes from accessing the mRNA.

Vaccination Schedule 1:

Vaccines were prepared on the day for which vaccination was scheduled to occur. Mice were randomly assigned to one of five groups (n=4 per group). Mice were immunized on day 0, 14 28 subcutaneously at the base of the tail. Each mouse received a total volume of 200µl of vaccine preparation per injection with the exception of the commercial vaccine group which received 50 µl. Table 3 below summarizes *in vivo* group assignment

Vaccination Schedule 2:

The second vaccination schedule was identical to the one described above. Exogenous adjuvants were not included in these vaccine preparations. Each mouse received a total volume of 200µl of vaccine preparation per injection with the exception of the recombinant protein group which received 50 µl. rSolomon Islands is the commercially available recombinant Solomon Islands HA (Immune-tech cat # IT-003-0011p). Table 4 below summarizes *in vivo* group assignment.

Mice Housing:

Female B6C3F1 mice, 6 to 8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA, USA). The animals were housed in accordance with the guideline set forth by the animal care committee of the University of Ottawa. The housing was in a specific pathogen-free, environmentally controlled facility under the Children's Hospital of Eastern Ontario-68-approved protocol. Mice were housed four per cage under a controlled light/dark cycle at a standard RT (22–24 °C).

Group	Immunogen	Adjuvant	Carrier	Dose mRNA	Dose MPL	Route	Schedule Days
Lipo-RNA-MPL	**HA**	**MPL**	**Lipo**	**10μg**	**50μg**	**S.C**	1, 14, 28
RNA-MPL	**HA**	**MPL**	**x**	**10μg**	**50μg**	**S.C**	1, 14, 28
Lipo-MPL	**X**	**MPL**	**Lipo**	**x**	**50μg**	**S.C**	1, 14, 28
Saline	**X**	**X**	**x**	**x**	**x**	**S.C**	1, 14, 28
Commercial	**Fluviral**	**X**	**x**	**2μg**	**x**	**I.M**	1, 14, 28

Table 3: Cationic liposome vaccine preparation and composition

Group	Immunogen	Adjuvant	Carrier	Dose mRNA	Dose KLK	Route	Schedule Days
mRNA/KLK	**HA**	**X**	**x**	**40μg**	**10μg**	**S.C**	0, 14
mRNA/KLK/NISV	**HA**	**X**	**NISV**	**40μg**	**10μg**	**S.C**	0, 14
KLK	**X**	**X**	**x**	**x**	**10μg**	**S.C**	0, 14
NISV/KLK	**X**	**X**	**NISV**	**x**	**10μg**	**S.C**	0, 14
Recombinant protein	**rSolomon Islands (Immune-Tech)**	**X**	**x**	**2μg**	**x**	**I.M**	0, 14

Table 4: NISV liposome vaccine preparation and composition

Serum Collection and Antibody Measurement:

Antibody titers against hemagglutinin were assessed in each mouse. Mice were bled ten days following each immunization and the humoral immune response was assessed by ELISA using the collected mouse serum. Blood was collected in a capillary type tube (Microvette, Sarstedt, Numbrecht, Germany) by puncturing the saphenous vein with a needle. Blood, 200 µl, was collected and samples were centrifuged at 14000 rpm for ten minutes to obtain serum, after blood sample was allowed to coagulate. ELISA was used to measure HA-specific IgG responses. 96 well plates (Corning COSTAR catalog # 2592) were coated overnight at 4°C with recombinant Solomon Islands 3/2006 (Immune-tech cat # IT-003-0011p) at a final concentration of 2.5µg/ml or with commercial seasonal inactivated flu vaccine (Fluviral 2007-2008). The next day, the wells were washed three times with 300 µl of PBS+0.05% Tween-20 (Sigma, Cat # P7949) and blocked for 3 hours at RT with 10% goat serum in PBS. The plates were then incubated with a 1:40 dilution of the sera at 37°C for 1 hour. Plates were then washed three times with 300 µl of PBS+0.05% Tween-20 and incubated with HRP conjugated anti-mouse anti-total IgG, anti-IgG1 or anti-IgG2 (Biorad) for 1 hour at 37°C. Plates were then washed three times with 300 µl of PBS+0.05% Tween-20 and developed with 100 µl of TMB substrate (BioFX Laboratories) for 20 min at RT. Then, 100 µl of TMB-Stop solution (BioFX Laboratories) was added to stop the reaction. Plates were then read on a microplate reader (Biorad) at 450nm.

MDCK Cell Line:

Madin-Darby Canine Kidney (MDCK) cells are of adherent cells of canine origin. They are derived from the kidney and bear an epithelial morphology. They were purchased from ATCC (Cat # CCL-34)

Microneutralization (MNA) Assay:

Animal serum samples were serially diluted 4-fold in 96-well flat-bottomed plates (Corning COSTAR cat # 2592) with a 50 µl final volume in IMDM media supplemented with 2% FCS and 1% penicillin/streptomycin. Wells designated as the virus control (virus alone) received 50 µl of 2% FCS-IMDM. Wells designated as the cell control (MDCK cells alone) received 100 µl of 2%FCS-IMDM. All remaining wells received 50 µl of diluted Solomon Islands influenza virus (1:100 of 1.10^5 pfu). Sera and virus were incubated together for 2 hours at 37°C, unless otherwise noted. $1x10^5$ MDCK cells/well were then added to all wells of a 96-well plate, and incubated overnight for 18-22 hours. The next day, cell media was decanted and plates were washed once in 200 µl of PBS and cells were fixed with 100 µl of cold (4°C) 80% acetone for 10 min, at RT. Acetone was then removed and plates were air-dried for 20 min to ensure complete evaporation of the fixative. Plates were then washed 5 times in PBS + 0.05% Tween-20 (Sigma) and incubated for 1 hour in the dark at RT with 100 µl of biotinylated influenza A NP (Chemicon) diluted 1:2000 in 5% FBS in PBS. Plates were then washed five times in PBS-Tween-20 and incubated for 1 hour in the dark at RT with 100 µl of streptavidin-HRP conjugated anti Influenza A NP secondary antibody (Upstate Cell Signaling, USA) diluted 1:10,000 in 5% FBS/PBS. Plates were then washed again five times in PBS-Tween-20 and developed for 12 min at RT with 100 µl of TMB substrate (BioFX Laboratories). 100 µl of TMB-Stop solution (BioFX Laboratories) was added then to stop the reaction. Plates were then read on a microplate reader (Biorad) at 450nm.

Preparation of Splenocytes from Mouse Spleen:

Mice were euthanized by injection of 6.5 mg of Euthansol (Schering-Plough, Canada) into the intra-peritoneal (I.P) cavity. Blood was taken from the heart by cardiac puncture (for sera analysis) and spleens were surgically removed and placed in eppendorf tubes containing sterile PBS. Spleens were then processed into cellular suspensions by gently pressing the spleen with a plastic pipette through a 100 μm nylon mesh (BD Biosciences) into a 50 ml sterile falcon tube (BD Biosciences): twenty ml of PBS (Wisent Bioproducts, Canada) was added to aid the passing of the cells through the mesh. The tubes were then centrifuged at 1600 rpm at 4°C for 5 min. The supernatant was removed and the cellular suspensions were depleted of red blood cells (RBC) by the addition of 10 ml of RBC lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) for 5 min at RT. The cells were then centrifuged at 1600 rpm for 5 min at 4°C after the addition of 10 ml of warmed RPMI 1640 (Wisent Bioproducts, Canada). The supernatant was then removed and the cells were washed two more times with 10 ml of complete RPMI. The cells were then counted using a haemocytometer and divided into several fractions and frozen at -80°C in freezing media containing 10% dimethyl sulphoxide (DMSO, Sigma-Aldrich, Canada cat # D1435) and 90% fetal calf serum (FCS, Wisent Bioproducts, Canada).

Confirmation of RNA Stability:

mRNA was extracted from NISV preparations using the RNAeasy extraction kit according to the manufacturer's protocol (Qiagen). mRNA was eluted from the column using 50 μl of RNAse free water and run on a 1% agarose gel in 1X MOPS (Sigma). RNA stability was defined as the maintenance of the expected size upon the agarose gel.

mRNA Quantification:

mRNA was extracted from the NISV and run on an agarose gel as described above. The kit used to extract mRNA from the gel was purchased commercially (QIAquick Gel Extraction Kit, Qiagen). The band corresponding to intact mRNA was then excised from the agarose gel with a scalpel. The gel slice was then soaked in TE buffer for 25 min at room temperature with gentle shaking. The gel slice was then removed from the TE buffer, and placed it in a colorless tube and mixed with six volumes of buffer QG based on the gel weight (W/V). The mixture was then incubated at 58°C for 25 min with vortexing every 2–3 min during the incubation. The mixture was then applied to the provided QIAquick column and centrifuged for 60 seconds at 13OOO rpm. The column was then washed with 750 µl of buffer PE and spun for 60 seconds at 13OOO rpm. The column was then placed in a 1.5 µl microcentrifuge tube. RNA was then eluted by adding 50µl of RNAse free water in the column and centrifugation at 13000 rpm for one minute.

mRNA concentration in the eluate was analyzed using the nanodrop 3000 (Thermo Scientific). 2µl of each sample were pipetted onto the measurement pedestal.

Virus Purification:

Virus was harvested from the allentoic fluid of eggs infected with Solomon Islands virus. The fluid was clarified by centrifugation at 6000 rpm for 15 min at which time 8% of PEG (W/V) (Sigma) was added followed by 1 hour of agitation at 4°C. Virus was pelleted at 7000 RPM for 20 min at 4°C. The pellet was then dissolved in 10 ml of PBS with 1mM EDTA then overlaid on a sucrose gradient (10 ml 30% sucrose, 5 ml 45% sucrose, 5 ml 60% sucrose). The tube was then centrifuged for 3 hours at 24,000 RPMs (without braking).The milky band at 45-60% interphase was drawn horizontally using a 22 gauge needle mounted on a 5 ml

syringe. The virus was dissolved in 30 ml of PBS and pelleted for 1 hour at 24,000 RPM. PBS was then drained and the pellet was resuspended in 3 ml of PBS and sonicated.

Splenocytes Proliferation Assay:

Splenocytes were isolated from the vaccinated mice as described above and dispensed at $2x10^6$ cells per ml in complete RPMI 1640 (Wisent Bioproducts, Canada) and cultured in U-bottom 96-well tissue culture plates at $2x10^5$ cells per well. The cells received 100 µl of media containing either 0.2µg of recombinant Solomon Islands HA protein, 0.2µg of Fluviral or 0.02 µg of phytohemagglutinin as a positive control (PHA-P, Sigma Cat # L1668) or media without any stimulant. The final volume per well was 200 µl. The cells were then incubated 120 hours at 37°C and 5% CO_2. After 120 hours, each well was pulsed with 50 µl of complete RPMI containing 0.4 µCi (14.8 kBq) of [*methyl*-^{3}H]thymidine (Amersham Pharmacia Biotech). The plates were then returned to the incubator for an additional 16 hours before assessment of the radioactivity by scintillation counting. The ΔCPM (counts per minute) were determined by subtracting the background activity of cells incubated with only medium from the activity of cells incubated with medium containing antigen.

$KLKL_5KLK$ (KLK) Assay:

One µg of mRNA was incubated for 5 min in 5 µl of PBS containing different amounts of KLK: 1µg, 0.5µg, 0.25µg and 0.125µg. Following 5 min, additional 5 µl of PBS containing 5% fetal bovine serum was added and incubated at RT. At 10, 30 and 60 minutes, samples were withdrawn and mixed with 10 U of RNase inhibitor (Promega) and frozen at -70 °C until analysis. 4 µl DEPC-treated vaccination buffer containing 10 U of proteinase K and 4 µl DEPC-treated PBS containing 20% SDS and 1µl of loading dye was added. The entire sample

was then thawed, loaded and analyzed on a 1% agarose gel in DEPC-treated Tris-Borate-EDTA buffer.

Effects of KLK on mRNA Translation:

2 μg of mRNA were incubated for 5 min with different amounts of KLK: 2 μg, 1 μg and 0.5μg. Then CHO-K1 cells were transiently transfected with HA mRNA to verify presence of the HA protein within the cells. Prior to this, CHO-K1 cells were plated on coverslips at $2x10^5$ cells/ml in a 12-well plate with complete Ham's F-12 (Wisent Bioproducts, Canada; media containing 10% heat-inactivated FCS, 100U/ml penicillin and 100 μg/ml streptomycin). Following a 48 hour incubation period at 37°C and 5% CO_2 cells were 80% confluent and ready for transfection. RNA transfection of CHO-K1 cells was carried out using a non-lipid cationic reagent (Transmessenger Transfection Reagent, Qiagen). Transfection buffer, 94 μl, was mixed with 4 μl of enhancer reagent and the RNA/KLK mixture (2 μl) for a total final volume of 100μl. After incubation for 5 min at RT, 8 μl of Transmessenger was added and incubation was continued for an additional 10 min at RT. Cells were then washed with PBS and resuspended in serum free Ham's F-12 containing the RNA-Transfection reagent mixture for 3 hours at 37 °C 5% CO_2. The cells were then washed twice with PBS followed by incubation for 18 hours at 37°and 5% CO_2 with fresh complete Ham's F-12. The cells were then washed three times in PBS then trypsinized and fixed for 30 min with 500ul of 2% paraformaldehyde in PBS containing 0.01% triton X-100 in 15ml tubes. The cells were then blocked in 1% BSA in PBS for 1 hour at RT at which time a 1:100 dilution of mouse anti-HA primary antibody (Affinity Bioreagents, Cat #MA1-83250) was added directly to the blocking solution for 1 hour at RT followed by three washes in PBS. Blocking solution containing 1μl of anti-mouse FITC-conjugated secondary antibody (Sigma,

Cat # F-4143) was then added and the tubes were incubated for 1 hour at 37°C and 5% CO_2 in the dark followed by three washes in PBS. The cells were then analyzed on a flowcytometer based on 50000 gated events.

Plaque Reduction Assay:

First, Solomon Islands/3/2006 influenza A virus was diluted to 600 pfu/ml. Serum was also diluted at final concentration of 1:40 and 1:80 then 100 µl of serum and 100 µl of virus were mixed together in a 96-plate for 1 hour. This procedure was repeated for each bleed from each animal. A virus control, which received 100 µl of PBS instead of serum, was also included. In the mean time MDCK cells plated two days prior to the experiment were prepared as follows. Media was removed from each well of each 6-well plate which were subsequently rinsed with PBS. Then 100 µl of virus and sera mixture was added to the corresponding well on a 6-well plate. A virus control and cell control (which receives 100 µl of PBS) were included on each plate. The cells were then incubated for 1 hour at 37° with 5% CO_2 The plates were rocked back and forth every 15 min to ensure proper distribution on the cells and to avoid drying of the cell layer. After 1 hour, each well was covered by a layer of 0.65% agarose in MEM media containing 2% Pen/ Strep, L-glutamine, carbonate buffer, and 2% TPCK trypsin. Tosyl Phenylalanyl Chloromethyl Ketone (TPCK) is an inhibitor of chymotrypsin. The presence of TPCK is necessary since the activity of chymotrypsin is detrimental to infection. The plates were then placed in incubator for two days at 37°C with 5% CO_2 After two days, the plates were fixed with a mixture of methanol and acetic acid (3:1) for 30 min. The fixative was then rinsed off and the agarose discs removed from each well. One milliliter of 0.1% Crystal violet stain solution was added to each well for 5 min. Plates were then washed and left to dry. Plaques were then counted.

C.Results

I) Confirmation of HA mRNA Expression:

1) Flow cytometry:

Before starting the vaccination studies, inherent aspects of the mRNA had to be confirmed. One central factor was to confirm that the synthetically designed mRNA was functional.

In this regard, Chinese hamster ovary (CHO) cells, which lack constitutive expression of HA, were transfected with the modified HA mRNA to confirm that the added stabilizing elements, consisting of the internal ribosomal entry site (IRES) and the poly A tail, did not impede HA expression. As depicted in Figure 5(A) below, two transfected cell samples were analyzed for the expression of HA protein by flowcytometry. The HA mRNA transfected sample yielded higher FITC fluorescence in comparison to the mock treated sample which received identical reagents with the exception of HA mRNA. This increase is representative of HA mRNA protein expression and confirms that the synthetic mRNA is capable of inducing HA protein.

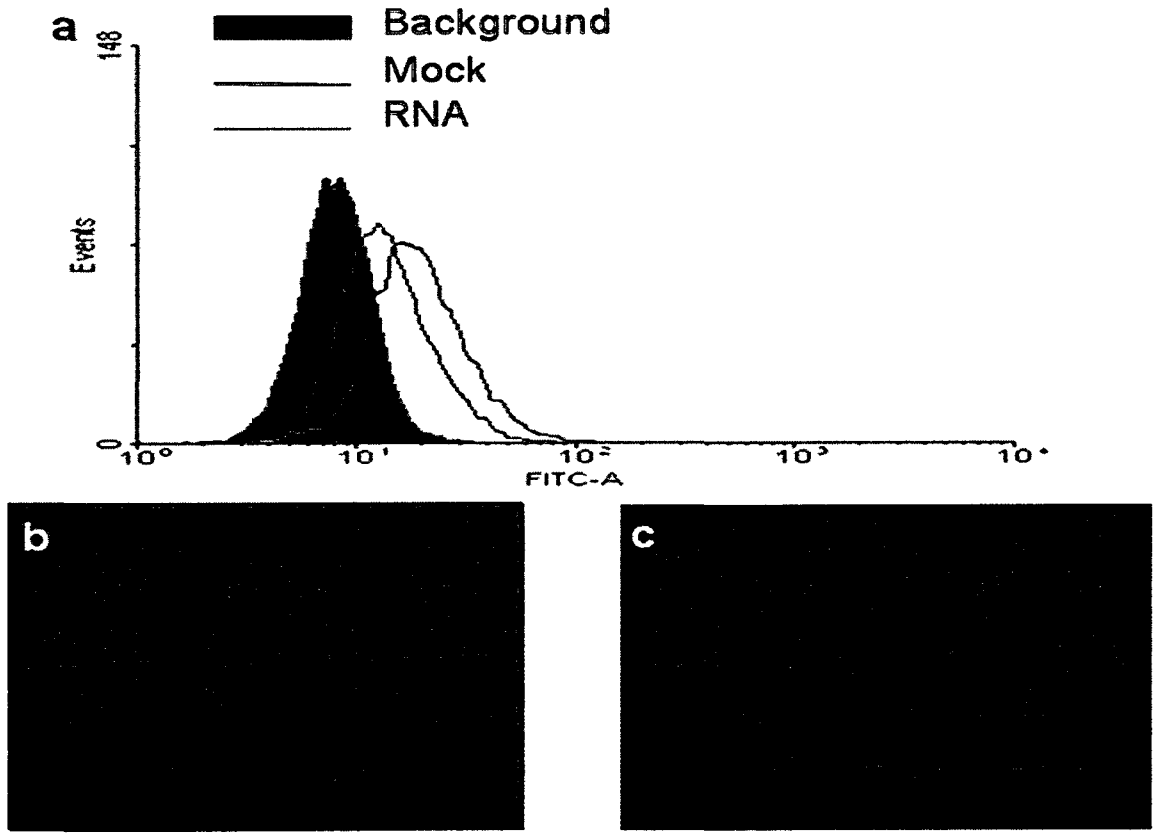
2) Immunocytochemistry:

As discussed above, flowcytometry results demonstrated the expression of HA protein which suggests that the mRNA was functional. Yet, the levels of protein expression were not of a great magnitude. Accordingly, another method of protein detection was examined to rule out any technique-specific artifacts. Mock and mRNA transfected cells were both analyzed under a microscope using immunofluorescence to detect HA protein expression. As observed in Figure 5(B) and Figure 5(C) below, mRNA transfected cells, in contrast to the mock transfected cells, clearly exhibit higher fluorescence levels indicative of HA protein expression.

Hence, both approaches confirmed the functionality of HA mRNA expression as evidenced by the presence of the HA protein.

Figure 5. Confirmation of HA protein expression in CHO-K1 cells

HA protein expression was verified in CHO-K1 cells 24 hours post-transfection with 2μg of mRNA. HA protein levels were assessed by **(A)** flowcytometry and **(B, C)** immunocytochemistry **(B)** mock transfected, 20x magnification; **(C)** HA mRNA transfected, magnification 20X.


a
Background
Mock
RNA
148
Events
0
10°
10¹
10²
10³
10⁴
FITC-A
b
c

II) Vaccination with mRNA in Cationic Liposomes

1) mRNA presence in cationic liposome's preparations

mRNA instability is a concern when it comes to its handling and especially when formulating it into a lipidic carrier. Characterization of the vaccine formulation that contained mRNA and the cationic liposome was necessary since the presence of the mRNA had to be ensured following the preparation of the liposome. Therefore, a quantification of the overall charge combined with a measurement of the carrier's stability was carried out. As summarized in Table 5 below, a one in fifty dilution from the liposome preparation containing mRNA, liposome and MPL was run through a zeta sizer. In addition, a sample containing the same components but without the mRNA was run in parallel as a direct point of comparison. This technique readily allows for the monitoring of the overall charge and stability of the lipid particles. As a point of reference, any particle, liposomes in this instance, with an overall charge greater than to 25mV is considered stable. Predictably, liposomes devoid of mRNA showed a charge of 43.6 mV indicative of a good stability (see Table 5 below). This charge was expected since these liposomes are made of cationic lipids, which confer a positive charge to the particle. More importantly, the sample that received the mRNA showed a decrease in the overall charge indicating that the binding of the negatively charged mRNA to the liposome was neutralizing some of its charge. Moreover, the addition of mRNA didn't appear to affect the overall stability of the liposomes since the 36 mV charge observed was still above the 25mV cutoff reference point.

Table 5. mRNA Coupling to cationic liposomes and stability

The attachment and stability behavior of a 1/50 dilution of cationic liposome preparations in water were assessed in the presence and absence of HA mRNA using a zetasizer. The overall charge was measured in millivolts (mV).

	Liposome	
	mRNA	empty
Amount (µg)	10	0
Charge (mV)	36	43.6

2) Immune responses in mRNA vaccinated mice

a) Anti-HA humoral immune responses

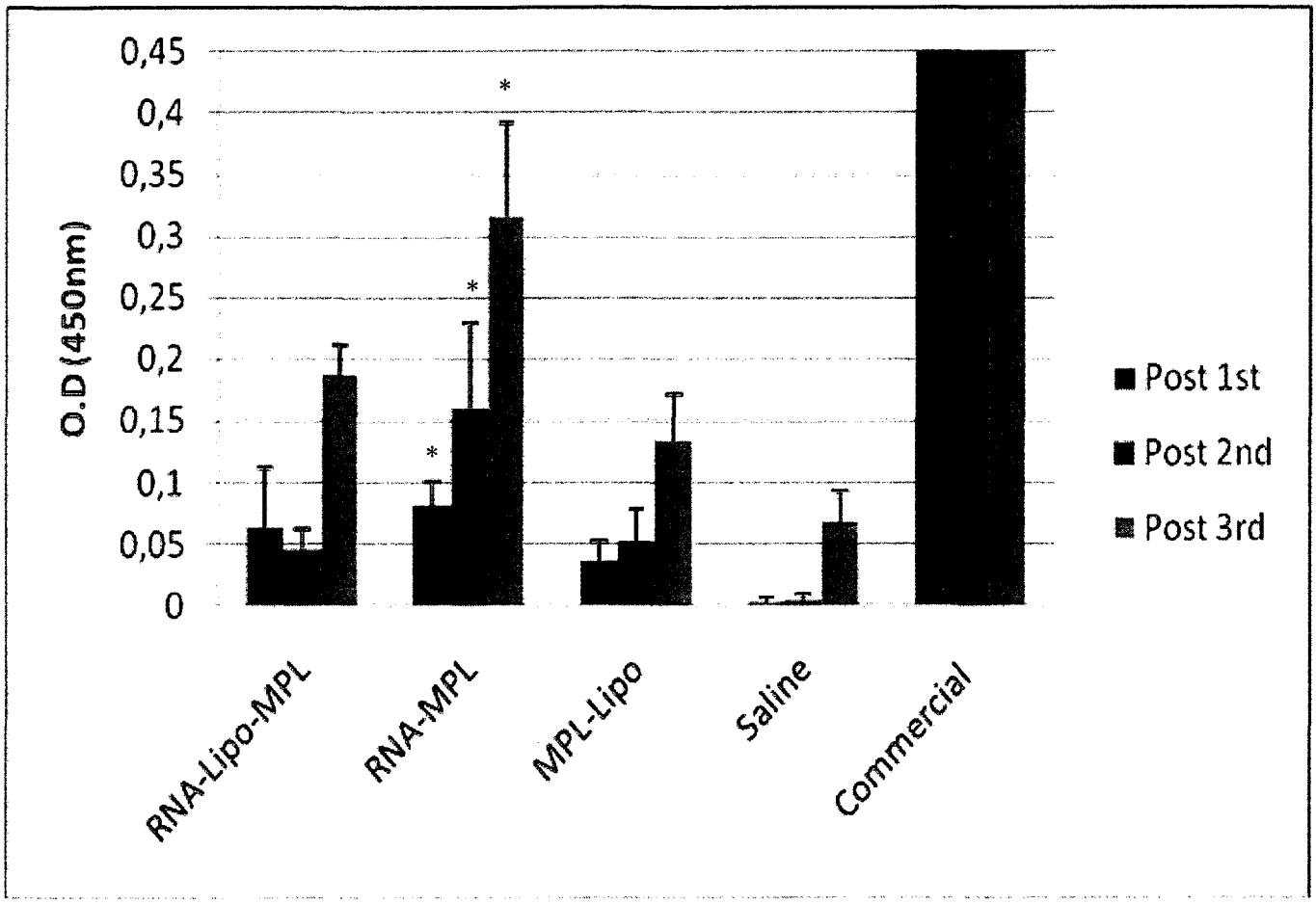
The next step was to demonstrate that an immune response could be raised against HA through mRNA vaccination. As described in the Method section, B6C3F1 mice were vaccinated three times with differing formulations. Ten days after each vaccination, blood was drawn and serum was isolated to be used in the ELISA detection of anti-HA IgG antibodies. The rationale underlying this experiment lies in the very nature of the virus itself. The virus requires an intact HA protein to attach and enter the host cell therefore antibodies directed against HA protein have the potential to block viral entry.

ELISA data suggested an increasing trend in anti-HA serum IgG levels in each of the groups that received a formulation containing HA mRNA (see Figure 6 below). Statistical analysis by T-test comparing the RNA-MPL to the saline group and the Lipo-RNA-MPL to the Lipo-MPL group did revealed statistical significance only for the RNA-MPL group.

Another measurement of the humoral responses triggered against HA is the quality of these antibodies with regard to their capacity to neutralize viral entry in target host cells. To examine this aspect, a microneutralization assay was conducted based on pooled samples from the vaccinated mice. In brief, the virus was pre-incubated with serum obtained from vaccinated mice for 1 hour and then added to MDCK cells. The next day, the presence of the virus inside the cells was detected by the presence of nucleoprotein (NP). A decrease in signal would indicate a decreasing amount of NP.

Figure 6. Measurement of anti-HA total IgG levels in the serum of vaccinated mice

Four vaccinated groups, RNA-Lipo-MPL, RNA-MPL, MPL-Lipo and Saline were analyzed post first, second and third round of vaccination for anti-HA total IgG levels in serum. IgG levels were then quantified as optical density (O.D). Absorbance was read at 450 nm with an ELISA plate reader. Each bar represents the average of four mice minus the average of the absorbance yielded by the pre-vaccination samples for each group along with the SEM. Statistically significant differences in comparison to the saline group, with $p \leq 0.05$, are indicated with an asterisk *.


0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
O.D (450nm)
*
*
*
RNA-Lipo-MPL
RNA-MPL
MPL-Lipo
Saline
Commercial
Post 1st
Post 2nd
Post 3rd

This decrease in signal would suggest that antibodies present in the serum are capable of binding the HA protein and preventing the virus from attaching and entering the cell. This is an indirect detection since the presence of nAb would prevent the infection of MDCK cells which would ultimately result in decreased amounts of NP inside the cells.

Immunized mice revealed no increase in neutralization in comparison to the negative controls while, as anticipated, the group which received the commercial vaccine showed high levels of neutralization (see Figure 7 below) paralleled by high levels of anti-HA IgG levels in ELISA (see Figure 6 above). The absence of significant neutralization in the groups that received mRNA was expected since low anti-HA titers were detected (in ELISA).

Overall, there were very weak antibody responses induced in animals which received naked mRNA or mRNA encapsulated in a lipidic carrier.

b) Anti-HA cellular responses

i) Lymphocyte proliferation

Two weeks after the final vaccination, vaccinated and control mice were sacrificed and splenocytes were isolated. Splenocyte memory responses were investigated by examining their capacity to proliferate in response to HA protein antigen stimulation. Splenocytes were stimulated with various sources of HA protein and cells were labeled with [^{3}H] thymidine. This assay used a radioactive form of thymidine which is a nucleoside essential for DNA synthesis. Splenocytes that proliferate in response to HA antigen stimulation will replicate and incorporate the radioactive thymidine. Splenocytes from each mouse were separately incubated with three sources of antigen: recombinant commercial HA protein, Fluviral or an in-house developed peptide comprised of a plurality of Solomon Islands HA epitopes. Fluviral

and the peptide were included because of the possibility that preservatives found in the recombinant protein might negatively affect splenocytes proliferation. Interestingly, the MPL-RNA group was the only one that showed significant ($p<0.01$) proliferation in response to the three different sources of antigen when stimulated with Fluviral in comparison to the negative controls (see Figure 8 below). Unexpectedly, liposome protected mRNA did not yield any lymphocyte proliferation responses.

3) Summary of the first vaccination strategy

mRNA vaccination was able to elicit antiviral humoral and cellular responses as demonstrated in figure 6 and 8. Vaccination with mRNA adjuvanted with MPL elicited significant increases in antibodies and splenocyte proliferation. Moreover, only small amounts of HA mRNA, typically considered a sensitive and unstable antigen, were required to induce immune responses against influenza. Incorporation of mRNA into a liposomal carrier, assumed to further protect its integrity, did not induce significant immunity. It remains unclear if the mRNA was damaged during formulation or, alternatively, if encapsulation of the mRNA had an inhibitory effect in the immune response or whether the mRNA was never captured by antigen presenting cells when entrapped in the type of liposomes used in this study. The data suggest that the provision of an additional activating signal, such as the TLR4-induced signal conferred by MPL, in combination with mRNA led to anti-HA humoral and cellular immunity. However, the assay used for detection of neutralization activity in the serum failed to reveal neutralizing antibodies post-immunization. It is possible that the neutralization assay requires refinement in order to be more sensitive or that this immunization strategy also requires refinement and that it is not robust enough to result in stronger humoral or cell-mediated antiviral immunity in its present state of development.

Figure 7. Detection of neutralizing antibodies against Solomon Island's influenza virus

Detection of HA neutralizing antibodies (nAb) was measured using a microneutralisation assay. Five vaccinated groups, RNA-Lipo-MPL, RNA-MPL, Lipo-MPL, saline and commercial were analyzed post first, second and third round of vaccination for anti-HA nAbs in serum. HA nAbs were then quantified indirectly through NP's absence as optical density (O.D). Absorbance was read at 450 nm with an ELISA plate reader.

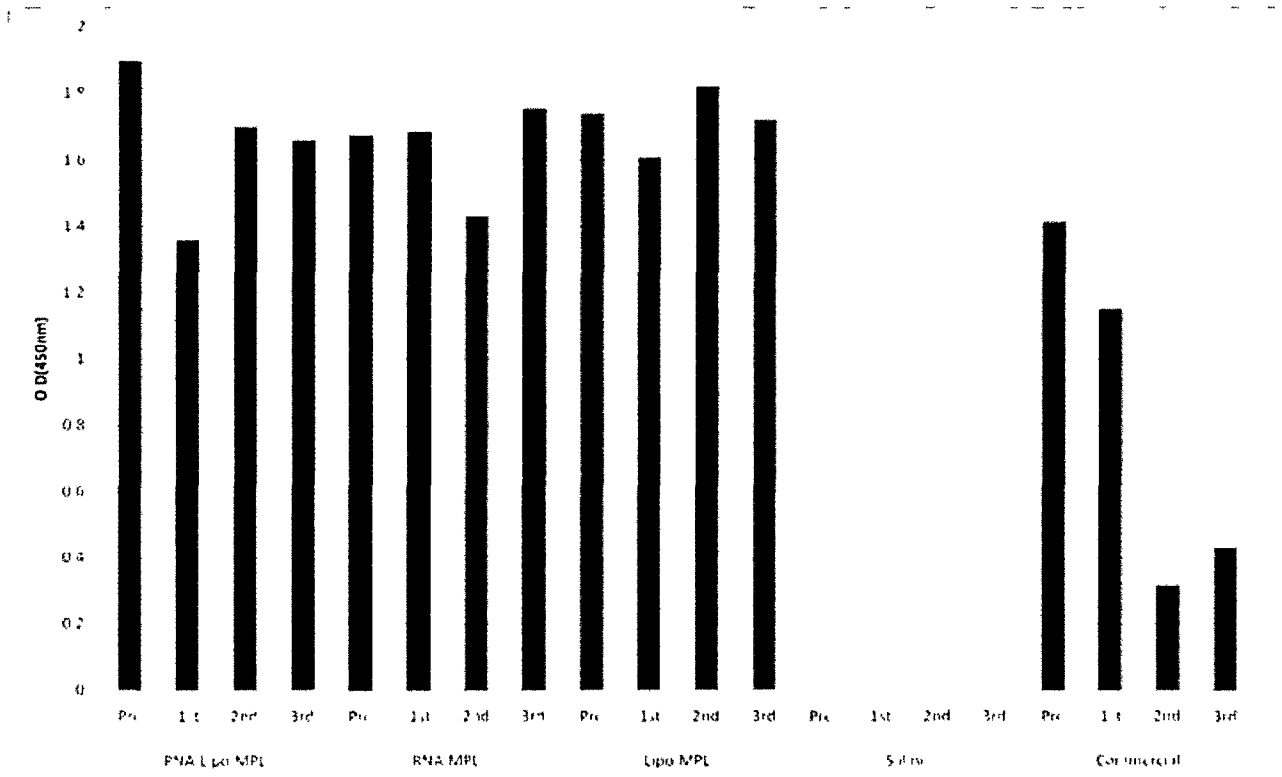
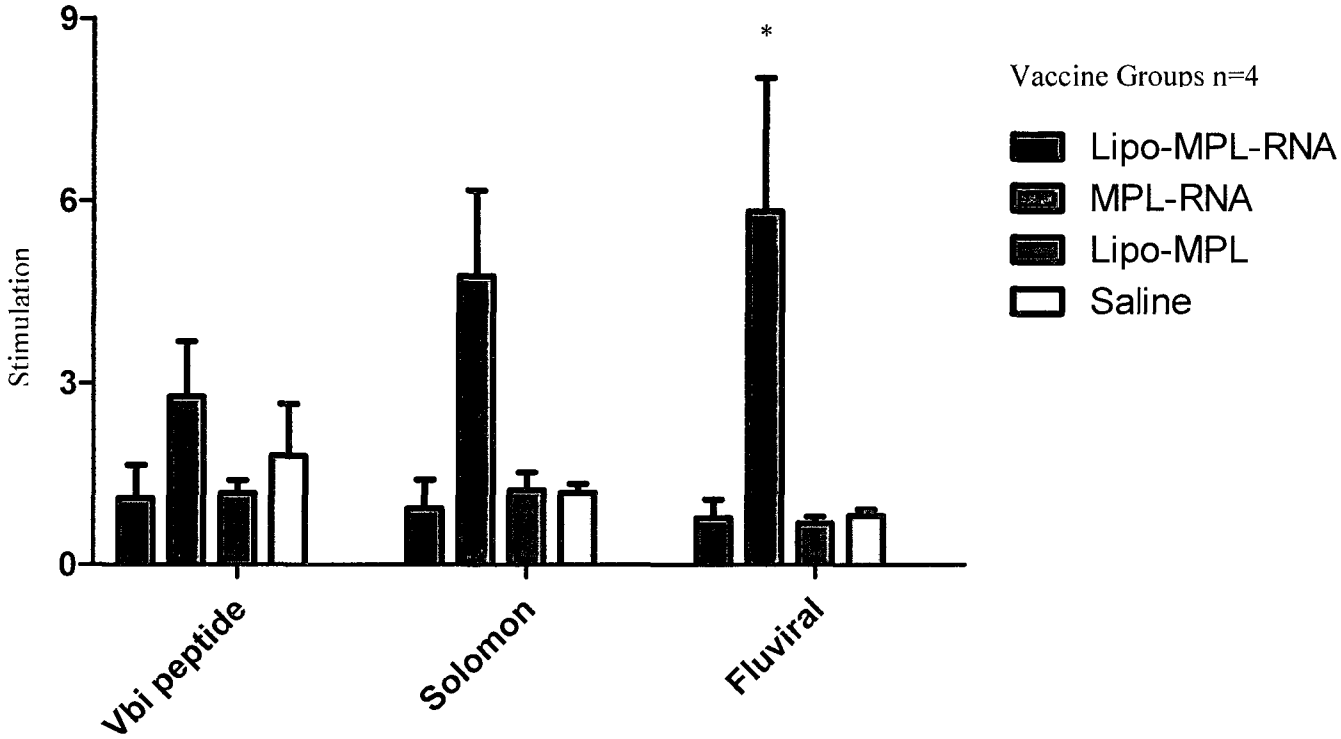

O D(450nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Pre
1st
2nd
3rd
Pre
1st
2nd
3rd
Pre
1st
2nd
3rd
Pre
1st
2nd
3rd
Pre
1st
2nd
3rd
PNA-Lipo-MPL
PNA-MPL
Lipo-MPL
Saline
Commercial

Figure 8. Hemagglutinin-specific spleenocyte proliferation responses in mice immunized with several vaccine formulation containing HA mRNA

Lymphocyte recall responses to various antigens were analyzed by thymidine incorporation for each of the Lipo-MPL-RNA, MPL-RNA, Lipo-MPL and Saline vaccinated groups. Spleenocytes from immunized animals were incubated with various antigens: VBI peptide, which consists of variable peptide sequences of HA epitopes, including H1N1 Solomon Island; recombinant H1N1 Solomon Island HA and Fluviral vaccine antigens. Results were expressed as stimulation index (S.I.) which was calculated by dividing the mean cpm of cells incubated with stimulants by the cpm of cells incubated with medium alone. Each bar represents the average S.I. from the groups of immunized mice and the standard error (SEM). A significant difference was only found between the immunized RNA-MPL group and saline control, which was stimulated with Fluviral antigens ($p < 0.01$ indicated with an asterisk*).



Antigens used for spleenocyte stimulation

III) Vaccination with Peptide and/or NISV Protected mRNA

Due to the relative failure of the first immunization strategy another vaccination approach was sought.

1) mRNA protection with KLK

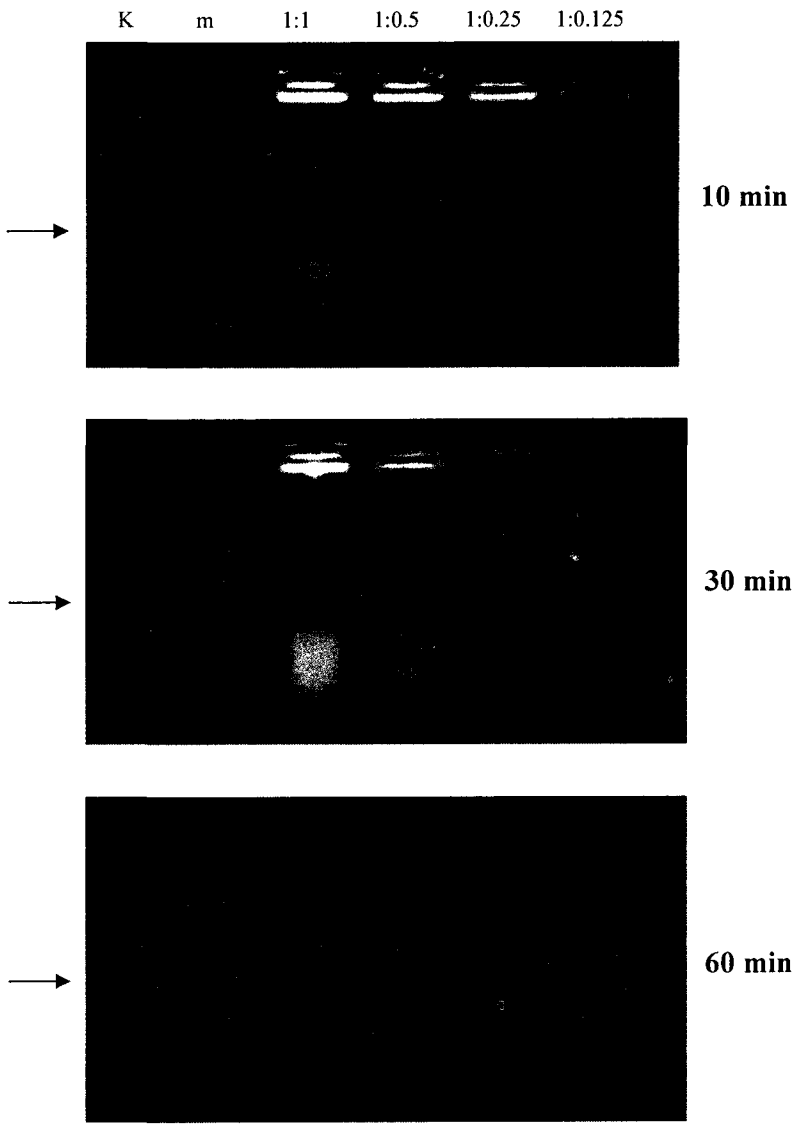
The KLK peptide is derived from a protein of the flesh fly (*Sarcophaga peregrina*) and is known for its antimicrobial effects. Recently, KLK has been shown to be a potent stimulator of Th-2 adaptive immune responses when co-administered with antigens (Agger et *al,* 2006; Schellak *et al,* 2006). Its overall positive charge, however, is what makes KLK an attractive option for these studies. mRNA, which is intrinsically negatively charged, can in theory be bound by the KLK peptide, by virtue of electrostatic interactions, and be protected from degradation by ubiquitous nucleases when injected into the animal.

As demonstrated, when 1 μg mRNA was mixed with decreasing amounts of KLK and subjected to PBS containing serum (which includes RNases), substantial protection from degradation was observed (see Figure 9 below). The protection was proportional to the amount of KLK used to shield the mRNA. Naked mRNA was degraded within 10 min whereas KLK protected mRNA was maintained for at least 30 min (see Figure 9 below). The presence of condensed mRNA at the top of each lane suggests that KLK was not efficiently separated from the mRNA prior to loading on the gel even though it was treated with a protease and heated to 85°C. This trend suggests a strong interaction between KLK and mRNA.

Interestingly, a second band underneath the intact mRNA band seemed to be present in all conditions. This band might correspond to degradation as mentioned above or to possible contamination of the mRNA preparations.

Figure 9. Influenza Hemagglutinin mRNA degradation and KLK-protection assay

mRNA degradation in serum was analyzed on an agarose gel. One μg of naked HA mRNA was incubated with varying amounts of KLK (W/W ratios) in the presence of 5% fetal calf serum. KLK transiently protected the HA mRNA at different KLK concentrations. **K**= KLK alone, **m**= mRNA alone, 1:1, 1:0.5, 1:0.25 and 1:0.125 represent the different ratios of mRNA to KLK in micrograms. The arrow points toward the expected migration site of the full-length hemagglutinin mRNA (~1.9 Kb).


K
m
1:1
1:0.5
1:0.25
1:0.125
10 min
30 min
60 min

2) Expression of KLK-protected HA mRNA

Before continuing with additional studies, it was necessary to determine if mRNA shielding by KLK prevented the expression of the mRNA. To investigate this question, CHO cells were transfected with 2μg of HA mRNA mixed with decreasing amounts of KLK. In addition, two groups were included as points of comparison: mock (no mRNA) and mRNA only (no KLK).

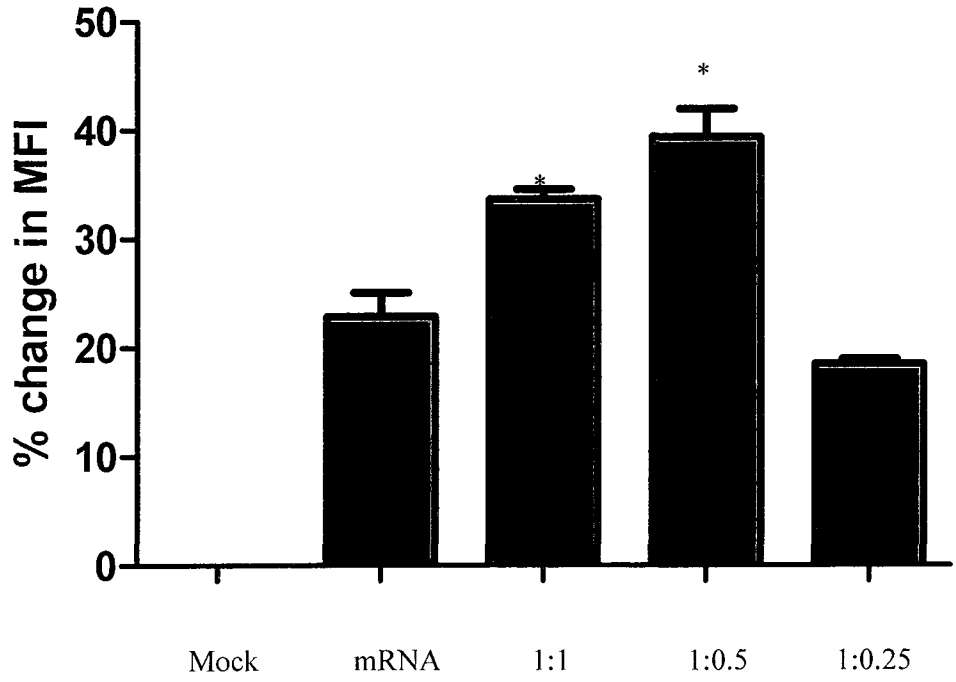
As expected, CHO cells which received naked mRNA displayed the presence of HA protein (see Figure 10 below). More importantly, cells which received KLK-protected mRNA in a 1:1 and 1:0.5 (mRNA:KLK) ratios contained significantly higher amounts of HA protein in comparison to mRNA alone. This may be due to the prolonged availability of mRNA in the cell which allows for its translation into protein for extended periods of time in comparison to the availability of the unprotected mRNA.

Figure 10. KLK protection of mRNA and effects on expression in CHO cells

The protective effects of KLK on mRNA expression were analyzed by flow cytometry. HA protein expression was verified in CHO-K1 cells 24 hours post-transfection with 2μg of mRNA and decreasing amounts of KLK. 1:1, 1:0.5 and 1:0.25 represent the different ratios of mRNA to KLK. a: raw data from one representative experiment, b: values presented as percentages of change in the mean fluorescence intensity (MFI) in comparison to the mock transfected sample (n=3). Statistically significant differences in comparison to the mRNA only transfected group, with $p \leq 0.01$, are indicated with an asterisk*

A	MFI
MOCK	6652
mRNA	7945
1:1	8777
1:0.5	8942
1:0.25	7840

F



3) mRNA presence after NISV preparation:

For the second vaccination strategy, a different type of lipid carrier vesicle, termed NISV, was investigated since cationic liposomes appeared to have detrimental effects on mRNA immunogenicity (see Figures 6 to 8 above). NISV, which are non-charged liposomes with low reactogenicity and toxicity, were prepared with mRNA that was previously adsorbed to KLK. It should be noted that the procedure for NISV preparation is harsh in regards to the stability of a fragile molecule, such as mRNA. The preparation involves chemicals that are not certified RNAse free and there is heating for prolonged periods which can be detrimental for a sensitive molecule like mRNA. Therefore, the first step in this second vaccination strategy was to ensure that the mRNA was still intact at the end of the NISV preparation procedure.

To test the integrity of the mRNA, NISV containing mRNA and KLK (as formulated for the vaccine preparation) were prepared and tested for mRNA degradation. mRNA was extracted from loaded NISVs using a commercial mRNA extraction kit then the RNA was run on an agarose gel. Figure 11a below, indicates the presence of HA mRNA.

The band corresponding to intact HA mRNA was cut from the gel and the nucleic acid was extracted for mRNA/KLK/NISV group in order to assess the entrapment efficiency of undamaged RNA. Figure 11b summarizes the entrapment efficiency of two mRNA/KLK/NISV preparations.

Going forward this data was critical, since it confirmed the presence of entrapped mRNA in the NISV and that this lipid/nucleic acid formulation could be evaluated as a potential vaccine candidate.

Figure 11. Confirmation of the integrity of entrapped influenza hemagglutinin mRNA in non-ionic surfactant vesicles (NISV) after its formulation

The influenza A hemagglutinin mRNA (40 μg) was entrapped in NISV following a standardized procedure. The mRNA was extracted from 50 μl of the mRNA-KLK-NISV (10 μg) and 50 μl of the KLK-NISV preparation containing no mRNA. Two extra groups which consisted of HA mRNA entrapped in NISV (10μg) and unentrapped HA mRNA were added as controls. A:The extracted nucleic acid was run on a 1% agarose gel in MOPS buffer (MW indicates the corresponding molecular weights in bases). The arrow indicates the predicted molecular weight of the mRNA (~1.9Kb). The mRNA lane corresponds to unprocessed mRNA. B: The band corresponding to intact HA mRNA was extracted from each lane and quantified. Entrapment efficiencies were also calculated (n=2).

A

B

	Amount (in 50µl)	Efficiency (%)
Sample 1	7.1	71
Sample 2	6.9	69

4) Immune responses in mice vaccinated with Influenza HA mRNA

a) Anti-HA humoral immune responses

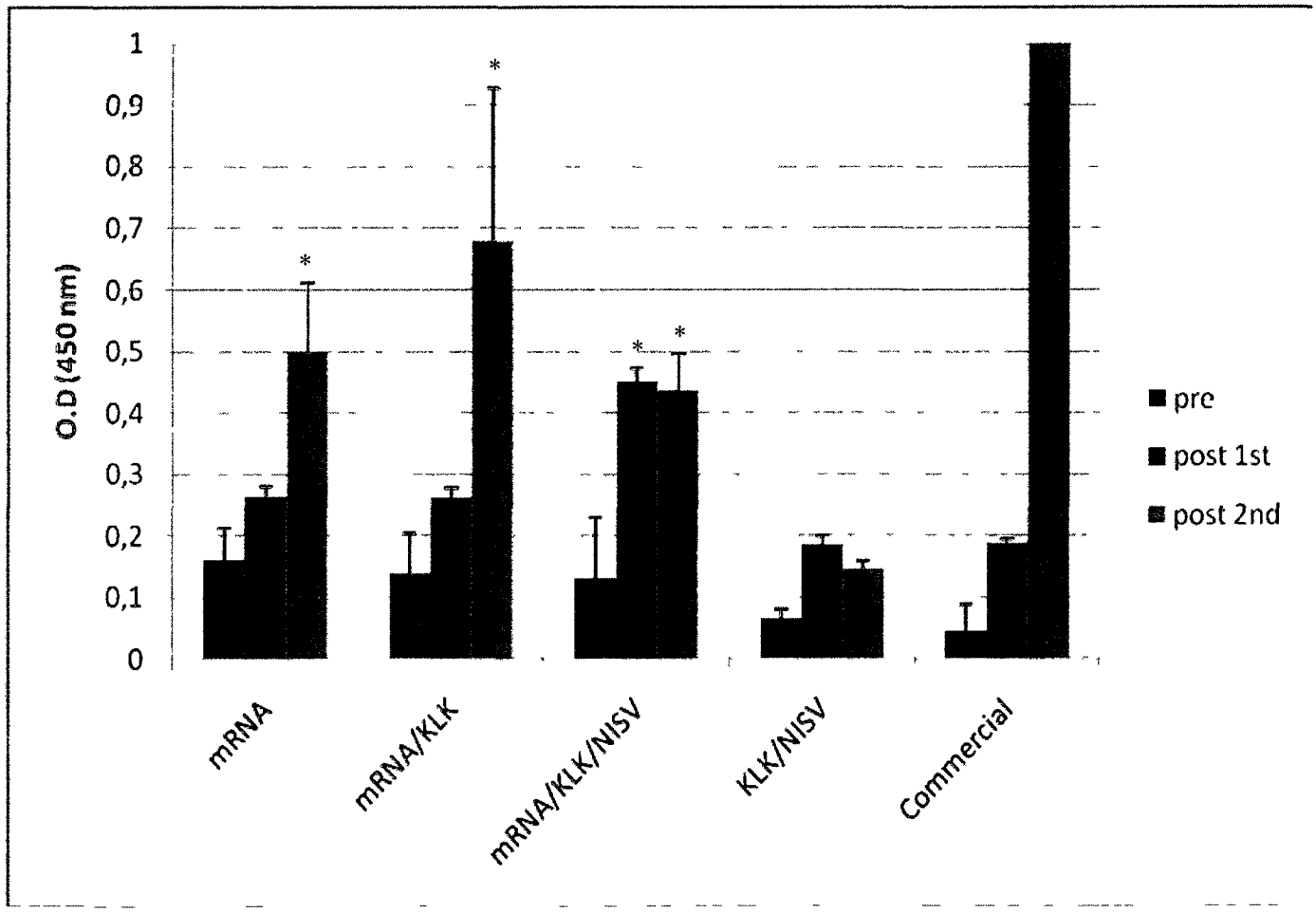
i) Total IgG

The first vaccination strategy showed that mRNA vaccination did not induce potent immunity derived from the antigen encoded by the mRNA. Hence, the vaccination strategy had to be modified in order to improve the overall immune response. For this purpose mRNA was either protected with a cationic peptide (KLK), or with NISV, and injected into B6C3F1 mice subcutaneously. Mice were divided into several groups and received two doses of a vaccine formulation as it is described in the Methods section (see Table 4). Ten days after each vaccination, blood was drawn and the serum was isolated to be used subsequently in ELISA for detection of anti-HA IgG antibodies.

All groups that received mRNA in their vaccine showed substantial increases in total anti-HA IgG as early as the first vaccination (see Figure 12 below). The KLK protected mRNA demonstrated the anticipated response that correlated with the KLK protection assay data described earlier as it showed the highest titers after the second vaccination (see Figure 9 and Figure 10 above). Interestingly, the NISV entrapped mRNA showed robust antibody titers as early as the first vaccination compared to the naked or KLK protected mRNA.

Figure 12. Measurement of anti-HA total IgG levels in the serum of vaccinated mice

Four vaccinated groups, mRNA, mRNA-KLK, mRNA-KLK-NISV and KLK-NISV were analyzed post first and second round of vaccination for anti-HA total IgG levels in serum which was then quantified as optical density (O.D.). Absorbance was read at 450 nm with an ELISA plate reader. Each bar represents the average of four mice along with the SEM. Statistically significant differences in comparison to the KLK/NISV group, with $p \leq 0.05$, are indicated with an asterisk *.


O.D (450 nm)
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
*
*
*
*
mRNA
mRNA/KLK
mRNA/KLK/NISV
KLK/NISV
Commercial
pre
post 1st
post 2nd

ii) IgG subtyping

The antibody subtype, specifically within the IgG antibody class, raised against a specific antigen is indicative of the bias of the triggered immune response (Th1 or Th2 bias). Therefore, the IgG subtype present was investigated after each mRNA vaccination. IgG1, which is considered to be an indication of a Th2-type humoral response, was found to follow the same kinetics as the total anti-HA IgG (see Figure 12 above) with a significant increase following second vaccination (see Figure 13 below).

As for IgG2a, this subtype indicates a bias towards a Th1-type immune response. A quantification of IgG2a was carried out and the outcome was similar to the trend seen in the total IgG analysis with a significant peak after the second vaccination (see Figure 14 below).

In addition to the analysis of the IgG kinetics, the IgG subtypes, including IgG1 and IgG2a, were also evaluated to determine whether there was a polarization of the immune response. mRNA vaccinated groups seem to favor the predominance of an IgG2a response. This bias indicates that immunization with mRNA-loaded NISVs elicit a Th1-dominated cellular immune response. Indeed, in comparison to the commercial vaccine, it is clear that in contrast to the Th2 type response generated with the commercial influenza vaccine, vaccination with mRNA induces predominantly a Th1-biased response.

Figure 13. Analysis of anti-HA serum IgG1 in immunized mice

Four vaccinated groups, mRNA, mRNA-KLK, mRNA-KLK-NISV, KLK-NISV and commercial were analyzed post first and second round of vaccination for anti-HA IgG1 levels in serum which was then quantified as optical density (O.D). Absorbance was read at 450 nm with an ELISA plate reader. Bars represent averages of IgG1 ELISA titers in serum samples of individual immunized mice along with the SEM. Statistically significant differences in comparison to the KLK/NISV group, with $p \leq 0.05$, are indicated with an asterisk *.

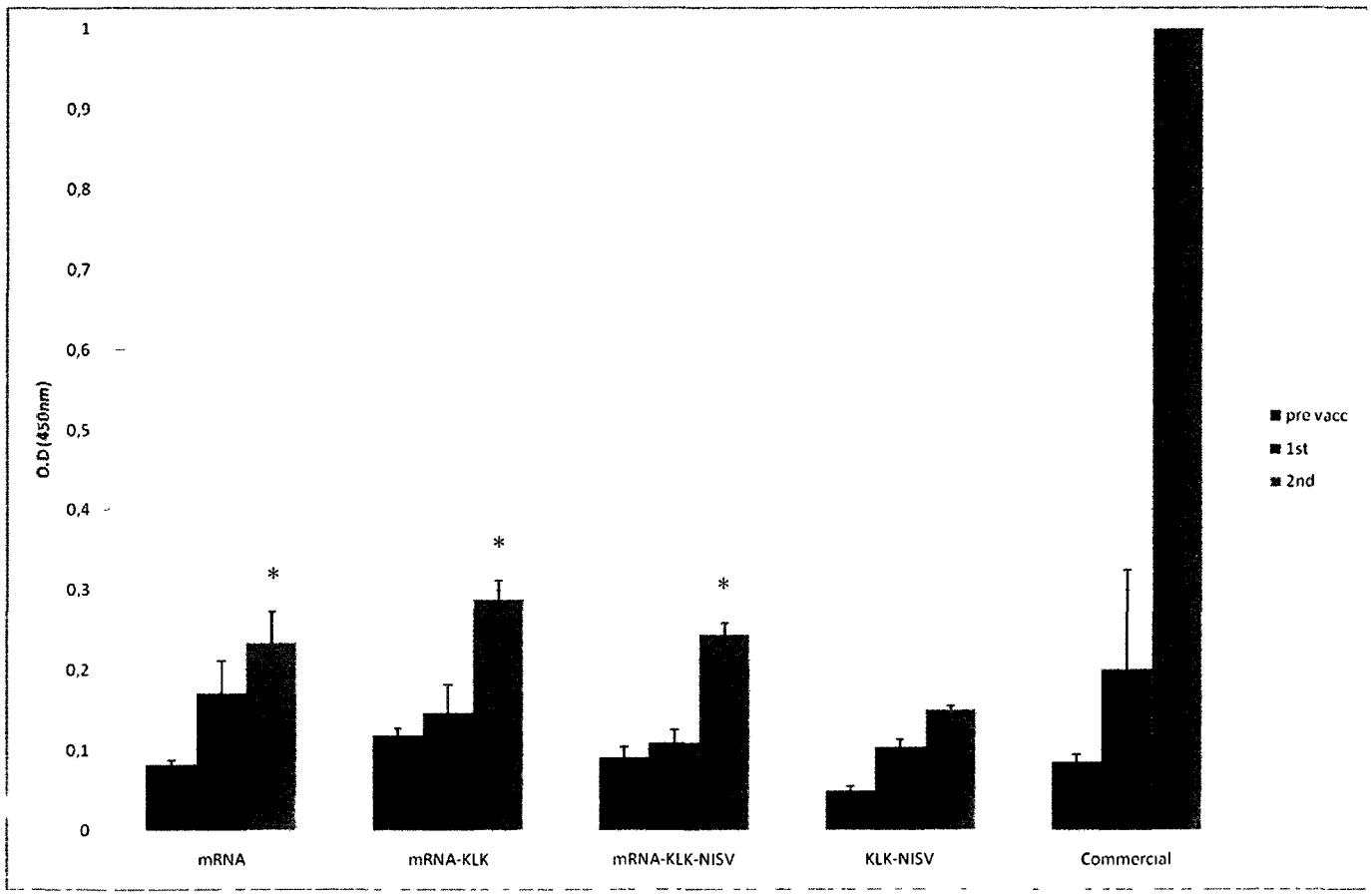
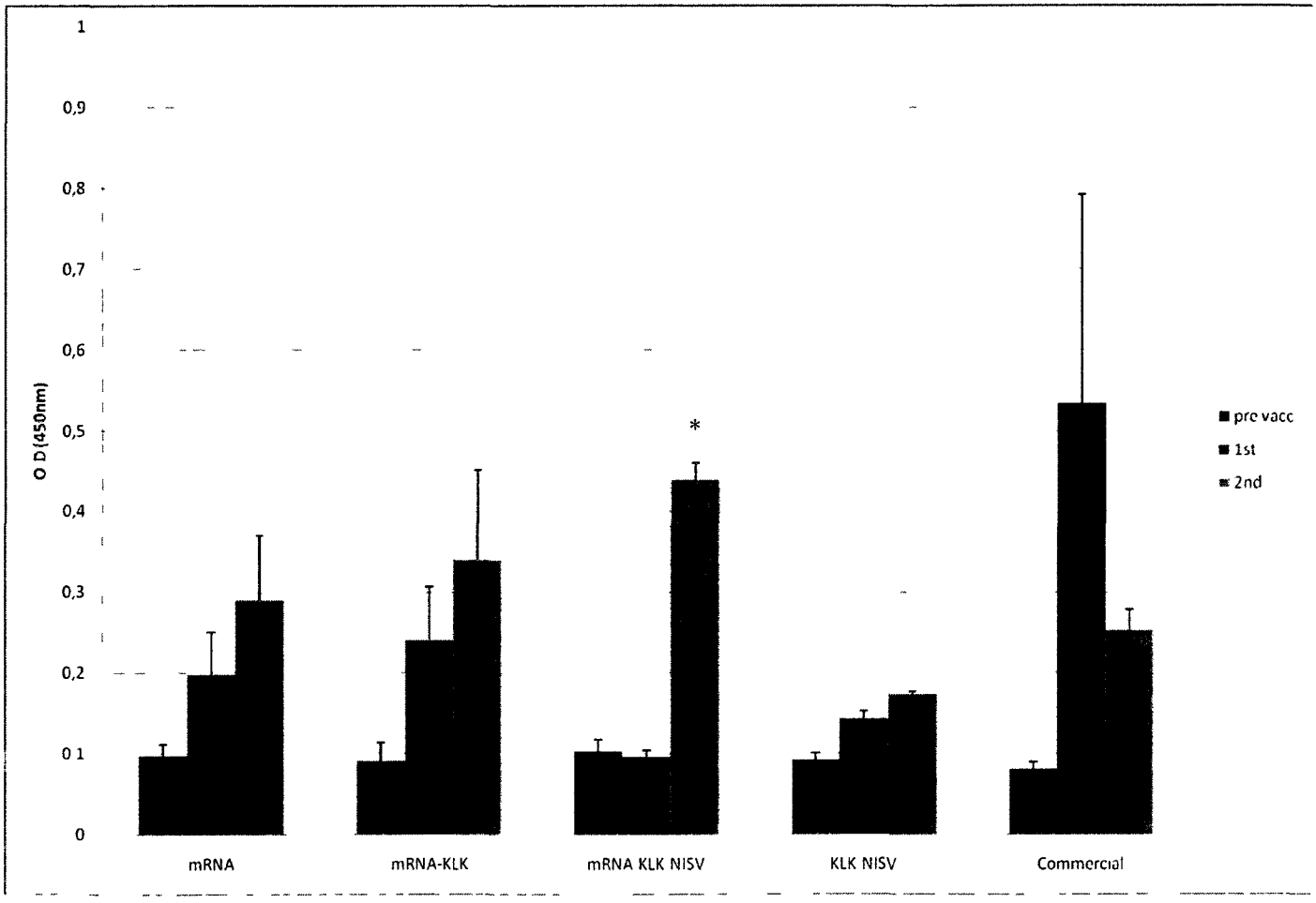

1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
O.D (450nm)
*
*
*
mRNA
mRNA-KLK
mRNA-KLK-NISV
KLK-NISV
Commercial
pre vacc
1st
2nd

Figure 14. Analysis of anti-HA serum IgG2a in immunized mice

Four vaccinated groups, mRNA, mRNA-KLK, mRNA-KLK-NISV, KLK-NISV and commercial were analyzed post first and second round of vaccination for anti-HA IgG2 levels in serum which was then quantified as optical density (O.D.). Absorbance was read at 450 nm with an ELISA plate reader. Bars are averages of IgG2a ELISA titers in serum samples of individual immunized mice along with the SEM. Statistically significant differences in comparison to the KLK/NISV group, with $p \leq 0.01$, are indicated with an asterisk *.

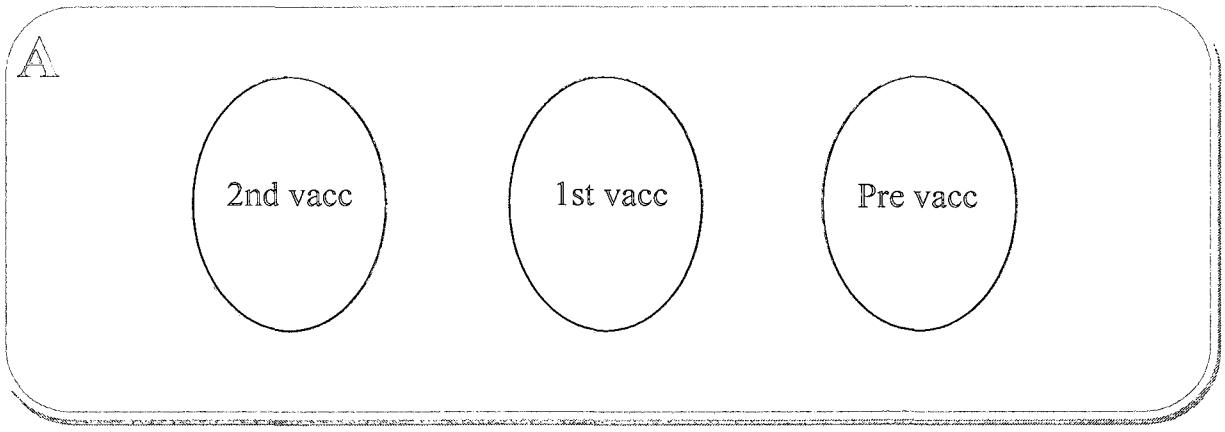

1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0 1
0
O D (450nm)
*
pre vacc
1st
2nd
mRNA
mRNA-KLK
mRNA KLK NISV
KLK NISV
Commercial

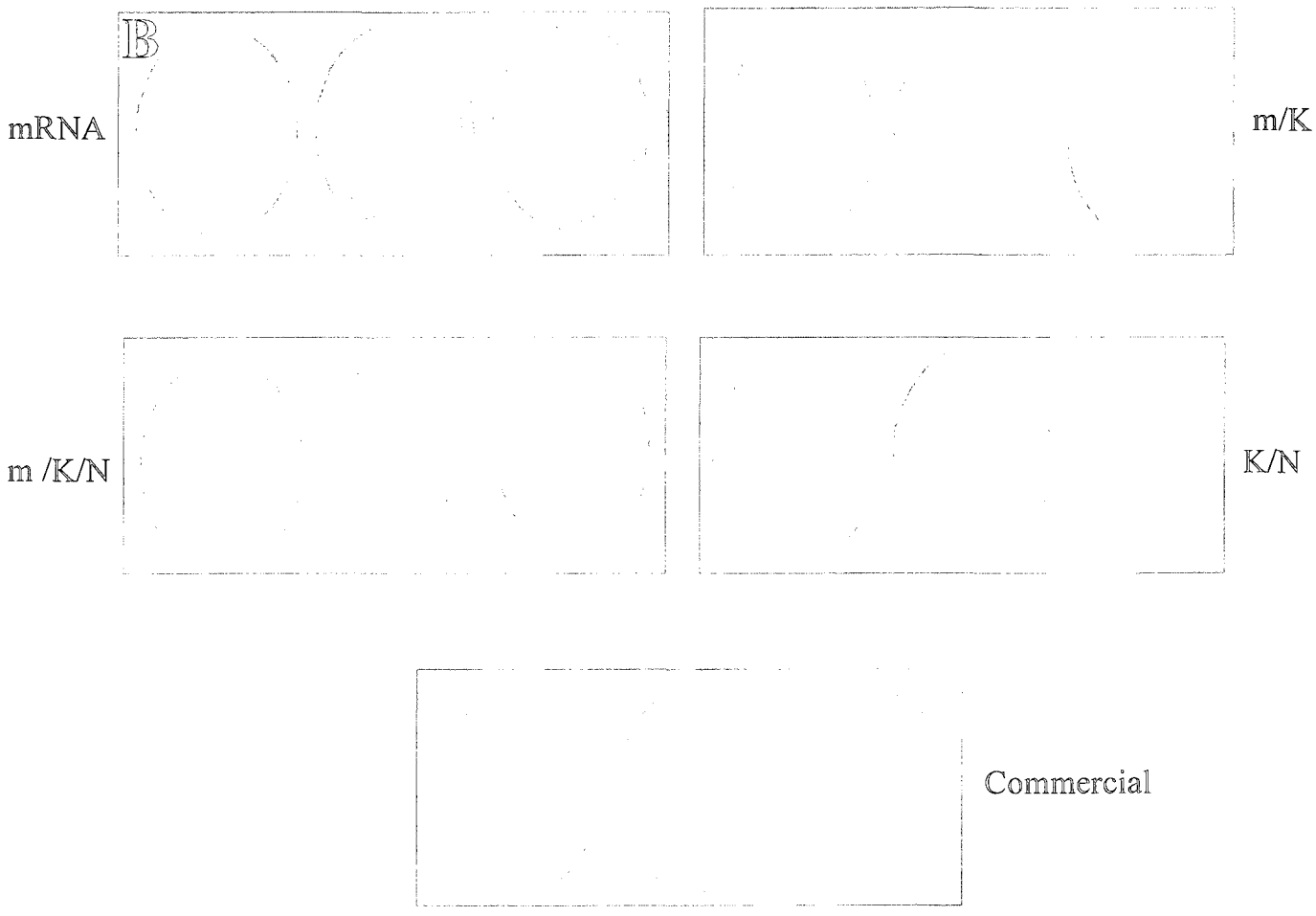
iii) Neutralization

The role of a specific class of anti-HA antibodies was mentioned earlier in reference to their capacity to prevent viral entry into the host cell. With the first vaccination strategy, nAbs were not detected in any of the groups examined (see Figure 7 above). The titers of antibodies, however, detected in the second vaccine approach were significantly higher which increased the chances of detecting nAbs if they were present. This class of antibodies was further examined using a different and more sensitive type of assay in contrast to the microneutralization assay used during the studies of the first vaccine approach: the plaque reduction assay. A decrease in plaques suggests a diminished infectivity of the virus which is mediated by antibodies present in the serum capable of binding the HA protein and preventing the virus from attaching and entering the cell. In keeping with these higher sensitivity characteristics and the appreciable amounts of antibodies detected by ELISA, easily detectable levels of neutralization was observed in each group which carried mRNA in its formulation as mirrored by the decreased amount of plaques (see Figure 15 below).

Figure 15. Solomon Islands virus-neutralizing activities of the serum antibodies from vaccinated mice

Detection of neutralizing antibodies (nAbs) was measured by a plaque reduction assay. Four vaccinated groups, mRNA, mRNA-KLK (m/K), mRNA-KLK-NISV (m/K/N), KLK-NISV (K/N) and recombinant Solomon protein were analyzed pre, post first and second vaccination for anti-HA nAb in serum at a serum dilution of 1:40. A: Plate Layout, B: Results


A
2nd vacc
1st vacc
Pre vacc


B
mRNA
m/K
m /K/N
K/N
Commercial

Overall, animals that received mRNA in any form (naked, with KLK or with NISV) showed marked increases in anti-HA antibodies that appeared to reach a maximum level of expression following the second round of vaccination. The use of a protective KLK peptide, entrapment in NISV and the use of higher amounts of mRNA induced humoral immunity after just one vaccination which also correlated with the presence of neutralizing antibodies. Interestingly, even though anti-HA antibodies levels were similar after one or two vaccination, neutralization was only detected after the second vaccination. In summary, the conditions and approaches used in the second vaccine study allowed for the detection of induced humoral immunity which was not detectable using the conditions and approaches of the first vaccine study.

b) Anti-HA cellular responses

i) Lymphocyte proliferation

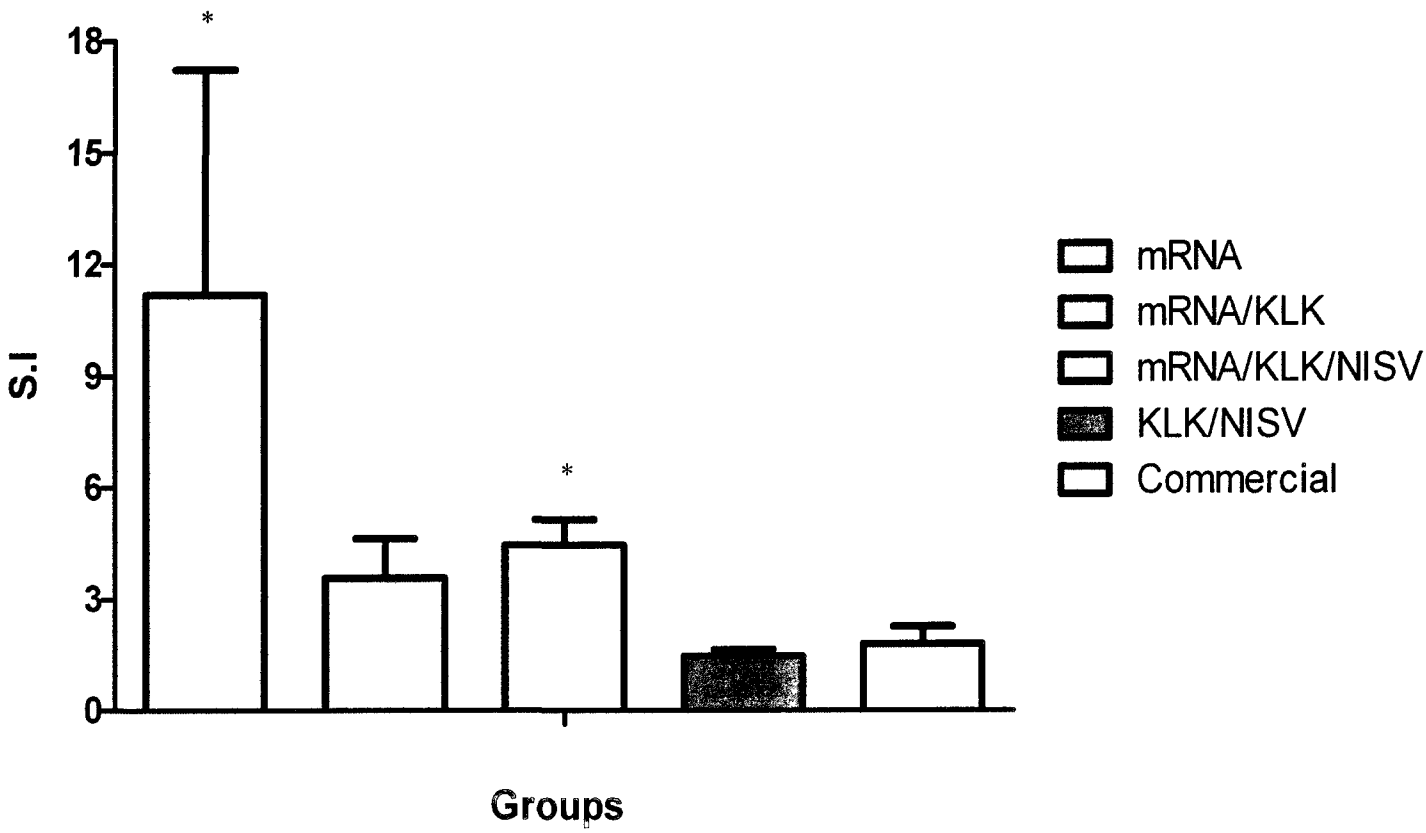
Two weeks after the final vaccination, vaccinated and control mice were sacrificed and splenocytes were isolated. Spleenocyte memory responses were investigated by their capacity to proliferate in response to HA protein antigen stimulation. This was carried out as described above using the thymidine incorporation method. In this study the whole inactivated Solomon Islands virus was used as a stimulant in place of recombinant protein. The reason behind this change is that the sodium azide contained in the commercial recombinant HA protein was thought to be detrimental to the stimulation of the cells.

Using this approach, all mRNA containing groups demonstrated enhanced HA-specific T-cell proliferative responses with particular significance for the naked and NISV complexed mRNA groups (see Figure 16 below). Vaccination with mRNA alone induced the strongest cellular response, but the weaker antibody response. In summary, use of the KLK and NISV

modalities together rapidly (after just 1 vaccination) induced positive antibody and cellular immune responses.

Figure 16. Solomon Islands specific splenocytes proliferation responses in immunized mice

T-cell lymphocyte recall responses to heat inactivated Solomon Islands virus were analyzed by thymidine incorporation for each of the following: mRNA, mRNA-KLK, mRNA-KLK-NISV, KLK-NISV and commericla. Results were expressed as a stimulation index (S.I.) calculated by dividing the mean cpm of cells incubated with stimulants by the cpm of cells incubated with medium alone. Each bar represents the average S.I. along with the SEM from the grouped immunized mice. Statistically significant differences in comparison to the KLK/NISV group, with $p \leq 0.01$, are indicated with an *.


*
18
15
12
9
6
3
0
S.I
*
Groups
mRNA
mRNA/KLK
mRNA/KLK/NISV
KLK/NISV
Commercial

D.Discussion:

The use of mRNA as a therapeutic continues to intrigue the scientific community due to the relatively untapped potential and sensitivity of mRNA. This sensitivity, however, constitutes a double edged sword: a short half-life is ideally sought for any foreign composition of matter introduced into the body but the foreign composition of matter also must remain present long enough to accomplish the desired effects. In the present studies, it was of primary importance that the mRNA be available long enough to raise an immune response against the antigen it was encoding. In this regard, optimized mRNA (e.g. improved for codon usage and stability) constitutes an ideal candidate capable of fulfilling these requirements. Several studies in the vaccine field have demonstrated that RNA, whether it be naked, coated on metal particles, in liposomes or transfected *in vitro* in dendritic cells, is a potent gene vaccination vehicle (Pascolo, 2008). Furthermore, mRNA can be produced in substantial GMP quality amounts which will readily allow for the further development and advancement of mRNA-based therapies. Recent clinical trials conducted in humans have demonstrated that the delivery of mRNA, naked or transfected, in dendritic cells is capable of inducing the expected antigen-specific immune response (Weide *et al,* 2009).

The full potential of mRNA, as an antigen coding vehicle, has only been investigated in relation to cancer therapeutic vaccines and never in the context of infectious diseases. The experiments of the present study were designed to examine the potential of mRNA as an adjuvant and as an antigen-coding vehicle through the use of an HA-encoding mRNA vaccine against Flu AV.

1) Overall conclusions:

The outcome of the vaccination approaches (naked, in lipidic carriers or complexed with cationic peptides) demonstrated the feasibility and efficacy of an mRNA vaccine against Flu AV. Vaccination with mRNA, free of any lipidic carrier, was able trigger both cellular and humoral responses. Complexing the mRNA, however, to a protective charged peptide and further entrapment within a non-ionic liposome induced the strongest immunity, which was balanced between humoral and cellular immunity, including induction of antibodies with neutralizing (functional) activity. This observed parallel between the humoral and cellular responses is of great importance since it is extremely rare that vaccines are found which stimulate both arms of the adaptive immune response. For example, the commercial seasonal inactivated Flu AV vaccine triggers significant serum anti-HA IgG titers and yet results in poor cellular immunity. The cellular arm of the adaptive immune response is suspected to be the key factor in providing cross protection against challenge by heterologous Flu AV strains. It is likely the presence of the cellular response which gives an edge to mRNA vaccination in contrast to the subunit and split particle vaccines which typically result in poor cellular immunity.

With regard to influenza, the efficacy of whole and live attenuated versus subunit and split particle vaccines has been mainly attributed to the presence of viral ssRNA which vastly improves immunity to the HA and NA components of the seasonal vaccine. In contrast, the vaccines of the present study differ from traditional vaccines by combining the advantageous aspects of both the attenuated/whole and the split/subunit vaccines which consist of a natural adjuvant (ssRNA) and the major protein antigen (HA). Theoretically, an mRNA-based vaccine could comprise all the advantages of the live attenuated vaccine while simultaneously

discarding the common fears associated with the use of a live attenuated vaccine. In addition to mRNA's intrinsic activity and benefits, a vaccine approach which consists of mRNA as a single component vaccine would facilitate any potential manufacturing, regulatory or licensing concerns.

One major aspect, which is often overlooked in the development of novel vaccine technology, is the importance of activating the innate immune system. mRNA binds naturally to TLR7 on the surface of professional antigen presenting cells (e.g. plasmacytoid dendritic cells) which in turn trigger the secretion of significant amounts of inflammatory cytokines and trigger an anti-viral response which is central in combating influenza infection, particularly Flu AV infection. Hence, mRNA vaccination as used in the present studies, in contrast to split and subunit Flu AV vaccines, is capable of simultaneously activating the two major components of the immune system (innate and adaptive).

The mRNA used in the present studies received structural modifications to improve its stability and expression. These modifications included an IRES to circumvent cap-dependent translation and an extended poly-A tail for translation and stability purposes. The mRNA was produced in a relatively large amount (approximately 10mg) and was of GMP quality. It was crucial to the advancement of the vaccination studies to demonstrate that the added IRES and extended poly-A tail did not impede the critical end point of mRNA expression. The transfection of CHO cells followed by the detection of HA protein by two complementary methods (flowcytometry and immunocytochemistry) demonstrated that the mRNA was effectively translated. These results were required for the progression of the experiments and as a future consideration for taking mRNA into the clinic. These results demonstrated that

mRNA can be reliably produced in large amounts without affecting the end point of mRNA expression.

Much has been written in the literature regarding the sensitivity of mRNA. A main concern upon initiation of these studies was the wide acceptance that mRNA is readily degraded in serum. Significant serum anti-HA IgG and anti-HA cellular responses, however, demonstrated that the mRNA was effective (see Figures 12 to 16 above). Indeed, naked mRNA was not only able to induce measurable amounts of anti-HA serum IgG, as observed by ELISA, but these antibodies were also able to neutralize virus infection of susceptible cells *in vitro* which is a correlate of protection (see Figure 15 above). A similar trend was seen regarding the T-cell response wherein mRNA was able to significantly induce T-cell proliferation in response to HA stimulation which further supports the potential of mRNA as a vaccine capable of inducing quantifiable cellular responses (see Figure 8 and Figure 16 above).

2) <u>mRNA in cationic liposomes study:</u>

For the first part of the study, readily available cationic liposomes were a logical choice since mRNA naturally associates with positively charged lipids (based on electrostatic interactions) that would then theoretically improve the stability and delivery of mRNA into target cells. Unexpectedly, mRNA complexed with cationic liposomes yielded poor anti-HA antibody titers in comparison to naked mRNA (see Figure 6 above). The same trend was mirrored by the observed T-cell lymphocyte proliferation levels (see Figure 8 above). The lipids upon which these liposomes were based and which confer an overall positive charge to the particle might explain some of these effects. Liposomes are known to be capable of

attaching to the negatively charged membranes of lymphocytes, solubilizing the membrqnes of lymphocytes and causing subsequent overall negative effects on the immune response (Jahnová *et al,* 1993). Possible toxicity effects of cationic liposomes to keep in mind include thrombosis and platelet aggregation, and molecular damage such as hemolysis and changes in membrane proteins (e.g. PKC) where these cationic liposome fuse (Bottega and Epand, 1992; Simoes *et al,* 2005). Some degree of toxicity is typically associated with the majority of compounds and biologics. Progressively decreasing the amount of cationic lipids in the vaccine to find an ideal threshold amount may ameliorate these concerns while retaining its overall positive effects.

The quality of the raised anti-HA antibodies, as measured by the microneutralization assay, did not demonstrate the presence of any nAbs (see Figure 7 above) which was consistent with the low antibody titers that were observed.

3) <u>mRNA with KLK and NISV study:</u>

The second vaccination approach was designed to improve on the findings described above while eliminating the possible negative disadvantages associated with the cationic liposomes. Accordingly, alternative approaches were pursued which included the use of a cationic peptide and NISV.

First, increasing the amount of antigen (4 times more) was sufficient to substantially increase the levels of anti-HA antibodies as reported by ELISA (see Figure 12 above). Second, complexing mRNA with KLK in the absence or presence of NISV seemed to have beneficial effects on the overall humoral response (see Figures 12 to 14 above). This is likely a consequence of the added protection provided by these two approaches against endogenous RNAses and also by the very nature of these components. As noted above, the KLK peptide

was originally derived from a protein found in the hemolymph of the flesh fly (*Sarcophaga peregrina*) and is known for its antimicrobial effects against *Staphylococcus aureus* (Alvarez-Bravo *et al*, 1994). KLK has recently been shown to be a potent stimulator of Th-2 adaptive immune responses when co-administered with antigens. This stimulatory effect is mainly by enhancing antigen to antigen presenting cells association and by inducing interleukin (IL)-4 and IL-5 cytokine responses and IgG1 antibodies (Schellak *et al*, 2006).

In the present studies, these effects on the stimulation of Th-2 responses were observed when mRNA was complexed with 10 μg of KLK (see Figures 12 to 15 above). These are important results which represent a new addition to the KLK literature since most cited formulations using KLK use at least 130 μg to observe enhanced antibody secretion unlike the present studies which used 10 μg. Overall; results suggest that mRNA and KLK may act in a synergistic fashion to improve the humoral response to HA.

One difficulty to consider for future studies is that KLK was difficult to dissociate from mRNA as seen on the agarose gel despite protease treatment and extreme heating (see Figure 8 above). KLK-complexed mRNA stayed stranded at the top of each well even at the 60 min mark indicating that intact mRNA was still available. A change in the pH of the solution in which mRNA and KLK are incubated might reverse the interaction freeing the mRNA and improve the *in vitro* results (mRNA trapped at the level of the well).

The other protection approach used involved NISV. The first step, before embarking on experiments using NISV containing mRNA, was to make sure that at the end of the procedure the immunogen was still present in sufficient amounts given that the preparation of NISVs involves high temperatures and the use of reagents which are not RNAse free.

Moreover, the procedure is lengthy which, as a consequence, exposes the mRNA to degradation. Conversely to Martinon *et al*, which reported an mRNA recovery rate of 5-10%, the presents results revealed that mRNA was present in easily detectable amounts after the NISV preparation procedure was completed with entrapment efficiencies of approximately 70% (see Figure 11 above). This result is highly important for future research in the field since NISVs are known to have extremely low toxicity, can act easily as adjuvants and are easily modifiable to include ligands for targeting (Brewer and Alexander, 1994; Sun *et al*, 2006). NISVs can also increase the possibilities for future mRNA therapy such as offering delivery routes which were previously inaccessible to naked mRNA (e.g. oral).

With regard to the animals that received NISVs with mRNA, antibody production was significantly enhanced (see Figure 12 above). An interesting point to note was the surge in anti-HA serum IgG antibodies detected as early as the first vaccination which was not observed with the mRNA alone or mRNA-KLK groups and which may reflect the intrinsic adjuvant properties of NISVs (see Figure 12 above). It would be of great interest to further examine the potential intrinsic adjuvant properties of NISVs (with or without mRNA) by studying the activation and maturation of bone-marrow derived dendritic cells (DCs) with formulations containing NISVs. This could be carried out by studying the expression of DCs surface markers which are associated with activation and maturation, such as MHCII and CD86.

One of the objectives of the second vaccination approach was to minimize the overall negative effects of cationic liposomes on mRNA. The use of NISV successfully delivered the mRNA to cells of the immune system without causing any apparent toxicity on T-cell lymphocytes as indicated by the noticeable T-cell proliferation levels (see Figure 16 above).

Interestingly, proliferation for this group was below the levels yielded by the mRNA alone group.

Protection against an influenza challenge is widely recognized as mediated by nAbs. All formulations containing mRNA showed great neutralization as supported by the plaque reduction assay (see Figure 15 above), particularly in animals that received the mRNA alone. This is in contrast to the nAbs examined using the first vaccine approach which revealed little to no detectable nAbs.

The demonstrated humoral response triggered by mRNA vaccination (see Figures 12 to 15 above) reveals that these vaccines are capable of triggering antibodies, against the encoded antigen, in adequate amounts and quality which might provide sufficient protection against a challenge by the homologous strain. This result is totally novel in view of the current literature because most known mRNA vaccination protocols require, at a minimum, 50 μg of mRNA, with 7 to 15 vaccination rounds (Scheel *et al,* 2006; Hoerr *et al,* 2000). The cellular immune response analysis yielded results consistent with the finding of the first study wherein the presence of mRNA in the vaccination formulation was sufficient to drive Th-1 type responses (see Figure 7 above).

After conclusively demonstrating that mRNA vaccination could trigger antigen specific antibodies, a subtyping of the antigen specific antibody response was carried out since it is known that the raised subtype is indicative of the nature of the immune response. Though not statistically significant, results revealed that IgG2a responses were slightly superior to IgG1 antibody levels indicating a slight Th-1 trend (see Figures 13 and 14 above). The differences between IgG1and IgG2, however, were not of great magnitude. This finding,

in combination with the detectable antibody levels, indicates that the trend is likely not significant therefore it is difficult to definitively conclude the predisposition of mRNA to trigger one type of response over another.

Overall, it appeared that the response elicited by mRNA vaccination appeared to be balanced between the Th-1 vs. Th-2 response. This finding is interesting since most mRNA vaccine studies demonstrated a clear Th-1 bias. These results add another level of complexity in that the nature of the mRNA (sequence) appears to play an important role in the type of response which is subsequently raised.

Another aspect of these studies which requires further investigation would be the elicitation of the full nature of the response. This could be examined by adding additional vaccination rounds to determine what the HA antibody levels would be following a third or fourth vaccination round which would still fall below the typical 7 to 15 vaccination rounds currently cited in the literature (Scheel *et al,* 2006; Hoerr *et al,* 2000).

Importantly, no visible adverse effects to the mice were noted during either of the vaccination studies. Visible adverse effects were defined as behavioral changes, loss of weight (known to be a major indicator of physiological imbalance) or signs of inflammation or irritation at the site of injection.

Recent international events surrounding the outbreak of the H1N1 pandemic flu have emphasized the importance of developing new approaches to vaccine technology. A basic vaccine development technique should be readily adaptable and responsive to whatever new strain appears. The current seasonal vaccine, based on the WHO predictions, has proven to be

inefficient in protecting against the virus while the vaccine against the H1N1 causative strain remains largely unexamined at the clinical level. With regard to the vaccine approaches disclosed in the present studies, the sequence of the HA from the H1N1 pandemic strain has been available for months and a homologous mRNA could have been synthesized in a short period of time providing a possible vaccine which would have alleviated the observed mortality, morbidity and social stigma associated with this most recent influenza outbreak.

In closing, these studies have shown that:

1) an mRNA vaccine is safe and could be readily scaled for use in larger mammals;

2) mRNA can be used to induce measurable immune responses;

3) mRNA can be used as a means for adaptable therapy against infectious diseases; and

4) mRNA can efficiently and effectively be combined with lipidic carriers for use in animals.

These studies have examined some aspects related to the potential of mRNA, as an adjuvant and an antigen-coding vehicle by studying the potential of an HA-encoding mRNA vaccine against Flu AV. Results suggest that this approach merits further research, especially with regards to the use of mRNA with KLK and NISV.

E. References:

Agger EM, Rosenkrands I, Olsen AW, Hatch G, Williams A, Kritsch C, Lingnau K, von Gabain A, Andersen CS, Korsholm KS, Andersen P. Protective immunity to tuberculosis with Ag85B-ESAT-6 in a synthetic cationic adjuvant system IC31. Vaccine. 2006 Jun 29;24(26):5452-60.

Alvarez-Bravo J, Kurata S, Natori S. Novel synthetic antimicrobial peptides effective against methicillin-resistant Staphylococcus aureus. Biochem J. 1994 Sep 1;302 (Pt 2):535-8.

Behbehani AM. The smallpox story: life and death of an old disease. Microbiol Rev. 1983 Dec;47(4):455-509

Beigel JH. Influenza. Crit Care Med. 2008 Sep;36(9):2660-6

Boczkowski D, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J Exp Med. 1996 Aug 1;184(2):465-72.

Bottega R, Epand RM. Inhibition of protein kinase C by cationic amphiphiles. Biochemistry. 1992 Sep 22;31(37):9025-30.

Bouvier NM, Palese P. The biology of influenza viruses. Vaccine. 2008 Sep 12;26 Suppl 4:D49-53.

Brewer JM, Alexander J. Studies on the adjuvant activity of non-ionic surfactant vesicles: adjuvant-driven IgG2a production independent of MHC control. Vaccine. 1994 May;12(7):613-9.

Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the United States. JAMA. 2006 Feb 22;295(8):891-4

Carralot JP, Weide B, Schoor O, Probst J, Scheel B, Teufel R, Hoerr I, Garbe C, Rammensee HG, Pascolo S. Spontaneous cellular uptake of exogenous messenger RNA in vivo is nucleic acid-specific, saturable and ion dependent. Genet Vaccines Ther. 2005 Aug 22;3:6.

Centers for Disease Control and Prevention (CDC). Interim within-season estimate of the effectiveness of trivalent inactivated influenza vaccine—Marshfield, Wisconsin, 2007–2008 influenza season. MMWR Morb Mortal Wkly Rep. 2008 Apr 18;57(15):393-8.

Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med. 2001 Dec;7(12):1306-12.

Cheung TK, Poon LL. Biology of influenza a virus. Ann N Y Acad Sci. 2007 Apr;1102:1-25.

Christensen D, Agger EM, Andreasen LV, Kirby D, Andersen P, Perrie Y. Liposome-based cationic adjuvant formulations (CAF): past, present, and future. J Liposome Res. 2009;19(1):2-11.

Conry RM, LoBuglio AF, Wright M, Sumerel L, Pike MJ, Johanning F, Benjamin R, Lu D, Curiel DT. Characterization of a messenger RNA polynucleotide vaccine vector. Cancer Res. 1995 Apr 1;55(7):1397-400.

Cooper CL, Davis H, Cameron DW. Influenza vaccination with 1/10th the full dose. N Engl J Med. 2004 Nov 25;351(22):2339-40.

Couceiro JN, Paulson JC, Baum LG. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium: the role of the host cell in selection of Hemagglutinin receptor specificity. *Virus Res* 1993;29(2):155–165

Cros JF, Palese P. Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses. Virus Res 2003;95 (Sep(1/2)):3–12.

Eickhoff TC, Meiklejohn G. Protection against Hong Kong influenza by adjuvant vaccine containing A2-Ann Arbor-67. Bull World Health Organ. 1969;41:562–3.

Hayden FG, Pavia AT. Antiviral management of seasonal and pandemic influenza. J Infect Dis. 2006 Nov 1;194 Suppl 2:S119-26.

Hilleman MR. Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. Vaccine 2002; 20:3068–87

Hoerr I, Obst R, Rammensee HG, Jung G. In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. Eur J Immunol. 2000 Jan;30(1):1-7.

Hollenbeck JE. An Avian Connection as a Catalyst to the 1918-1919 Influenza Pandemic. Int J Med Sci. 2005;2(2):87-90

Ito T, Couceiro JN, Kelm S, *et al.* Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J Virol* 1998;72(9):7367–7373

Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004 Oct;5(10):987-95

Jahnová E, Ferencík M, Nyulassy S, Devínsky F, Lacko I. Amphiphilic detergents inhibit production of IgG and IgM by human peripheral blood mononuclear cells. Immunol Lett. 1993 Dec;39(1):71-5.

Kaiser J. One size fits all flu-vaccine? Science. 2006 Apr 21;312(5772):380-2.

Karlsson Hedestam GB, Fouchier RA, Phogat S, Burton DR, Sodroski J, Wyatt RT. The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. Nat Rev Microbiol. 2008 Feb;6(2):143-55.

Kilbourne ED. Influenza pandemics of the 20th century. Emerg Infect Dis. 2006 Jan;12(1):9-14

Kilbourne ED, Arden NH. Inactivated influenza vaccines. In: Plotkin SA, Orenstein WA, editors. Vaccines. Philadelphia: Saunders, 1999. p. 531–51.

Klenk HD, Rott R, Orlich M, *et al.* Activation of influenza A viruses by trypsin treatment. *Virology* 1975;68(2):426–439.

Koh G, Wong T, Cheong S, Koh D. Avian Influenza: a global threat needing a global solution. Asia Pac Fam Med. 2008 Nov 13;7(1):5.

Krug RM. Priming of influenza viral RNA transcription by capped heterologous RNAs. *Curr Top Microbiol Immunol* 1981;93:125–149.

Lamb RA, Choppin PW. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proc Natl Acad Sci U S A. 1979 Oct;76(10):4908-12.

Lamb RA, Choppin PW. Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. Virology. 1981 Jul 30;112(2):729-37

Lazarowitz SG, Choppin PW. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the Hemagglutinin polypeptide. *Virology* 1975;68(2):440–454

Li X, Palese P. Characterization of the polyadenylation signal of influenza virus RNA. J Virol 1994;68(Feb (2)):1245–9.

Luo C, Nobusawa E, Nakajima K. An analysis of the role of neuraminidase in the receptor-binding activity of influenza B virus: the inhibitory effect of Zanamivir on haemadsorption. *J Gen Virol* 1999;80(Pt 11):2969–2976

Maassab HF. Adaptation and growth characteristics of influenza virus at 25 degrees c. Nature. 1967 Feb 11;213(5076):612-4

Martin K, Helenius A. Transport of incoming influenza virus nucleocapsids into the nucleus. J Virol 1991;65(Jan (1)):232–44

Martinon F, Krishnan S, Lenzen G, Magné R, Gomard E, Guillet JG, Lévy JP, Meulien P. Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. Eur J Immunol. 1993 Jul;23(7):1719-22.

Matlin KS, Reggio H, Helenius A, *et al.* Infectious entry pathway of influenza virus in a canine kidney cell line. *J Cell Biol* 1981; 91(3 pt 1): 601–613

Nobusawa E, Sato K. Comparison of the mutation rates of human influenza A and B viruses. J Virol. 2006 Apr;80(7):3675-8.

Palese P, Shaw ML. Orthomyxoviridae: The viruses and their replication. In: Knipe DM, Howley PM, editors. Fields Virology. 5th Ed. Philadelphia: Lippincott Williams & Wilkins; 2006. pp. 1648–1689.

Palucka AK, Ueno H, Connolly J, Kerneis-Norvell F, Blanck JP, Johnston DA, Fay J, Banchereau J. Dendritic cells loaded with killed allogeneic melanoma cells can induce objective clinical responses and MART-1 specific CD8+ T-cell immunity. J Immunother. 2006 Sep-Oct;29(5):545-57.

Pascolo S. Vaccination with messenger RNA. Handb Exp Pharmacol. 2008;(183):221-35.

Probst J, Weide B, Scheel B, Pichler BJ, Hoerr I, Rammensee HG, Pascolo S. Spontaneous cellular uptake of exogenous messenger RNA in vivo is nucleic acid-specific, saturable and ion dependent. Gene Ther. 2007 Aug;14(15):1175-80.

Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, Holmes EC. The genomic and epidemiological dynamics of human influenza A virus. Nature. 2008 May 29;453(7195):615-9

Romagne F. Current and future drugs targeting one class of innate immunity receptors: the Toll-like receptors. Drug Discov Today. 2007 Jan;12(1-2):80-7

Schellack C, Prinz K, Egyed A, Fritz JH, Wittmann B, Ginzler M, Swatosch G, Zauner W, Kast C, Akira S, von Gabain A, Buschle M, Lingnau K. IC31, a novel adjuvant signaling via

TLR9, induces potent cellular and humoral immune responses. Vaccine. 2006 Jun 29;24(26):5461-72.

Simões S, Filipe A, Faneca H, Mano M, Penacho N, Düzgünes N, de Lima MP. Cationic liposomes for gene delivery. Expert Opin Drug Deliv. 2005 Mar;2(2):237-54.

Simonsen L. The global impact of influenza on morbidity and mortality. Vaccine. 1999 Jul 30;17 Suppl 1:S3-10.

Simonsen L, Viboud C, Grenfell BT, Dushoff J, Jennings L, Smit M, Macken C, Hata M, Gog J, Miller MA, Holmes EC. The genesis and spread of reassortment human influenza A/H3N2 viruses conferring adamantane resistance. Mol Biol Evol. 2007 Aug;24(8):1811-20

Steitz J, Britten CM, Wolfel T, Tuting T. Effective induction of anti-melanoma immunity following genetic vaccination with synthetic mRNA coding for the fusion protein EGFP.TRP2. Cancer Immunol Immunother. 2006 Mar;55(3):246-53

Stegmann T, Morselt HW, Scholma J, *et al.* Fusion of influenza virus in an intracellular acidic compartment measured by fluorescence dequenching. *Biochim Biophys Acta* 1987;904(1):165-170.

Stevens J, Corper AL, Basler CF, *et al.* Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* 2004;303(5665):1866–1870.

Sun H, Pollock KG, Brewer JM. Analysis of the role of vaccine adjuvants in modulating dendritic cell activation and antigen presentation in vitro. Vaccine. 2003 Feb 14;21(9-10):849-55.

Tamura S, Tanimoto T, Kurata T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. Jpn J Infect Dis. 2005 Aug;58(4):195-207

Treanor JJ, Hayden FG, Vrooman PS, Barbarash R, Bettis R, Riff D, Singh S, Kinnersley N, Ward P, Mills RG. Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. US Oral Neuraminidase Study Group. JAMA. 2000 Feb 23;283(8):1016-24.

Viboud C, Grais RF, Lafont BA, Miller MA, Simonsen L; Multinational Influenza Seasonal Morbidity Study Group. Multinational impact of the 1968 Hong Kong pandemic: evidence for a smoldering pandemic. J Infect Dis. 2005;192:223–48.

Voltaire, Lettres Philosophiques. Lettre XI: Sur l'insertion de la petite vérole. 1743

Van Loon FP, Holmes SJ, Sirotkin BI, *et al.* Mumpssurveillance—United States, 1988-1993. *MMWR CDC Surveill Summ* 1995; **44:** 1–14.

Weide B, Carralot JP, Reese A, Scheel B, Eigentler TK, Hoerr I, Rammensee HG, Garbe C, Pascolo S. Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. J Immunother. 2008 Feb-Mar;31(2):180-8.

WHO, Fact sheet N°211, 2003

Wilson IA, Skehel JJ,Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. *Nature* 1981;289(5796):366–373

Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle in vivo. Science. 1990 Mar 23;247(4949 Pt 1):1465-8.

Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, Zheng NY, Mays I, Garman L, Helms C, James J, Air GM, Capra JD, Ahmed R, Wilson PC. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature. 2008 May 29;453(7195):667-71

Zebedee SL, Lamb RA. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J Virol. 1988 Aug;62(8):2762-72.

Zhong B, Tien P, Shu HB. Innate immune responses: crosstalk of signaling and regulation of gene transcription.Virology. 2006 Aug 15;352(1):14-21