DEVELOPMENT OF NOVEL VACCINE CANDIDATES FOR MEASLES

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ABSTRACT

Despite the availability of live attenuated measles virus vaccines, a large number of measles-associated deaths occur among infants in developing countries during the "window of susceptibility" (age 4-9 months). During this period declining maternal antibody titers are no longer protective against wild-type measles virus (MV) and impede successful immunization with the live attenuated vaccines. Therefore, the development of a safe vaccine that would induce protective immunity in the presence of maternally derived MV-specific antibodies in young infants and would close the "window of susceptibility" is desirable. Since adenoviruses have been shown as suitable vaccine candidates capable of eliciting potent protection against mucosal infectious diseases, the ability of an adenovirus-vectored anti-measles vaccine to elicit robust immune responses against MV was assessed in this study. Mice immunized intramuscularly or intranasally with a combination of human adenovirus serotype 5 (Ad5) recombinants expressing MV hemagglutinin (H) and fusion (F) glycoproteins developed MV-specific neutralizing antibody titers similar for both routes of immunization. However, intramuscular immunization of mice with Ad5 recombinants resulted in induction of a predominant T helper type (Th1) immune response, whereas intranasal immunization induced a balanced Th1/Th2 immune response. Furthermore, intranasal immunization resulted in increased titers of MV-specific immunoglobulin A (IgA) in lungs in comparison to intramuscularly immunized animals. The ability of the Ad5 recombinants to induce protective immune responses in cotton rats by different routes of administration was also evaluated. Cotton rats that received a single

dose of the Ad5 recombinants intramuscularly or intranasally experienced a rise in MV-specific neutralizing antibody titers and reduction of the viral RNA load in the lung tissue after intranasal MV challenge. In addition, the largest reduction in viral replication was observed in the group of cotton rats inoculated with the Ad5 recombinants intranasally. Based on these observations, the Ad5-based vaccine appears to be a suitable candidate against measles. Furthermore, a capability of purified globular head domain of MV H protein produced in a human cell line to induce MV-specific immune responses in mice was tested. Subcutaneous immunization of mice with the recombinant protein alone resulted in both humoral and cell-mediated immunity, characterized by the production of MV-specific serum IgG and neutralizing antibodies, as well as interferon gamma (IFN-γ) and interleukin 5 (IL-5) production by *in vitro* restimulated splenocytes. The former responses were further enhanced by formulation of the protein with aluminium hydroxide. However, very low numbers of INF-γ secreting cells and low levels of IgG2a in the serum suggested a Th2 immune response. Novel adjuvants (Th1-directing), as well as MV F protein should be considered for the inclusion into the vaccine formulations to induce more balanced Th1/Th2 immune responses against measles.

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LIST OF ABBREVIATIONS

ΔE1	deletion of the early 1 region of the adenovirus genome
ΔE3	deletion of the early 3 region of the adenovirus genome
Ad	human adenovirus
Ad5-Empty	control recombinant replication-deficient human adenovirus type 5
Ad5-MV-F	recombinant replication-deficient human adenovirus type 5 expressing the fusion protein of measles virus
Ad5-MVvacH	recombinant replication-deficient human adenovirus type 5 expressing the hemagglutinin of measles virus
Ad5-F/H	a combination of Ad5-MV-F and Ad5-MVvacH
AP	alkaline phosphatase
ATCC	American Type Culture Collection
BAL	bronchoalveolar lavage
BHGpolyA	bovine growth hormone polyA signal
cDNA	complimentary deoxyribonucleic acid
CMV	human cytomegalovirus
CPE	cytopathic effect
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
E region	early region
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FIMV	formalin-inactivated measles vaccine
F	fusion protein
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	human immunodeficiency virus
H	hemagglutinin protein
HRP	horseradish peroxidase
Ig	immunoglobulin
IFN	interferon
IL	interleukin
IM	intramuscular/intramuscularly
IN	intranasal/intranasally
ISCOM	immune stimulating complex
kDa	kilodalton
LAV	live attenuated vaccine
L	large protein
mAb	monoclonal antibody
MEM	minimum essential medium
MHC	major histocompatibility complex
MLP	major late promoter
M	matrix protein
mRNA	messenger ribonucleic acid
MV	measles virus
MV-H156/617	recombinant globular head domain of MV H (amino acids 156-617)
N	nucleoprotein
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PLG	polyactide co-glycolide
P	phosphoprotein
PRN	plaque reduction neutralization
PRRS	pathogen recognition receptors

Quil A	an adjuvant, purified saponin fraction extracted from the cortex of the South American tree *Quillaja saponaria molina*
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SC	subcutaneous
SDS	sodium dodecyl sulphate
SINCP	Sindbis virus-based replicon plasmid
TEV	tobacco etch virus
$TCID_{50}$	median tissue culture infective dose
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TLR	toll-like receptor
VN	virus neutralization/neutralizing
VSV	vesicular stomatitis virus
UV	ultraviolet
WHO	World Health Organization

1.0 LITERATURE REVIEW

1.1 MEASLES VIRUS

1.1.1 Measles virus genome and proteins

Measles virus (MV) is a member of the *Morbillivirus* genus in the subfamily of *Paramyxovirinae* of the *Paramyxoviridae* family. Humans are the only reservoir for MV, but non-human primates can also be infected with MV and develop a disease similar to measles in humans. MV is a 15 kb, enveloped, non-segmented negative-strand RNA virus replicating entirely in the cytoplasm of the host (Fig. 1.1B). The RNA of MV has a short leader sequence at the 3' end and a trailer sequence at the 5' end, which do not have any coding capacity and are thought to function as regulators of transcription and replication. The 3' noncoding region contains a recognition site for the RNA polymerase complex, which sequentially initiates transcription of the viral genes (Horikami and Moyer, 1991). Furthermore, there is evidence that this region is involved in triggering the early interferon response (Plumet et al., 2007). Six non-overlapping genes encoding the six structural proteins, namely nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H), and large (L), are located between the leader and trailer regions (Fig. 1.1A). In addition, the P gene encodes two nonstructural proteins, C and V (Griffin, 2007). In the viral particle, N is the most abundant structural protein. Its primary function is to form a helical nucleocapsid around the genomic RNA. This nucleocapsid is associated with P and L proteins to form ribonucleoprotein complex that is the template for both transcription

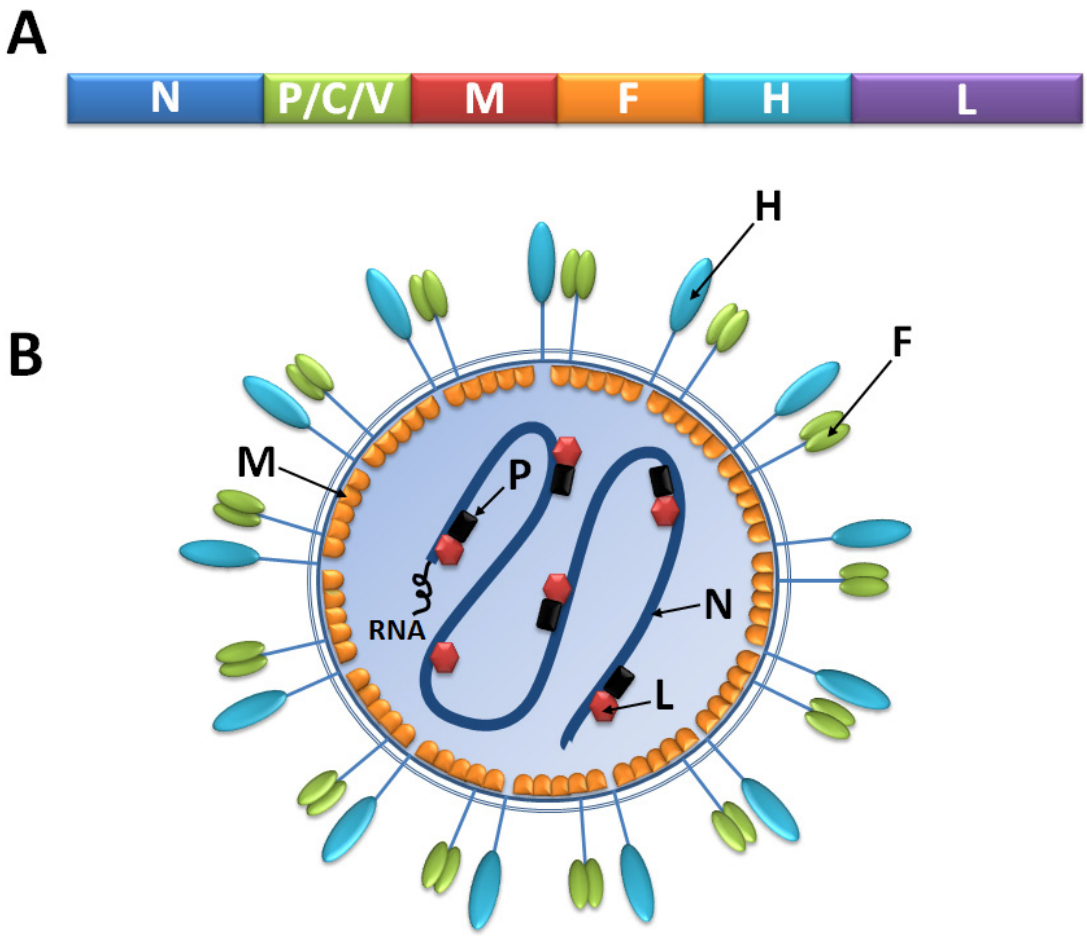

Figure 1.1. Measles virus. Schematic diagram of measles virus genome (A). *N*, *P*, *M*, *F*, *H*, *L*, genes encoding the six structural proteins, namely nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H), and large (L), respectively. *P*, gene encodes two nonstructural proteins, C and V that are alternatively translated from the RNA. Schematic diagram of measles virus (B). *M*, *H*, *F*, *N*, *L*, *P*, proteins of measles virus.

and replication. The RNA-dependent RNA polymerase composed of the P and L proteins carries out the latter processes. The L protein functions as the catalytic component of the polymerase complex, whereas the P protein binds to L and N proteins as well as RNA to form the replicase complex. The H, F and M proteins, together with lipids from the host cell membrane, form the viral envelope (Griffin, 2007). Transmembrane glycoproteins, F and H, are found at the outer surface of the lipid envelope and needed for fusion and entry (Cattaneo and Rose, 1993; Wild, Malvoisin, and Buckland, 1991; Zhang et al., 2005). The M protein lines the interior of the envelope and mediates the contact between the nucleocapsid and the glycoproteins during virus assembly (Iwasaki et al., 2009). Two non-structural proteins, C and V, are not essential for virus replication in tissue culture (Radecke and Billeter, 1996; Schneider, Kaelin, and Billeter, 1997), but believed to regulate transcription and replication and interact with cellular proteins modulating the intracellular environment (Devaux et al., 2007; Liston, DiFlumeri, and Briedis, 1995; Tober et al., 1998).

1.1.2 Hemagglutinin protein

The hemagglutinin protein mediates receptor attachment of MV and is an important determinant of morbillivirus cellular tropism (Griffin, 2007). In addition, it supports fusion of the infected cells mediated by F protein, as specific F-H protein interactions are required for the cell fusion (Cattaneo and Rose, 1993; Wild, Malvoisin, and Buckland, 1991). Two major receptors mediating the MV entry into the cell are CD46 and CD150 or SLAM (Griffin, 2007). There is evidence suggesting that an unknown epithelial cell receptor can also mediate the virus entry (Takeda et al., 2007; Takeuchi et al., 2003). Wild-type strains

of MV use SLAM as the primary cellular receptor (Erlenhoefer et al., 2001; Hsu et al., 2001; Ono et al., 2001), which is expressed on cells of the immune system including immature thymocytes, activated T and B lymphocytes, activated monocytes, and mature dendritic cells (Griffin, 2007). CD46 is the ubiquitous regulator of complement activation, which is present on all nucleated cells (Liszewski and Atkinson, 1992). This receptor is used efficiently by tissue culture-adapted and vaccine strains of MV for adhesion (Bartz et al., 1998; Condack et al., 2007). The latter strains also interact with SLAM, but exhibit a decreased tropism for lymphocytes.

MV H is a 617 amino acid (78-kDa) type II transmembrane glycoprotein (Fig. 1.2B), which is located on the surface of virions and infected cells as a homotetramer consisting of two disulfide-linked homodimers (Griffin, 2007). This protein is comprised of an N-terminal cytoplasmic tail (1-34 aa), a transmembrane domain (36-58 aa), an extracellular membrane-proximal stalk domain (59-154 aa) containing two disulfide bonds formed by cysteine residues C139 and C154, and a large C-terminal globular head (157-607 aa) (Alkhatib and Briedis, 1986; Colf, Juo, and Garcia, 2007; Griffin, 2007; Hashiguchi et al., 2007; Plemper, Hammond, and Cattaneo, 2000). Crystal structure of MV-H (Edmonston) head domain was determined, and binding regions on the H head domain for the different cellular receptors were identified (Colf, Juo, and Garcia, 2007; Hashiguchi et al., 2007). In addition, it has been shown that N-linked sugars at N-linked sites 200 and 215 cover wide areas of MV H protein (Hashiguchi et al., 2007), and glycosylation plays an important role in the processing and antigenicity of MV H protein (Griffin, 2007).

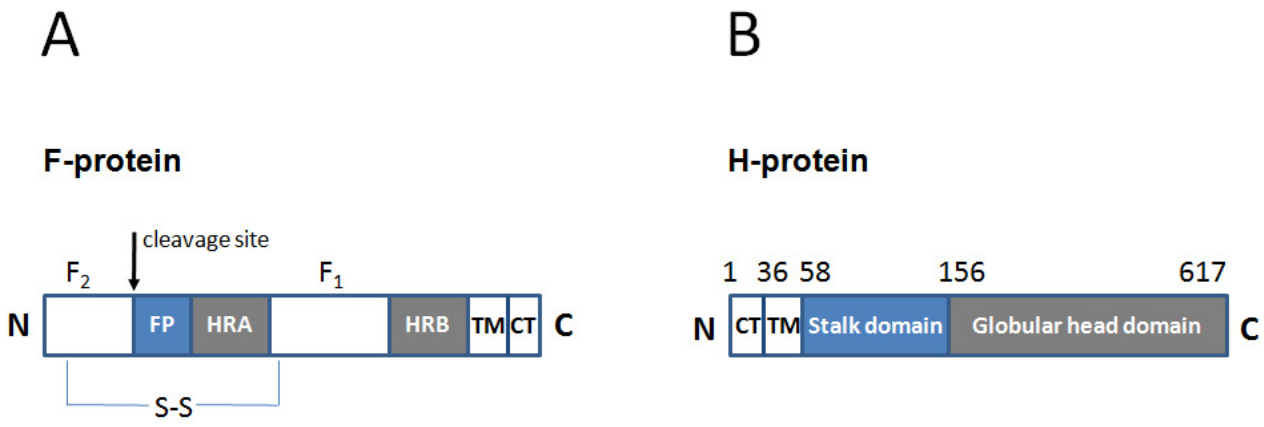

Figure 1.2. The MV envelope glycoproteins. Schematic of the MV F-protein (A). The disulfide bond (S-S) holding cleavage fragments F_1 and F_2 is indicated. *FP*, fusion peptide; *HRA, HRB*, heptad repeats A and B, respectively. *TM*, transmembrane segment; *CT*, cytoplasmic tail; *N* and *C*, N- and C-terminus, respectively. Schematic of the MV H-protein (B). *CT*, cytoplasmic tail; *TM*, transmembrane segment; *N* and *C*, N- and C-terminus, respectively.

1.1.3 Fusion protein

The fusion protein facilitates fusion of the virion and host cell membranes during virus infection and mediates fusion of infected cells (Lamb, 2007). The MV F protein (Fig. 1.2A) is a 553 amino acid type I transmembrane glycoprotein located on the surface of virions and infected cells as a trimer and comprised of a globular head domain, a helical stalk region consisting of membrane-proximal heptad repeats A and B, a transmembrane domain, and a cytoplasmic tail (Griffin, 2007; Yin et al., 2006).

The F protein is synthesized as an F_0 precursor (F_0, 60 kDa). In the trans-Golgi, F_0 is cleaved by the ubiquitous intracellular protease furin into F_1 (a membrane-spanning subunit, 40 kDa) and F_2 (a membrane-distal subunit, 20 kDa) subunits, which are covalently linked by a disulfide bond (Bolt and Pedersen, 1998; Griffin, 2007). After cleavage, the fusion peptide located at the N-terminus of the F_1 fragment is ready to be inserted into the target membrane to initiate fusion (Bolt and Pedersen, 1998; Watanabe et al., 1995). The F_2 subunit of the MV F protein is glycosylated and has three N-linked carbohydrate chains that have important roles in processing, cell surface expression and function of the MV F protein (Griffin, 2007; Hu et al., 1995). In addition, it was shown that single tyrosine residues in the cytoplasmic tails of the glycoproteins are responsible for basolateral targeting of F and H in polarized epithelial cells, and their basolateral expression is important for the cytopathic properties (syncytia formation) of MV infection *in vivo* and *in vitro* (Moll et al., 2004). Moreover, in polarized epithelial cells, interaction of M protein with the glycoprotein cytoplasmic tails allows retargeting of F and H proteins to the apical surface for the virion release (Naim, Ehler, and Billeter, 2000).

1.2 MEASLES

1.2.1 Pathogenesis of measles infection

MV is a human-restricted pathogen. It is highly contagious in nature and transmitted from person to person by respiratory droplets. The clinical pathogenesis of measles is divided into four phases: incubation, prodromal, exanthematous (rash), and recovery. After the incubation period of 10 to 14 days, the patient undergoes a 2- to 3-day prodromal stage with fever, coryza, cough, conjunctivitis and small white spots inside the cheeks (Koplik's spots). The maculopapular rash develops in a few days, and its onset coincides with the appearance of adaptive immune responses and initiation of virus clearance. Measles is typically a self-limiting disease, and recovery is followed by lifelong immunity to the virus. However, the high mortality and complication rates associated with the MV infection are mainly due to secondary infections that arise during measles-induced immunosuppression. The disease can be very severe for malnourished children and immunocompromised individuals. Serious complications include blindness, diarrhea, otitis, laryngotracheobronchitis, pneumonia, measles inclusion body encephalitis and subacute sclerosing panencephalitis (Griffin, 2007).

Two models of MV pathogenesis have been proposed. The current model implies that the initial event is established in the respiratory tract with the virus replication in tracheal and bronchial epithelial cells, followed by infection of lymphoid cells, perhaps pulmonary macrophages or dendritic cells (DCs), that transport MV to the regional lymph nodes where it is amplified and gives rise to primary viremia (Griffin, 2001; Rall, 2003; Schneider-Schaulies, Meulen, and Schneider-Schaulies, 2003; Sips et al., 2007). The

primary viremia, typically of brief duration and low titer, occurs two to six days after infection. In this phase virus enters the bloodstream and spreads to other lymphoid tissues including spleen, liver and bone marrow that are typical sites of secondary viral replication. The second phase of viremia begins 10 days after exposure. This phase is accompanied by characteristic lymphopenia and dissemination of virus to multiple organs including the skin, gastrointestinal tract, liver, central nervous system and thymus (Tyler, 2001). In these various sites, MV antigens are detected in endothelial and epithelial cell as well as monocytes and macrophages (Moench et al., 1988) In blood, monocytes are the primary infected cells, but B and T cells can be infected as well (Esolen et al., 1993; Grivel et al., 2005; McChesney et al., 1989). In the thymus and spleen, epithelial cells and macrophage-rich areas are sites of MV replication, respectively (Moench et al., 1988). On the contrary, an alternative model suggests that initial targets of wild-type MV are SLAM-expressing lymphatic cells that cause systemic spread of the virus (de Swart et al., 2007; von Messling, Svitek, and Cattaneo, 2006; Yanagi, Takeda, and Ohno, 2006). In this alternative model, MV infects certain epithelial cells from the basolateral side via the unknown epithelial receptor and buds from the apical side when it leaves the host (Leonard et al., 2008). To conclude, MV infection starts in the respiratory tract; however, the type of cell of initial viral replication, if any, has not been definitely determined (de Swart, 2008).

1.2.2 Immune responses to measles infection

Innate and adaptive immune responses are both involved in MV clearance, recovery from infection and the establishment of long-term immunity. Activation of innate immunity is

essential for the control of viral infection and initiation of adaptive immune responses. During the incubation period MV replication is controlled by nonspecific innate host responses. The innate immune system detects pathogens through interaction of pathogen recognition receptors (PRRs) with pathogen-associated molecular patterns. Various research groups have reported that MV can trigger the activation of signalling pathways activating the production of proinflammatory cytokines such as interferons, interleukins, and tumor necrosis factor-α *in vivo* and *in vitro* (Helin et al., 2001; Sato et al., 2008; Sato, Miura, and Kai, 2005).

IFN-α/β plays an important role in the initiation of innate immunity by a number of different mechanisms. Type I IFNs induce an antiviral state in the cell, maturation of DCs, as well as enhance cytotoxic T cell responses and stimulate production of chemokines, which recruit inflammatory cells to the site of infection (Hahm et al., 2005; Helin et al., 2001; Leopardi, Hyypia, and Vainionpaa, 1992; Vidalain et al., 2002). However, the role of type I IFNs in MV infection is still unclear due to discrepancies between various studies. For instance, it has been shown that wild-type MV activates signalling pathways involving interferon regulatory factor-3 and nuclear factor-κB involved in the initiation of innate immune responses in the human lung epithelial cells (Helin et al., 2001). In addition, MV infection also resulted in an efficient IFN-α/β and IL-6 production. On the contrary, Naniche et al. (2000) demonstrated that wild-type MV is able to suppress the synthesis of IFN-α/β in peripheral blood mononuclear cells (PBMC). In addition, a comparison of vaccine strains with wild-type MV led to the conclusion that wild-type MV induces very low levels of IFN-α/β and possesses mechanisms to suppress the type I IFN production (Naniche et al., 2000; Shingai et al., 2007). Furthermore, production of IL-12 that participates in linking the

innate and adaptive immune responses can be inhibited by MV *in vitro* and *in vivo* (Atabani et al., 2001; Karp et al., 1996; Polack et al., 2002). Finally, *in vivo*, there is little evidence of IFN production in response to natural infection (Griffin et al., 1990; Shiozawa et al., 1988; Zilliox, Moss, and Griffin, 2007). While the mechanism of inhibition remains unclear, some data suggest that C and V proteins of MV interfere with IFN signalling (Palosaari et al., 2003; Shaffer, Bellini, and Rota, 2003).

Studies performed to demonstrate the role of natural killer cells in early anti-viral defences revealed that natural killer cell activity in children with acute measles is reduced compared to values for children without measles infection (Griffin et al., 1990). The role of DCs as the primary professional antigen presenting cells for initiation of the immune response to MV has also been investigated. DCs can be infected with wild-type MV, as well as the vaccine strains, but the infection of DCs is strain dependent and more efficient by wild-type strains of MV (Murabayashi et al., 2002; Ohgimoto et al., 2001). MV-infected DCs co-cultured with lymphocytes cause lymphocyte infection and eventually undergo apoptosis (Fugier-Vivier et al., 1997; Murabayashi et al., 2002; Servet-Delprat et al., 2000). Moreover, it was shown that MV-infected DCs failed to promote T cell expansion (Shishkova et al., 2007). Nevertheless, in spite of the deficiencies identified in innate system function, robust humoral and cellular immune responses are mounted to MV infection.

The humoral arm of the immune response has been extensively studied to determine vaccine efficacy and to assess protection against measles. Measles-specific antibodies appear within several days after the onset of the rash and their titers rise rapidly thereafter (Bech, 1959). Measles-specific IgM antibodies are the earliest antibody type to develop and can be detected in serum by 3 days and for 28 days after the onset of the rash

in most individuals (Helfand et al., 1997; Rossier et al., 1991), followed by a switch first to IgG2 and IgG3 and then, in the memory phase, to IgG1 and IgG4 (Isa et al., 2001). IgG responses are induced shortly thereafter, peak at 3-4 weeks, and are maintained for a long time (Stokes et al., 1961).

Antibodies are generated to most MV proteins. The majority of rapidly produced and abundant antibody is directed against the N protein. Antibodies against N and H proteins increase notably during the second week after the onset of the rash and also antibodies against M and F proteins can be detected by that time but only in small amounts (Graves et al., 1984). The majority of neutralizing antibodies are directed against the H protein of MV, but up to 10% of neutralizing antibodies are specific for the MV F protein (de Swart et al., 2009). Furthermore, it has been revealed that human convalescent sera show reactivity to linear and conformational MV H epitopes and recognize linear epitopes in six to seven regions over much of the F protein (Griffin, 2007).

The importance of neutralizing antibodies in protection at the time of virus exposure has been illustrated in multiple studies. It has been shown that passively acquired maternal antibodies confer protection of infants against MV infection (Griffin, 1995). Another indication of the importance of neutralizing antibodies is the fact that susceptible individuals can be protected by post-exposure administration of anti-MV immunoglobulin if given within the first three days after exposure (Norrby, 1995; Wyde, 1999). Correlates of protection against measles have also been defined. MV-specific neutralizing antibody titers at the time of exposure to virus correlate with protection from disease. Serum plaque reduction neutralizing (PRN) titers of 200 mIU/ml are considered protective (Chen et al., 1990; Samb et al., 1995; WHO, 1993). Furthermore, although quantitative antibody responses are

important in preventing measles infection, qualitative or functional parameters of humoral immunity have been shown to correlate with protection (Bouche, Ertl, and Muller, 2002; Griffin, 2007). Recent study, which was undertaken to enlighten mechanisms involved in atypical measles, demonstrated that immunization with the formalin-inactivated measles vaccine (FIMV) resulted in anamnestic production of nonprotective, low-avidity, complement-fixing antibodies, immune complex deposition and atypical measles pathology in macaques upon natural measles infection (Polack et al., 2003a). These findings show the importance of the quality of the antibody response in conferring protection to re-infection.

Although antibodies provide protection and prevent measles infection following natural infection, vaccination, or passive transfer of antibodies (Albrecht et al., 1977; Black, 1989; Chen et al., 1990; Halsey et al., 1985; Permar, Griffin, and Letvin, 2006), their role in clearance of MV remains unclear. Recent studies using non-human primates demonstrate that antibodies have a limited role in the control of MV replication and clearance (Permar et al., 2004; Permar et al., 2003). However, several studies suggest the relative contributions of humoral immunity to the clearance of MV. In one study it was shown that the failure of children to mount sufficient hemagglutinin-inhibiting antibody titers was associated with a poor prognosis (Wesley, Coovadia, and Kiepiela, 1982). Moreover, correlation between antibody-dependent cellular cytotoxicity antibody titers and reductions in viremia was also demonstrated (Forthal et al., 1994). Finally, antibody-induced antigenic modulation affects intracellular viral replication (Fujinami and Oldstone, 1979; Fujinami and Oldstone, 1980; Schneider-Schaulies et al., 1992).

While the role of antibody responses in the clearance of MV is still unclear, the importance of cellular immunity in the control of MV infection was demonstrated in humans and confirmed by observation in the rhesus monkey model. Clinical data from patients indicate that children with B-cell deficiencies (e.g. agammaglobulinemia) recover from measles and develop lifelong immunity (Bruton, 1953; Good and Zak, 1956), whereas individuals with T-cell deficiencies develop a severe or fatal disease (Mitus et al., 1965; Nahmias et al., 1967). Clinical observations in rhesus monkeys indicate that the CD8(+) lymphocyte-depleted animals exhibited a more severe, extensive rash and prolonged high-titer viremia compared to the control animals (Permar et al., 2004; Permar et al., 2003), indicating a central role for CD8(+) lymphocytes in recovery from illness by controlling viral replication and dissemination (de Vries et al., 2010). Moreover, the contribution of cell-mediated immunity was also demonstrated in rhesus macaque studies, when there was no cytotoxic T cell response induced after FIMV immunization and antibodies did not undergo affinity maturation (Polack et al., 1999; Polack et al., 2003a). Importantly, atypical measles primed by FIMV immunization was associated with immune complex deposition in affected tissues, eosinophilia, and a Th2 polarization of the immune response (Polack et al., 1999).

Cellular immune responses have been shown to peak at the time of rash as plasma levels of soluble CD8 and β-2 microglobulin increase (Griffin, 2007) and activated T cells are detected in circulation (Ward et al., 1990), as well as in areas of MV-infected epithelial cell of the skin (Permar et al., 2003). Viremia is cleared within a few days after the onset of rash and MV is no longer detectable in PBMCs by co-cultivation (Permar et al., 2003). However, viral RNA is still detected in PBMCs as well as in respiratory secretions and urine for several weeks after apparent recovery (Riddell et al., 2007). Following virus clearance,

numbers of CD8(+) T cells in circulation and plasma levels of soluble CD8 and IFN-γ decline (Moss et al., 2002; Ohga et al., 1992; Ryon et al., 2002), whereas numbers of activated CD4(+) T cells decrease more slowly (Ryon et al., 2002). During the acute phase of measles infection, production of IFN-γ and IL-2 (Th1 type cytokines) by CD4(+) T cells is increased, while the convalescent phase is characterized by elevations in levels of the type 2 cytokines, IL-4 and IL-5. This type 2 cytokine skewing may last for several weeks after clearance of the virus and resolution of the rash. This pattern of cytokine production is consistent with the activation of CD8 (+) (IFN-γ) and type 1 CD4 (+) (IFN-γ and IL-2) T cells early in the immune response, followed by activation of type 2 CD4 T cells (IL-4, IL-13) and then regulatory T cells (IL-10) during recovery (Griffin, 2007). Furthermore, MV infection causes severe and prolonged immunosuppression, which is thought to be responsible for the occurrence of secondary infections. Manifestations of immune suppression include impairment of humoral and cellular immune responses to new antigens and polarization of effector CD4 T cells to produce Th2 cytokines, as well as suppression of delayed type hypersensitivity skin test responses and lymphoproliferative responses to mitogens (Griffin, 2007). Similarly, immunosuppression and type 2 cytokine skewing induced by measles vaccination was also documented, but none of these effects appear to be clinically significant (Smedman et al., 1994; Ward and Griffin, 1993). However, months to years after natural infection or vaccination, measles-specific IFN-γ- and IL-4- secreting cells were detected among PBMCs stimulated with MV antigens, suggesting that measles immunity is maintained by both Th1/Th2 cells (Dhiman et al., 2005; Ovsyannikova et al., 2003).

Findings in murine and non-human primate models indicate that all main structural proteins are recognized by T cells in the context of either MHC class I or class II molecules

(van Els and Nanan, 2002). A number of these epitopes were also confirmed in humans during natural infection or vaccination, including epitopes in H, M, and C proteins (Jaye et al., 2003; Ota et al., 2007).

1.2.3 Animal models of measles infection

The first animal model for measles was non-human primates. The two species, rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*) have been used as animal models for immunopathogenesis and measles vaccination studies (El Mubarak et al., 2007). These primates are highly susceptible to MV infection and develop similar disease manifestations to those associated with measles in humans after experimental inoculation with this virus. Clinical manifestations include Koplik spots, leucopenia, immunosuppression and a morbilliform rash (Auwaerter et al., 1999; Kobune, Sakata, and Sugiura, 1990; Kobune et al., 1996). Similar to humans, wild-type MV strains cause disease in the macaques, whereas vaccine strains do not (Auwaerter et al., 1999). Infectious virus can be isolated from PBMCs, lung lavages and pharyngeal epithelial cells (El Mubarak et al., 2007; van Binnendijk et al., 1994). However, the use of these animals is restricted due to the high cost, the limited availability, the lack of inbred population and ethical reasons.

Other research aimed to the study of MV infection and pathogenesis has been focused on rodent-adapted MV. Rodent-adapted neurotropic strains of MV induce encephalitis in nontransgenic mice, rats, or hamsters after intracerebral infection, but the infection does not spread to the periphery (Duprex et al., 1999; Moeller-Ehrlich et al., 2007; Schubert et al., 2006). Recently, identification of two human receptors, CD46 and CD150,

for the MV entry, has allowed researchers to study MV in new transgenic animal models (Manchester and Rall, 2001; Sellin and Horvat, 2009). Although none of them mimic MV infection in humans, they have provided valuable information and new insights into the pathogenesis of measles.

The cotton rat model is also used to investigate measles vaccination and pathogenesis. The cotton rat (*Sigmodon hispidus*) is a new world rodent that belongs to the order *Rodentia*, family *Muridae*, and subfamily *Sigmodontinae*. Since 1939, cotton rats have been used extensively as laboratory animals and found to be susceptible to a variety of human viruses including influenza A and B, parainfluenza, adenovirus, respiratory syncytial virus (Faith et al., 1997). Cotton rats are also naturally susceptible to MV infection after intranasal inoculation. They can be infected with both vaccine and wild-type MV strains, but they are semi-permissive for MV infection (the titers of virus obtained from the lung tissue are proportionate to the titer of the inoculum) (Wyde et al., 1992; Wyde et al., 1999). After intranasal infection with MV, cotton rats demonstrate reduced activity without overt clinical signs. Wild-type viruses are found in lung tissues, the mediastinal draining lymph nodes and spleen (Pfeuffer et al., 2003; Wyde et al., 1999), whereas vaccine strains of the virus replicate in lungs, but rarely spread to the lung-draining lymph nodes (Pfeuffer et al., 2003). Differences in the virus spread between wild-type and vaccine strains might be related to differences in the receptor usage (Pfeuffer et al., 2003). In addition, studies with the use of recombinant viruses to investigate the relevance of receptor usage in cotton rats revealed that in these rodents, as in humans, MV uses two receptors that homologous to the human molecules, CD46 and CD150 (Pfeuffer et al., 2003). Infectious virus induces an atypical pneumonia with interstitial infiltrates and can be detected in lung epithelial cells

(Moll et al., 2004), as well as in macrophages isolated by bronchoalveolar lavage from intranasally infected animals (Pfeuffer et al., 2003; Wyde et al., 1999). The Edmonston strain of MV replicates in lungs of cotton rats for at least 7 days after virus inoculation with peak pulmonary titers occurred on day four (Niewiesk et al., 1997; Wyde et al., 1992). Importantly, to recover virus from lung homogenates in a reproducible fashion, a minimal infectious dose of 5 X 10^3 PFU was needed (Niewiesk et al., 1997). It has also been shown that MV RNA is detectable by RT-PCR in many organs of cotton rats infected with this strain of MV for 4 or 7 days (Niewiesk et al., 1997). Similar to humans, suppressive effect of MV was also found in cotton rats. Proliferation of splenocytes stimulated with mitogens and keyhole limpet hemocyanin-specific T cells was suppressed *ex vivo* (Niewiesk et al., 1997; Niewiesk, Gotzelmann, and ter Meulen, 2000). Moreover, it was found that suppressive effect of wild-type and vaccine viruses differs. For instance, wild-type virus suppresses proliferation of splenocytes to mitogens at all titers tested (10^5 or 10^4 $TCID_{50}$) for up to 20 days (Pfeuffer et al., 2003), whereas the Edmonston vaccine strain inhibits proliferation of splenocytes only at high titers ($\geq 10^6$ PFU) for up to 10 days (Niewiesk et al., 1997). Further studies investigating the inhibitory effect of MV on B or T cells of cotton rats revealed that proliferation of B and T cells stimulated with B cell and/or T cell mitogens was inhibited by MV *ex vivo* (Niewiesk, Gotzelmann, and ter Meulen, 2000). In addition, Niewiesk et al assessed the influence of MV infection on B and T cell responses *in vivo*. The results have demonstrated strong inhibition of antigen-specific primary and secondary T cell responses. In contrast, primary and secondary B cell responses were not altered by MV infection (Niewiesk, Gotzelmann, and ter Meulen, 2000). MV infection induces protective immune responses in cotton rats and is cleared by day 10. The T cell responses

develop earlier during infection. They are first seen on day 5, peaks on day 7 and 8, and remain detectable for 90 days (Pueschel et al., 2007). The animals mount MV-specific neutralizing antibody immune responses which can be detected 6 days after infection by ELISA and 12 days after infection by the neutralization assay (Niewiesk&Germann, 2000). As in humans, passively acquired or actively induced serum neutralizing antibodies against MV protect animals against infection (Schlereth et al., 2000b). Besides, cotton rats vaccinated with the live attenuated vaccine virus (Edmonston strain) are protected against disease, but the efficacy of immunization by different routes of inoculation varies (Schlereth et al., 2003).

Cotton rats have also been used to test putative vaccine candidates, as clearance of MV infection in these animals correlates with the development of neutralizing antibodies. Several vector systems expressing the F, H, and/or N proteins have been shown to induce protective levels of neutralizing antibodies. Among these systems are adenovirus (Fooks et al., 1998), immune stimulating complexes (ISCOM) (Wyde et al., 2000), modified virus Ankara (Weidinger et al., 2001), canarypox virus vectors (Wyde et al., 2000), plasmid immunization (Schlereth et al., 2000a), Salmonella and Shigella-mediated plasmid transfer (Pasetti et al., 2003) and recombinant vesicular stomatitis virus (VSV) (Schlereth et al., 2003). Moreover, cotton rat provides a suitable animal model for studying the inhibition of vaccine-induced seroconversion by maternal antibodies. By immunization of dams and measuring the level of MV-specific antibodies transferred to pups, Schlereth et al. (2000b) demonstrated that maternal antibodies inhibited vaccine-induced seroconversion in cotton rats after immunization with the vaccine strain of MV. Nevertheless, this approach has a few disadvantages, including variability of maternal antibody titers between pups and

inability to distinguish between actively generated and passively transferred antibodies (Niewiesk, 2001). To overcome these problems, a new approach with the use of human MV-specific antibodies as a source of 'maternal' antibodies has been developed (Schlereth et al., 2000b). Using this system it has been demonstrated that a recombinant VSV expressing MV H protein induces high titers of neutralizing antibodies to MV in the presence of pre-existing MV-specific antibodies (Schlereth et al., 2000b).

1.3 MEASLES VIRUS VACCINES

1.3.1 Live attenuated measles virus vaccines

The Edmonston B strain of MV licensed in 1963 was the first attenuated live measles vaccine (Enders, Katz, and Holloway, 1962). However, due to induced fever and rash in a large proportion of children (Katz, Enders, and Holloway, 1960) its further attenuation in chick embryo fibroblasts at reduced temperature was implemented. This led to the development of live attenuated vaccine (LAV) strains, Schwarz and Moraten. The Moraten vaccine is the only measles vaccine used in the United States, whereas Schwarz and Edmonston-Zagreb are widely used throughout the world (Moss and Griffin, 2006).

LAV administered subcutaneously or intramuscularly with a dose between 10^3 and 10^4 PFU induces both neutralizing antibody and cellular immune responses, which are qualitatively similar to those induced by the natural infection, although neutralizing antibody titers are lower (Krugman, 1971; Ovsyannikova et al., 2003). Antibodies first appear 12-15 days after vaccination and peak at 1-3 months. LAV is used as a single-virus vaccine or in combination with other LAVs such as those for mumps, rubella and varicella. The

recommended age of vaccination varies from 6 to 15 months. However, LAVs are generally well tolerated and highly effective in infants 9 months of age or older (Markowitz and Nieburg, 1991). Besides, this type of vaccine is recommended for administration to human immunodeficiency virus (HIV)-infected children who do not have severe immunosuppression (Moss, Clements, and Halsey, 2003). LAVs against measles have an excellent safety record and have saved the lives of millions of children (Wolfson et al., 2007). For instance, prior to the development and widespread use of LAVs, measles was estimated to result in 5-8 million deaths annually (Moss and Griffin, 2006), however, nowadays, with the use of LAVs significant progress has been made in measles control, reducing a number of deaths attributed to measles from an estimated 733,000 in 2000 to 164,000 in 2008 (MMWR, 2009).

Even though major progress has been made in interrupting endemic MV transmission, measles remains a major cause of infant and young child morbidity and mortality for a vaccine-preventable disease in a number of developing countries (Grais et al., 2007; Moss, 2007). Several factors converge to facilitate the transmission of MV, ranging from low vaccination coverage to limitations of LAV. First, a reconstituted LAV is very unstable as it is readily inactivated by heat and light and loses about half of its potency at 20^{o}C and almost all potency at 37^{o}C within 1 h (Melnick, 1996). Therefore, a cold chain must be maintained to support measles immunization activities. Second, LAV should be administered intramuscularly or subcutaneously to young infants, as aerosolized vaccine induced lower primary immune responses compared to subcutaneously delivered vaccine (Low et al., 2008). This requirement implies that there is a need for professional healthcare workers, sterile needles and proper disposal of contaminated waste material. Besides, due

to high infectivity of MV and primary and secondary vaccine failures, two doses of LAV must be administered to achieve sufficient levels of population immunity (95%) to interrupt MV transmission (Cutts, Henao-Restrepo, and Olive, 1999). Importantly, the presence of maternal antibodies and immunological immaturity of the recipient hamper effective immunization of infants (Gans et al., 1998; Leuridan and Van Damme, 2007). Interference due to passively acquired antibodies among infants has been observed since the live attenuated measles vaccine was introduced in the 1960s. For instance, it was shown that 12 month-old children immunized against measles failed to seroconvert in the presence of high maternal antibody titers, whereas children with low maternal antibody titers seroconverted. Additionally, the resulting antibody titers were significantly lower than those in children without pre-existing immunity (Albrecht et al., 1977). In addition, vaccination efficacy in infants may be also impaired due to the immaturity of their immune system (Gans et al., 1998). Thus, the proportion of children that develop protective levels of antibodies is 67% below 9 months (Gans et al., 1998), 85% at 9 months (Diaz-Ortega et al., 1994), 95% at 12 months of age (Cutts, Grabowsky, and Markowitz, 1995). Considering the optimum age for seroconversion and the probability of acquiring measles before that age the World Health Organization (WHO) recommended measles vaccination at 12-15 months in areas with low measles burden or at 9 months in areas where measles remains prevalent (WHO, 2002). However, many children are no longer protected by maternal antibodies at the age of 3 months and earlier, especially in low-income developing countries (Dabis et al., 1989), and thereby susceptible to wild-type MV infection. Therefore, there are several requirements that would be advantageous for a new vaccine to meet. First, it would eliminate the need for a cold chain, and avoid the use of needles and

syringes (Mitragotri, 2005). Second, it would be efficacious in the presence of maternal antibodies allowing vaccination of infants before 6 months of age to close the window of susceptibility between the decay of maternal antibodies and vaccination. Third, induction of protective immunity overcoming immunologic immaturity of young infants would be desirable. Finally, it would be safe for immunocompromised individuals.

1.3.2 Experimental vaccines

Several approaches to protect children against measles and close the gap of susceptibility, even in the presence of maternal antibodies, are being evaluated, including alternative routes of administration and alternative dosages of live attenuated measles vaccines or alternative vaccines. First attempt to increase seroconversion rates in infants was immunization with 'high-titer' Edmonston-Zagreb live attenuated measles vaccine, when 100-fold higher doses were used. Some studies revealed that these vaccine candidates were more immunogenic in infants with maternal antibodies than standard 'low-titer' vaccines (Aaby et al., 1988; Markowitz et al., 1990; Whittle et al., 1990). Therefore, WHO recommended these vaccines for use in countries with significant measles transmission where the risk of death by measles was high (WHO,1990). However, 'high-titer' measles vaccines were associated with an increased mortality in girls over the subsequent 2-3 years that may be related to long-term suppression of immune responses to other pathogens (Seng et al., 1999) or to changes in the sequence of vaccine administration (Aaby et al., 2006). Consequently, the WHO recommendation was withdrawn.

Alternative non-percutaneous routes of vaccine administration have also been under intensive investigation as simplifying the delivery of measles vaccine could increase the coverage, acceptance, safety, and efficiency of measles elimination efforts (Cutts, Clements, and Bennett, 1997). The aerosol route is especially attractive for mass vaccination, and was originally proposed and promoted by Albert Sabin. In the 1980s, in Mexico, Albert Sabin and his colleagues were vaccinating children against measles with aerosolized measles vaccine (Sabin et al., 1984; Sabin et al., 1983). By the mid-1990s it was clear that this was an effective method for measles vaccination. One of the earliest meta-analysis reviewed intradermal, conjunctival, oral, intranasal, and aerosol delivery routes found that the administration of measles vaccine by aerosol and intranasal routes appeared to be the most promising (Cutts, Clements, and Bennett, 1997). Recent systematic reviews of published studies to examine the immunogenicity and safety of aerosolized route of measles vaccine administration revealed varying results. One meta-analysis concluded that the respiratory route of delivery of measles vaccine is at least as efficacious as measles vaccine administered through the subcutaneous route (Hiremath and Omer, 2005). A more recent meta-analysis found that aerosolized measles vaccine appears to be equally or more immunogenic than subcutaneous vaccine in children aged 10 months and older, and, in contrast, seroconversion rates were lower with aerosolized than with subcutaneous vaccine in children below 10 months (Low et al., 2008). However, it was suggested that observed differences between these routes were attributable to more than 25-fold lower dose administered by aerosol route, and that an improvement of aerosol delivery or increasing the dose may enhance the immunogenicity of primary measles vaccination by the aerosol route in this age group (Wong-Chew et al., 2006). Other trials

have demonstrated that measles vaccine administered via aerosol provides a superior boosting response, compared to vaccination by injection (Castro et al., 2005; Dilraj et al., 2007; Sepulveda-Amor et al., 2002). As a result, aerosol administration of currently licensed measles vaccine is a component of the WHO measles morbidity reduction and elimination strategy (WHO, 2004). WHO is currently evaluating three aerosol devices in clinical trials with the expectation that at least one can be licensed for use with the Edmonston-Zagreb measles vaccine. In addition, assessment of a measles vaccine in a powder form has also been supported. However, establishing the safety of aerosolized measles vaccines is a priority as theoretical concerns, such as exacerbation of asthma in young children or increased risk of vaccine-induced pneumonia or encephalitis, associated with these vaccines have not been addressed (Valdespino-Gomez et al., 2006).

Among the approaches currently considered to develop second generation measles vaccines, are the use of recombinant viruses expressing the relevant measles proteins as well as the use of DNA vaccines and viral subunits formulated with new adjuvants (de Vries et al., 2008). Investigation of the efficacy of LAV and killed measles vaccines has advanced our knowledge of the determinants of protective immunity. Therefore, development of a new vaccine against measles with certain characteristics would be desirable. A new vaccine should induce neutralizing antibodies to the H and F proteins in addition to stimulating the cellular immune response, should not prime individuals for atypical measles on exposure to wild-type MV, and should not be associated with prolonged immunosuppression.

Due to their simplicity and versatility DNA vaccines became a promising strategy for developing immunity against several infectious diseases. DNA vaccines are chemically and

biologically stable, easy to produce at comparatively low cost, induce strong T cell responses, and do not induce anti-vector immunity (Ingolotti et al., 2010). Early studies on DNA vaccine immunization against measles showed that these vaccines could induce good humoral and cellular responses in mice and cotton rats (Cardoso et al., 1996; Etchart et al., 1997; Yang et al., 1997). Further, DNA vaccines encoding either or both of MV H and F proteins could confer protection from measles in naive, juvenile rhesus macaques without evidence of atypical measles (Polack et al., 2000). Moreover, partial protection from MV challenge in the presence of maternal antibodies was observed in macaques vaccinated with a DNA plasmid encoding MV F, H and N (Premenko-Lanier et al., 2003). However, the vaccination did not lead to seroconversion in all animals, and low levels of neutralizing antibodies were induced.

More recent studies have focused on the use of Sindbis replicon-based DNA vaccines and adjuvants to enhance immunogenicity of DNA vaccines. Sindbis replicon-based DNA approach makes use of the propensity of alphaviruses to generate multiple copies of mRNA encoding structural proteins (Polo et al., 2000). As a result of efficient RNA amplification, high levels of the recombinant protein are produced (Boorsma et al., 2003), which along with activation of innate immune pathways by these vaccines (Leitner et al., 2003) increase their immunogenicity. Promising results were shown in a rhesus macaque study with the use of Sindbis virus-based replicon plasmid (SINCP)-based vaccines, where monkeys primed with the DNA plasmid expressing MV H protein developed high PRN titers, durable responses after boosting with LAV given by aerosol or subcutaneously or with Protollin-MV given intranasally, and were 100% protected from measles (Pasetti et al., 2007). Besides, Pasetti et al investigated immunosuppression and

predisposition to atypical measles in infant macaques primed with DNA vaccines and found that there was no apparent dampening of either antibody or INF-γ-secreting T-cell responses to tetanus toxoid in vaccinated/challenged animals versus the unvaccinated/unchallenged controls and no evidence of atypical measles (Pasetti et al., 2007).

A number of DNA adjuvants have also been studied. One current trend in DNA vaccination is the use of biodegradable cationic polylactide co-glycolide (PLG) microparticles. Their utility for delivery and enhanced immunogenicity has been shown in mice (Pan et al., 2008b). However, the same PLG/SINCP vaccine expressing MV H protein partially protected intramuscularly vaccinated macaques from challenge or showed no protection in monkeys vaccinated intradermally that had more severe rashes and higher viremias, suggesting exacerbated disease (Pan et al., 2008b). Another example of polymers that have been explored for measles DNA vaccination is Vaxfectin® (Hartikka et al., 2001). It was shown that infant and juvenile macaques vaccinated with Vaxfectin formulated codon-optimized DNAs encoding MV H and F glycoproteins developed sustained neutralizing antibody and MV-specific T cell responses and were protected from an intratracheal challenge (Pan et al., 2008a).

Subunit vaccines formulated with novel adjuvants represent another promising and viable strategy to the production of vaccines against measles. They possess characteristics that make them a safe and effective vaccine platform. Recently, some advances were made with a Protollin-MV vaccine candidate. Protollin-MV is a vaccine produced by mixing split MV antigen, which includes H and F proteins, with the novel intranasal adjuvant

Protollin™ (Lowell GH, 2004) comprising *Neisseria meningitides* outer membrane proteins non-covalently complexed with *Shigella flexneri* 2a lipopolysaccharide. It was shown that intranasal immunization of mice with two or three doses of Protollin-MV induces both systemic and mucosal neutralizing antibody responses as well as elicits a balanced Th1/Th2-type response (Chabot et al., 2005). Moreover, some promising results were reported in rhesus macaques, where MV seronegative juvenile macaques received three doses of Protollin-MV had a strong neutralizing antibody response and were also protected from MV challenge, exhibiting neither viremia nor signs of illness and atypical measles (Pasetti et al., 2007). Another promising adjuvant for measles immunization that has the ability to induce strong antigen-specific humoral and cellular immune responses is immunostimulatory complexes (ISCOMs) (Sun, Xie, and Ye, 2009). ISCOMs are delivery systems comprised of antigen, cholesterol, phospholipid and saponin. Varsanyi et al reported that mice inoculated subcutaneously with either H- or F-ISCOMs (preparations of H and F proteins obtained from F-, or H-depleted MV-lysates) were fully protected against a lethal MV challenge (Varsanyi et al., 1987). Moreover, cynomolgus monkeys vaccinated with the same MV H and F protein-ISCOM formulations developed MV-specific humoral and cellular immune responses in the presence and absence of passively transferred antibodies and were partially protected from intratracheal challenge with wild-type MV (van Binnendijk et al., 1997). Although these preparations have been shown to be highly immunogenic and are able to induce humoral and cell-mediated immunity, their development has been hampered by problems related to the toxicity of crude Quil A preparations (Cox, Sjolander, and Barr, 1998). However, purified Quil A components without undesired side effects have been confirmed as potent adjuvants in mice (Stittelaar

et al., 2000a). These improved vaccine candidates have been also tested in cotton rats that were completely protected from pulmonary virus infection and extrapulmonary dissemination (Wyde et al., 2000). Furthermore, additional study to evaluate the longevity of protective levels of MV neutralizing antibody in macaques showed that the same Quil A-based vaccine candidates induced long-lasting protective levels of VN antibodies in a “one-shot” regimen (Stittelaar et al., 2002). Therefore, the properties of ISCOMs make them suitable for use in vaccines but their safety needs further clinical investigation for the development of novel vaccines against measles (Sun, Xie, and Ye, 2009).

The idea of using viruses as gene-delivery systems to combat diseases stems from a documented immunogenicity and safety profile of the majority of existing live-attenuated vaccines. Recent developments in genetic engineering coupled with the accumulated data on the nucleotide structure of viral genomes and functions of viral genes allowed precise manipulations of viral genomes cloned as bacterial plasmids (Takeda et al., 2000) or bacterial artificial chromosomes (Cui et al., 2009) and led to the development of a wide range of different DNA (Dudek and Knipe, 2006) and RNA (Brandler et al., 2007; Wu, Kim, and Kang, 2009) viruses as vectors for efficient delivery of vaccine antigens. This strategy has been applied for the development of vaccines against many dangerous pathogens, including measles. Among the most promising viral vectors delivering MV antigens are adenovirus vectors (Fooks et al., 1998; Fooks et al., 1995), recombinant modified vaccinia virus Ankara (El Kasmi and Muller, 2001; Stittelaar et al., 2001; Stittelaar et al., 2000b), alphavirus (Pan et al., 2010), parainfluenza virus (Skiadopoulos et al., 2001), and vesicular stomatitis virus (Schlereth et al., 2003). Other vectors under research include recombinant bacterial vectors, the Bacille Calmette-Guerin (Zhu et al., 1997), Shigella flexneri and

Salmonella enteric serovar Typhi (Pasetti et al., 2003). Each of these vectors has unique properties that should be considered for the selection of the optimal vector for the vaccination strategy of choice.

Vaccination using replication-defective (ΔE1/ΔE3) recombinant Ad5 vector vaccines have been reported to be promising for vaccine applications because of their ability to induce robust humoral and cellular immune responses in mice, dogs, non-human primates and humans (Appaiahgari et al., 2006; Barratt-Boyes et al., 2006; Catanzaro et al., 2006; Gomez-Roman and Robert-Guroff, 2003; See et al., 2006; Tims et al., 2000). Alkhatib and Briedis reported the construction and initial characterization of replication-defective Ad5 expressing MV H under the control of an adenovirus major late promoter (MLP) *in vitro* (Alkhatib and Briedis, 1988). Their study demonstrated that biologically active MV H protein expressed by the Ad recombinant was properly glycosylated and transported efficiently to the cell surface. Similarly, the construction of the Ad5MVF recombinant with the MV F gene under the control of the adenovirus MLP resulted in expression of biologically active MV F protein that was glycosylated, cleaved and transported to the cell surface (Alkhatib, Richardson, and Shen, 1990). Later, the protective efficacy of Ad5 recombinants expressing MV H and F proteins under the control of the highly efficient human cytomegalovirus (CMV) immediate early promoter was investigated *in vivo* (Fooks et al., 1998). This study demonstrated that oral or intraperitoneal immunization of mice with the recombinant Ad5 expressing MV F protein failed to protect mice, whereas immunization with the recombinant Ad5 expressing MV H protein elicited a significant protective response in mice challenged with MV. Moreover, oral or intraperitoneal administration of replication-defective Ad recombinants could not elicit humoral immune responses,

suggesting that the protection was mediated by cell-mediated immunity. This research also revealed that immunization of cotton rats by the intraperitoneal route with the Ad5 viruses expressing the MV H or F proteins led to a reduction of MV titers in lungs after challenge, whereas intranasal immunization did not result in significant levels of protection (Fooks et al., 1998).

2.0 HYPOTHESES AND OBJECTIVES

The overall hypothesis of this study was that new vaccine candidates based on a replication-defective Ad5 vector and MV H protein subunit vaccine will be able to elicit protective immune responses against MV. To evaluate this hypothesis two sets of studies were carried out.

The hypothesis for the first set was that immunization with a combination of recombinant adenoviruses expressing the MV glycoproteins will induce potent anti-measles immune responses to protect cotton rats against MV challenge. Thus, the first objective here was to generate recombinant replication-defective E1/E3-deleted adenoviruses expressing either MV F or H proteins. The next objective was to examine immunogenicity of the Ad5-based vaccine candidate in mice and protective efficacy in cotton rats.

The hypothesis for the second set was that immunization with a parenterally delivered MV H protein subunit vaccine will result in induction of MV-specific immune responses in mice. Thus, the objective was to develop a stable human cell line expressing a recombinant MV H protein as well as to assess the ability of this protein to elicit MV-specific immune responses in mice.

3.0 ADENOVIRUS-VECTORED VACCINE FOR MEASLES

Published in *Vaccine* (Lobanova et al., 2010).

3.1 INTRODUCTION

Measles is a human-restricted, highly contagious infectious disease and causes severe health complications, such as pneumonia, blindness, diarrhea and encephalitis. Malnourished and un-immunized children under five years of age are most vulnerable to illness and death from this disease. Prior to the introduction of measles live attenuated vaccines measles was estimated to result in 5-8 million deaths annually. Nowadays, despite the global coverage of 83% with a single dose vaccination, measles remains a leading cause of mortality among children in developing countries (MMWR, 2009).

Among factors that facilitate MV transmission are low vaccine coverage and several limitations associated with the use of live attenuated measles vaccines. One of the drawbacks of current measles vaccines is a technical and financial burden for developing countries to maintain an uninterrupted cold chain, as the vaccine efficacy depends on its thermal stability. In addition, the presence of maternal antibodies and the immunological status in newborns and young infants have the most significant influence on the efficacy of LAVs (Albrecht et al., 1977; Gans et al., 1998). The invasive nature of the measles vaccine

administration implies the necessity of medical personnel and the availability of needles and syringes. The latter is associated with an additional safety issue, such as transmission of infectious diseases through unsafe injections. According to the WHO data, more than 50% of all injections administered in developing countries are unsafe (Miller and Pisani, 1999). Finally, to disrupt measles transmission two doses of vaccine as a part of routine childhood vaccination programs should be delivered (Cutts, Henao-Restrepo, and Olive, 1999). However, in 2008, only 16 of 47 priority countries with the highest burden of measles conducted two doses measles-containing vaccine supplemental immunization activities (MMWR, 2009). Therefore, thermally stable vaccines that could overcome the pre-existing maternal immunity and be administered in a safe, cost effective and non-invasive way suitable for mass vaccination campaigns are desirable. In addition, a novel measles vaccine should meet certain immunological requirements that are the correlates of protection, including the induction of certain levels of neutralizing antibodies in addition to stimulation of the cellular immune response (Krugman, 1971; Ovsyannikova et al., 2003; Polack et al., 1999; Polack et al., 2003a).

Adenoviruses possess several features that make them a suitable vaccine candidate capable of eliciting potent protection against mucosal infectious diseases. Due to low virulence, ample safety profile, genetic flexibility, and gene transfer potency replication-defective Ads have been widely used as vectors for vaccination against many infectious diseases (Appaiahgari et al., 2006; Barratt-Boyes et al., 2006; Catanzaro et al., 2006; Gomez-Roman and Robert-Guroff, 2003; See et al., 2006; Tims et al., 2000; Volpers and Kochanek, 2004). In addition, Ads can induce potent long-term humoral and cellular immune responses directed to the expressed product (Juillard et al., 1995; Seder and Hill,

2000). Furthermore, the natural tropism of Ads to the respiratory epithelium allows their mucosal application (Santosuosso, McCormick, and Xing, 2005), which can be superior in eliciting protection from measles (Neutra and Kozlowski, 2006). By exploiting the mucosal route of administration of replication-defective Ad vectors, maternal pre-existing immunity against measles may also be overcome. For instance, mucosal administration of measles and measles-rubella vaccines was shown to be more efficient than subcutaneous administration in pre-immunized humans (Bennett et al., 2002; Dilraj et al., 2000). Finally, the improvement of the stability profile of Ad5-based vaccine formulations should enhance the utility of Ad5 as a vector for vaccines, as an optimized liquid formulation can be stored for at least 2 years at 4^{o}C and has adequate short-term stability at ambient temperatures (Evans et al., 2004). In the present study, the ability of Ad5 recombinants expressing the MV glycoproteins to elicit balanced and protective immune responses against MV in mice and cotton rats was investigated.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines and viruses - Human embryonic kidney (HEK) 293 cells (ATCC, CRL-1573) were propagated in minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO) supplemented with 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA), 10 mM HEPES buffer (Invitrogen), 50 μg/ml gentamicin (Invitrogen) (complete medium), and 10% heat-inactivated fetal bovine serum (FBS, Lonza). Edmonston strain of

MV (ATCC, VR-24) was propagated in Vero (African monkey kidney, ATCC, CCL-81) cells, maintained in complete medium supplemented with 2% heat-inactivated FBS. Briefly, subconfluent monolayers of Vero cells were infected at a multiplicity of infection (MOI) of 0.05-0.1 $TCID_{50}$ per cell. After 1 h adsorption at 37^{o}C, the complete medium/2% FBS was added to the cells. Four to five days after infection, infected cells were harvested with the medium when approximately 80-90% of the cells were involved in cell fusion. Two freeze-thaw cycles were performed to release the virus from the infected cells, followed by clarification at 500 g at 4^{o}C for 10 min.

3.2.2 Plasmids and generation of Ad5-MV-F and Ad5-MVvacH recombinant viruses -

The pH5-R plasmid contains the right end viral sequences of the Ad5 genome with the 1878 bp deletion in the E3 region. The transfer plasmid pH5-L contains the left end viral sequences (nt 1-6100) of the Ad5 genome with early E1 region substituted by transcriptional control elements, including the CMV promoter and the bovine growth hormone polyA (BHG polyA) signal (Zakhartchouk et al., 2005). pUC-MV-F, ordered from GeneScript Corporation (Piscataway, NJ), contains a codon optimized sequence of the MV F gene (the codon adaptability for human cells and the entire mRNA structure were taken into consideration). pCG-MVvacH, provided by Dr. Roberto Cattaneo (Mayo Clinic, Rochester, USA), contains the native sequence of the MV H gene (Muhlebach, Leonard, and Cattaneo, 2008). Methodology of the construction of replication-defective recombinant adenoviruses has been previously described (Bett et al., 1994). Briefly, pCG-MVvacH was used as a template to amplify the full length MV H gene using primers (MVvacH-FOR) 5'-

CCCAAGCTTATGTCACCACAACGAGACCG-3' and (MVvacH-REV) 5'-ATTTGCGGCCGCCTATCTGCGATTGGTTCCAT-3'. PCR were carried out using a Phusion High-Fidelity PCR kit (New England Biolabs, Pickering, ON). The amplified PCR fragment was digested with *Hind*III and *Not*I and then ligated into the expression cassette of pH5-L cut with the same enzymes, resulting in the transfer vector pH5L-MVvacH. pH5L-MV-F was constructed by excision of the MV F gene from pUC-MV-F by *Sal*I/*Kpn*I digestion and ligation of the fragment into pH5L cut with the same enzymes. Modified left-hand fragments of the Ad5 genome were released from the transfer vectors pH5L-MVvacH and pH5L-MV-F by *Pac*I digestion. The resulting transfer fragments were separately co-transfected in HEK 293 cells with the right-hand portion of the Ad5 genome excised from the pH5-R plasmid by *Pac*I digestion. Co-transfections were carried out using a Calcium Phosphate Transfection Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two replication-defective recombinant adenoviruses generated by homologous recombination in HEK 293 cells (Graham et al., 1977) were named Ad5-MV-F and Ad5-MVvacH**.** Ad5-MV-F was rescued after the co-transfection followed by three blind passages in HEK 293 cells, whereas Ad5-MVvacH was rescued after the co-transfection and two blind passages in HEK 293 cells. Replication-defective Ad5 rescued using unmodified transfer fragments excised from pH5-R and pH5-L was named Ad5-Empty (control adenovirus).

3.2.3 Virus purification - To purify adenoviruses HEK 293 were plated in 150-cm^2 Corning flasks and infected with high titer stocks of Ad5-MV-F or Ad5-MVvacH when

monolayers reached approximately 80% confluency. The cells along with culture medium were harvested at 48 h post-infection when full cytopathic effect (CPE) was observed. Cell suspensions were clarified by centrifugation at 3,000 rpm for 10 min. After centrifugation the culture medium was aspirated off, and the pellets were combined and resuspended in phosphate buffer with 7% glycerol. Samples were stored at -80^{o}C. Subsequently, 1.2 ml of 5% sodium deoxycholate was added to 12 ml of thawed cell lysate. After 30 min incubation at room temperature (RT), 120 μl of 2 M $MgCl_2$ and 60 μl of DNase I solution were added. The samples were incubated at 37^{o}C for 30 min and then centrifuged at 3,000 rpm for 15 min at 5^{o}C. In an ultracentrifuge tube, 4 ml of 1.25 g/ml CsCl was overlaid with 1.5 ml of 1.35 g/ml CsCl. This was overlaid with 6 ml of the viral supernatant and centrifuged at 35,000 rpm (Beckman SW41 rotor) for 2 h at 10^{o}C (accelerate/decelerate set to 1). Viral bands, located at the 1.25g/ml-1.35g/ml interface, were collected by puncturing the side of the tube with a syringe. The withdrawn virus was transferred to an ultracentrifuge tube and the volume was increased with 1.35 g/ml CsCl. A second ultracentrifugation was performed at 35,000 rpm (Beckman SW50.1 rotor) for 16-20 h at 4^{o}C. The single band was collected, and 50% sucrose was added to the virus suspension to bring the sucrose concentration to 1%. Finally, the virus suspension was dialyzed against three changes of 1,500 ml of phosphate-buffered saline containing 1%, 2% and 5% sucrose, respectively, at 4^{o}C, and stored at – 80^{o}C until use.

For MV purification, Vero cells were infected at a MOI of 0.05 and incubated at 37^{o}C in humidified atmosphere with 5% CO_2. After 48 h, the culture medium was aspirated and 2.5 ml of MEM without FBS were added into each 150-cm^2 flask. Infected cells were collected by scraping and subjected to two freeze-thaw cycles. Cell debris was removed by

centrifugation at 500 g for 10 min at 4^{o}C. Concentrated virus was centrifuged at 4^{o}C for 4 h at 25,000 rpm (Beckman SW32 rotor) through 20% (w/v) sucrose onto a cushion of 60% (w/v) sucrose. Subsequently, the virus was collected at the interface, dialysed overnight in a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) against 1,500 ml of phosphate buffered saline (PBS) at 4^{o}C, and stored at - 80^{o}C until further use in ELISA. Protein concentration of the purified MV was determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), based on the Bradford dye-binding procedure (Bradford, 1976).

3.2.4 Virus titration – MV titers were determined by 50% tissue culture infective dose ($TCID_{50}$) method on Vero cells. Shortly, ten-ford serial dilutions of MV stock in MEM without FBS were added to 70-80% confluent Vero monolayers in 96-well tissue culture plates (Corning Inc., Corning, NY). Cells were incubated for 5 days at 37^{o}C in a humidified atmosphere with 5% CO_2, and wells were scored for the presence of viral cytopathic effect (CPE). Viral titer was expressed as $TCID_{50}$/ml (Karber, 1931; Spearman, 1908). Viral stocks ($1x10^6$ $TCID_{50}$/ml) were stored at – 80^{o}C until further use in virus neutralization assay or challenge trials.

Titration of the adenovirus vectors was carried out by $TCID_{50}$ method using HEK 293 cells. Briefly, HEK 293 cells were collected by trypsinization, and $1x10^5$ cells/ml cell suspension was prepared in complete medium supplemented with 10% FBS. One hundred μl of the cell suspension were plated into each well of a 96-well plate (Corning Inc.) along with 100 μl of 10-fold virus dilutions made in serum-free medium. After 14 days of incubation, wells were scored for the presence of viral CPE. Spearman-Karber statistical

method was used for determining the $TCID_{50}$ value of recombinant adenoviruses (Karber, 1931; Spearman, 1908).

3.2.5 Isolation of adenoviral DNA from infected cell lysates and PCR - HEK 293 cells were infected with high titer stocks of Ad5-MV-F, Ad5-MVvacH, or Ad5-Empty, and cultured in complete medium supplemented with 5% FBS. After 48 h.p.i., when full CPE was observed, the cell suspension was harvested and subjected to three cycles of freezing and thawing. Cell debris was removed by centrifugation at 1,500 rpm for 5 min. Two hundred and seventy-five μl of clarified virus supernatant were used for the DNA isolation. This procedure included incubation of the supernatant with 10 μl of DNase I (10 mg/ml) at 37°C for 30 min, followed by addition of 6 μl of 0.5 M ethylenediaminetetraacetic acid (pH 8), 7.5 μl of 20% sodium dodecyl sulfate (SDS), 1.5 μl of Proteinase K (20 mg/ml) and incubation at 50°C for 1 h. Total DNA was further extracted using the BIO101 GeneClean (GC) Spin Kit (Integrated Sciences, NSW, Australia) according to the manufacturer's instructions. For detection of insertion of the MV F and H genes into the Ad5 genome the total DNA extracted from virus-infected or mock-infected cells was subjected to the amplification using 2X PCR Master Mix (Fermentas, Burlington, ON). Primers, (ADAPT R) 5'-GCCACGCCCACACATTTCAG-3' and (CP52) 5'-CGCCCCATTGACGCAAA-3', were designed to anneal in the sites flanking the deleted E1 region of the Ad5 genome. PCR was carried out in 50 μl containing 25 pmol of each primer and ~0.1 μg of DNA, using 30 cycles each consisting of 10 sec at 98°C, 30 sec at 56°C,

and 1 min at 72°C. Amplification products were size-separated by electrophoresis in 1% agarose gels.

3.2.6 Western blotting - Non-complementing Vero cells grown in a six-well plate (10^6 cells/well) were infected with Ad5-MV-F (MOI 100 $TCID_{50}$/cell), Ad5-empty (MOI 100 $TCID_{50}$/cell), MV Edmonston (MOI 0.1 $TCID_{50}$/cell) or mock-infected. Total cell extracts were prepared at 72 h.p.i. by resuspension of cell pellets in RIPA buffer (50 mM Tris-HCl, pH7.4, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS and 150 mM NaCl) supplemented with aprotinin and phenylmethanesulfonylfluoride, followed by incubation on ice for 30 min and two short cycles of sonication. Solubilized proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Samples were boiled at 96°C for 5 min in 2X loading buffer containing 4% β-mercaptoethanol (Sigma, St. Louis, MO). The proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked by overnight incubation with 5% skim milk in TBS/0.1% Tween-20 at 4°C, followed by incubation at RT for 4 h with MV F-specific mouse monoclonal antibody (mAb) (Fost-2 antibody was kindly provided by Dr. Waku, INSERM, Lyon, France) diluted 1:500. Bound antibodies were detected by incubating the blot with goat anti-mouse HRP-conjugated secondary antibody (GE Healthcare, Baie d'Urfe, QC) (1:3,000) at RT for 1 h, followed by visualization of specific protein bands using ECL-plus reagent (GE Healthcare).

3.2.7 Fluorescence activated cell sorting (FACS) - Vero cells, cultured in a 12-well plate (10^5 cells/well) were mock-infected or infected with either 100 $TCID_{50}$/cell of Ad5-

Empty or Ad5-MVvacH. After 48 h.p.i., the cells were trypsinized, washed three times with PBS, and subjected to immunostaining with the clone CV_1CV_4 anti-measles mAb (1:5,000) (GenWayBiotech, San Diego, CA) along with goat Cy2-conjugated anti-mouse IgG (1:100) (Jackson Immunoresearch, West Grove, PA) at $4^{O}C$ for 1 h. To confirm the specificity of the assay, a protein A-specific mAb (1:15,000) (IgG1 isotype; Sigma-Aldrich, St. Louis, MO) was taken as an isotype control. After washings with PBS, the cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson). The acquired data were analyzed using CellQuest software.

3.2.8 Immunoprecipitation - Vero cells grown in a six-well plate (10^6 cells/well) were infected with 100 $TCID_{50}$/cell of Ad5-MVvacH or Ad5-Empty. For positive control, the cells were infected with MV Edmonston at a MOI of 0.1. After virus absorption for 1 h, the cells were incubated in complete medium containing 5% FBS. After 36 h.p.i., the medium was replaced to methionine/cysteine-free MEM (Sigma-Aldrich) supplemented with 2% dialysed FBS (Invitrogen) and 100 μCi of [^{35}S]methionine/cysteine (100 μCi/well, Perkin Elmer, Woodbridge, ON). Following 12 h of labelling, the cells were harvested and lysed with 200 μl of RIPA buffer for 30 min on ice, followed by two short cycles of sonication. The supernatants were incubated overnight at $4^{o}C$ with 10 μl of the clone 166 anti-measles mAb (GenWay Biotech). Subsequently, 100 μl of Protein A sepharose beads pre-incubated with a rabbit anti-mouse polyclonal antibody were added to the supernatants. After incubation at $4^{o}C$ for 6 h, the beads were washed four times with RIPA buffer, and the

samples were subjected to SDS-10%PAGE. The gel was vacuum-dried at 80^{o}C for 2 h and analysed by autoradiography.

3.2.9 Syncytium formation assay - Vero cells cultured in a 48-well plate (10^5 cells/well) were infected with 100 $TCID_{50}$/cell of Ad5-MV-F and Ad5-MVvacH or Ad5-MVvacH alone. Vero cells infected with MV (MOI=0.01) and uninfected Vero cells were taken as a positive and negative control, respectively. After 48 h.p.i., the cells were fixed with 75% ethanol/25% glacial acetic acid at RT for 15 min, and stained with Giemsa stain (Ricca Chemical, Arlington, TX). After washing with distilled water, plates were air-dried and examined under a microscope.

3.2.10 Mouse trial - Purified Ad5-MV-F and Ad5-MVvacH were used in this study. Six-week-old female C57BL/6 mice were randomly allocated to three groups of five animals each and vaccinated twice intramuscularly (IM) or intranasally (IN) at a 4-week interval. The first group of mice was inoculated IM with a combination of Ad5-MV-F and Ad5-MVvacH (collectively called Ad5-F/H) with a dose of $5x10^8$ $TCID_{50}$ of each virus per mouse. The second group was inoculated IN with the same dose of combined recombinants. Before intranasal vaccination, each mouse was anaesthetized with isoflurane. The third group was left as a negative control. Blood was collected prior to the first immunization as well as at week 4 (prior to the boost) and week 7. Blood samples were allowed to clot at RT for 2 h before centrifuging for 20 min at 2,000 g, and the resulting sera were stored at -

80^0C. Subsequently, sera were tested for total MV-specific IgG and IgG subclasses by ELISA and for the presence of MV-specific neutralizing antibodies. Mice were euthanized three weeks after the boost, and lungs were removed for preparation of bronchoalveolar lavages (BAL) that were sampled for the presence of MV-specific IgA by ELISA. To prepare BAL, lungs were washed with 0.5 ml of PBS and the fluids were collected into 15-ml Falcon conical tubes (BD Biosciences). Clarified supernatants were transferred into new tubes and stored at –80^oC. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council for Animal Care.

3.2.11 Cotton rat trial - Six-week-old cotton rats of either sex were randomly allocated into four groups of five animals. Before vaccination, each rat was anaesthetized with isoflurane. Groups 1 and 2 were immunized either IM or IN with Ad5-F/H with a dose of $5x10^8$ $TCID_{50}$ of each virus per rat. Groups 3 and 4 were inoculated either IM or IN with a control virus, Ad5-Empty (10^9 $TCID_{50}$ per animal). One rat died during the course of the study and was excluded from all analyses. Four weeks after vaccination, all cotton rats were anaesthetized with isoflurane and challenged IN with Edmonston strain of MV ($5x10^4$ $TCID_{50}$ per rat). Blood was collected prior to the first immunization and at week 4 prior to the challenge. Blood samples were allowed to clot at RT for 2 h before centrifuging for 20 min at 2,500 rpm, and the resulting sera were stored at -80^0C. Subsequently, sera were tested for the presence of MV-specific neutralizing antibodies. Total RNA was extracted from the homogenates of lungs collected on day 4 after challenge. Viral RNA was detected by RT-PCR with primers specific for the N gene of MV. All procedures involving animals

were performed in accordance with the guidelines of the Canadian Council for Animal Care.

3.2.12 Enzyme-linked immunosorbent assay (ELISA) - Sera were assayed for MV-specific IgG, IgG1, and IgG2a, whereas BAL samples were tested for MV-specific IgA. Ninety-six-well IMMULON 2 microtiter plates (Thermo Scientific, Hudson, NH) were coated overnight at 4^{o}C with 100μl/well of 1 μg/ml purified and UV-inactivated MV. The plates were washed five times in PBS containing 0.05% Tween-20, and incubated at room temperature for 2 h with samples serially diluted with PBS containing 0.5% gelatine and 0.05% Tween-20, beginning at 1:100, and continuing in 4-fold dilutions (sera) or 2-fold dilutions (BAL). After five washes biotinylated goat anti-mouse antibodies (Caltag Laboratories, Buckingham, UK) diluted 1:5,000 were added, followed by incubation at RT for 1 h and five washes. Alkaline phosphatase (AP)-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA) at dilution of 1:5,000 was used to detect bound IgG, IgG1, IgG2a and IgA. Following 1-h incubation at RT, the plates were washed eight times with distilled water. Reactions were visualized with *p*-nytrophenyl phosphate (Sigma-Aldrich), stopped with 1% HCl, and analyzed at O.D. 405 nm using an ELISA plate reader (Molecular Devices Corporation, Sunnyvale, CA). Results were expressed as the reciprocal of the highest dilution resulting in a reading of 2 standard deviations above the value of the negative control serum.

3.2.13 Virus neutralization assay - Serum samples were heat-inactivated at 56^{o}C for 30 min and were then diluted in MEM (no FBS), beginning at 1:20 dilution and

continuing in two-fold dilutions. Each serum dilution was incubated with 100 $TCID_{50}$ of MV Edmonston strain for 1 h at 37^{o}C. Sample-virus mixtures were added to sub-confluent Vero cell monolayers cultured in ninety-six-well plates and incubated at 37^{o}C for 5 days. Cell cultures were examined on day 6 for characteristic CPE. Virus-neutralizing titers are expressed as the reciprocal of the highest dilution that completely inhibited virus-induced CPE.

3.2.14 RNA isolation and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) - Four days after challenge, cotton rats were euthanized and lungs were removed into 2-ml screw-cap tubes (VWR International) containing 2.4-mm zirconia microbeads (BioSpec Products, Bartlesville, OK) and 1 ml of Trizol (Invitrogen), and were homogenized in a mini-beadbeater (BioSpec Products) for 10 s. RNA was isolated from 100µl of lung homogenates using additional 400 µl of Trizol reagent (Invitrogen) according to the manufacturer's instructions. Quantification of RNA was performed using the Agilent RNA analysis kit (Agilent Technologies, Santa Clara, CA) and 2100 Bioanalyzer. The purified RNA samples were subjected to RT-PCR using the OneStep RT-PCR kit (Qiagen, Hilden, Germany). Briefly, RT-PCR was performed in a final volume of 50 µl, containing 1 µg of RNA and MV-specific primers, NPB1 and NPB2 (Nakayama et al., 1995), flanking a 169 bp fragment of the MV N gene. A 128 bp fragment of cotton rat β2-microglobulin gene was amplified with primers (1) 5'-CGCACCATGGCTCGTACCGT-3' and (2) 5'-TGGGGTGGGTGGAACTGGGA-3' as a housekeeping gene control. Thermal cycler parameters were as follows: reverse

transcription for 30 min at 50°C; enzyme activation for 15 min at 95°C; followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 56°C, and extension for 1 min at 72°C; final extension for 10 min at 72°C. PCR products were separated by electrophoresis through 2% agarose gel and stained with ethidium bromide. Average intensities of DNA bands were quantified using Bio-Rad Quantity One software. The intensities of MV-specific DNA bands were normalized to intensities of β2-microglobulin cDNA fragments that were separated on the same gel. Normalized intensities of bands were compared to the highest normalized band intensity value which was set at 100%.

3.2.15 Statistical analysis - All data were analyzed using GraphPad Prism Version 5.03 software. Differences among all groups were examined using the Mann-Whitney U test. Differences were considered significant if $P<0.05$.

3.3 RESULTS

3.3.1 PCR analysis of genomes of the recombinant adenoviruses - To confirm the insertion of MV genes into Ad5 genomes, adenoviral DNA was isolated from HEK 293 cells infected for 48 h with Ad5-MV-F, Ad5-MVvacH, Ad5-Empty or mock-infected. The isolated total DNA samples were subjected to PCR with primers ADAPT R and CP52 that were designed to anneal in the areas flanking the deleted E1 region of the Ad5 genome. As predicted, a 418 bp PCR fragment was amplified from the Ad5-Empty DNA (Fig. 3.1A and

B), whereas the insertion of MV H and F genes led to the increase of the size of amplified fragments to 2,233 bps (Fig. 3.1A) and 2,097 bps (Fig. 3.1B), respectively. No fragments were amplified in no template controls and the sample of DNA extracted from mock-infected cells (Fig. 3.1A and B).

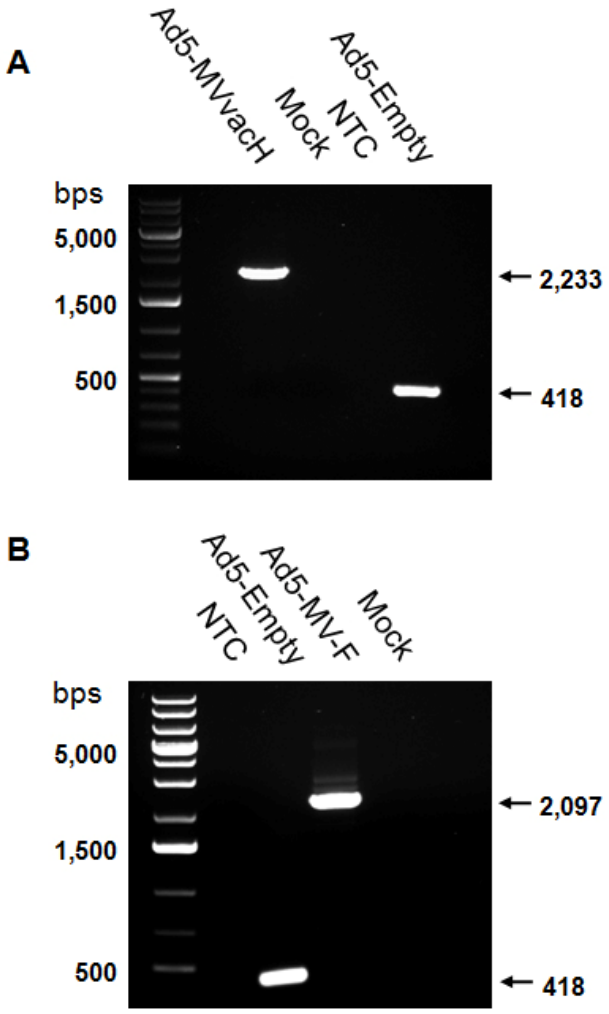

Figure 3.1. Validation of the insertion of expression cassettes for MV H and F proteins in place of the deleted E1 region of Ad5. Viral DNA was isolated from HEK 293 cells infected with Ad5-MVvacH, Ad5-MV-F, Ad5-Empty or mock-infected, and a fragment encompassing the expression cassette was amplified using the same primer pair. (A) Expected PCR fragments 2,233 bps and 418 bps amplified from Ad5-MVvacH and Ad5-Empty DNA, respectively. (B) As predicted, a 2,097 bp fragment was amplified from Ad5-MV-F DNA. NTC, no template control. Values on the left of the panels are fragment sizes in base pairs.

3.3.2 Characterization of the expression of MV F and H proteins by the recombinant adenoviruses -

Validation of MV F protein expression by Ad5-MV-F was performed on non-complementing Vero cells that were either uninfected or infected with Ad5-Empty, Ad5-MV-F or MV. The cells were collected at 72 h.p.i. and total cell extracts were prepared and examined by Western blotting using the F protein-specific mAb. As anticipated, no MV F protein-specific bands were detected in lysates of uninfected or Ad5-Empty-infected cells (Fig 3.2).The antibody recognized 60 kDa and 40 kDa protein bands, corresponding to uncleaved F_0 and cleaved F_1 forms of the F protein, respectively, only in whole-cell extracts of Ad5-MV-F and MV-infected cells (Fig. 3.2). In addition, a high molecular mass protein band was detected by the antibody. This band may correspond to the F protein trimers (Lee et al., 2007).

The expression of H protein in Ad5-MVvacH-infected cells was determined by immunoprecipitation. Vero cells were mock-infected or infected with Ad5-Empty, Ad5-MVvacH, or MV. At 36 h.p.i., the cells were metabolically labeled for 12 h with [^{35}S] methionine/cysteine. Following labeling, total lysates of the infected cells were immunoprecipitated with the MV H-specific mAb and analyzed by SDS-PAGE under reducing conditions. The immunoprecipitation of Ad5-MVvacH-infected lysates revealed a band with molecular mass about 78 kDa (Fig. 3.3), which co-migrated with the H protein produced in the MV-infected cells. No similar band was observed in mock-infected or Ad5-Empty infected cell lysates.

To test whether the H protein expressed by the recombinant adenovirus was present at the surface of infected cells, Ad5-MVvacH-infected Vero cells were labeled with the MV H-specific mAb and analyzed by FACS. As shown in Figure 3.4, the antibody

stained more than 97% of Vero cells infected with Ad5-MVvacH. In contrast, only background levels of staining were detected in the samples of Ad5-Empty-infected or uninfected cells. Moreover, less than 1% of the Ad5-MVvacH infected cells were stained with the isotype control antibody, confirming the specificity of the assay.

The biological activity of the recombinant F and H proteins was tested by examining the ability of these recombinants to cause cell fusion through F protein and subsequent syncytium formation in Vero cells, which are characteristics of MV infection. It has been demonstrated that fusion requires the expression of H and F proteins, as well as F-H protein interactions (Cattaneo and Rose, 1993; Wild, Malvoisin, and Buckland, 1991). In agreement with previously published results (Fooks et al., 1998), Vero cells infected with the combination of Ad5-MV-F and Ad5-MVvacH developed characteristic syncytia (Fig. 3.5, panel C), whereas the H-expressing adenovirus alone did not show any syncytium formation (panel B). As anticipated, no syncytium formation was detected in uninfected cells (panel A), whereas the infection with MV resulted in the development of characteristic cell fusions (panel D).

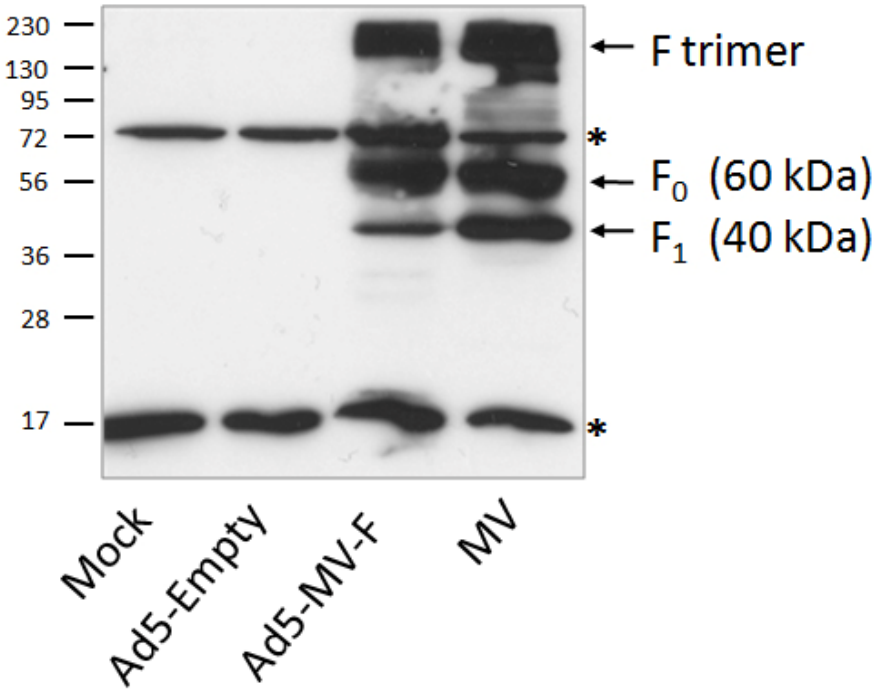

Figure 3.2. Detection of the MV F protein expression in Vero cells infected with the Ad5-MV-F recombinant. Non-complementing Vero cells were infected with Ad5-MV-F, MV, Ad5-Empty or mock-infected. At 72 h post-infection, the total cell extracts were prepared and analyzed with F-specific mAb, Fost 2, by Western blotting. F_0, F_1 species and F trimers are indicated on the right of the panel. Non-specific cellular proteins detected by the antibody are indicated by asterisks. Values on the left of the panel are molecular masses in kDa.

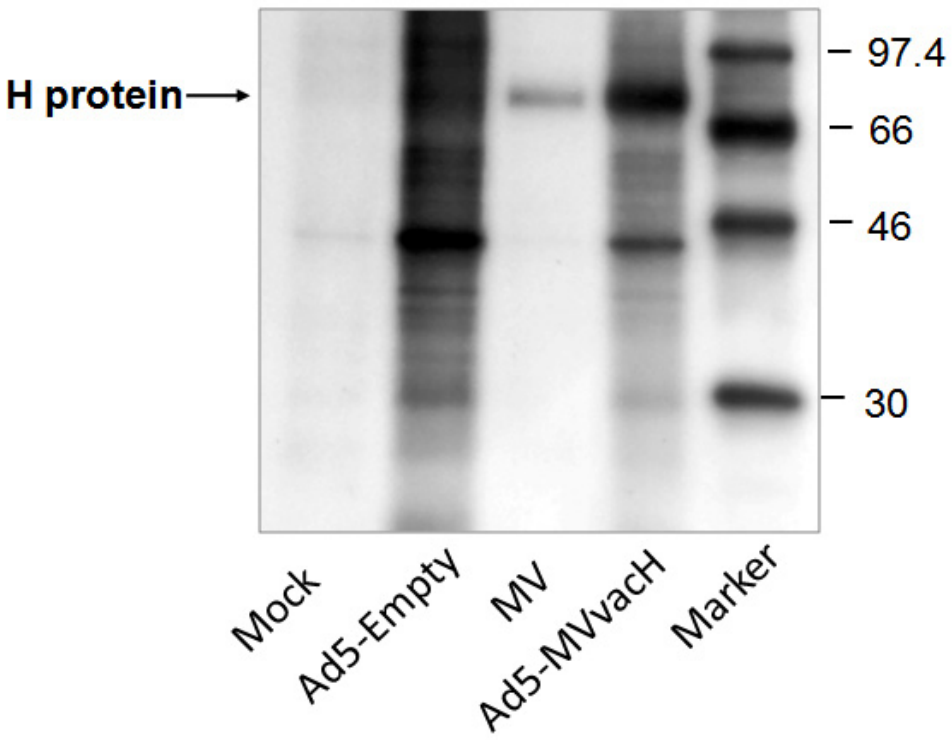

Figure 3.3. Detection of the MV H protein expression in Vero cells infected with the Ad5-MVvac H recombinant. Vero cells were mock-infected or infected with Ad5-Empty, Ad5-MVvacH, or MV. At 36 h.p.i., the cells were pulse labelled for 12 h with [^{35}S] methionine/cysteine. Total lysates of the infected cells were immunoprecipitated with the MV H-specific mAb and analyzed by SDS-10%PAGE under reducing conditions. Proteins from the lysates of radiolabelled Vero cells, uninfected, infected with Ad5-empty, MV or Ad5-MVvacH, a molecular mass marker containing [^{14}C] methylated proteins. The migration of molecular mass standards (in kDa) is shown on the right of the panel.

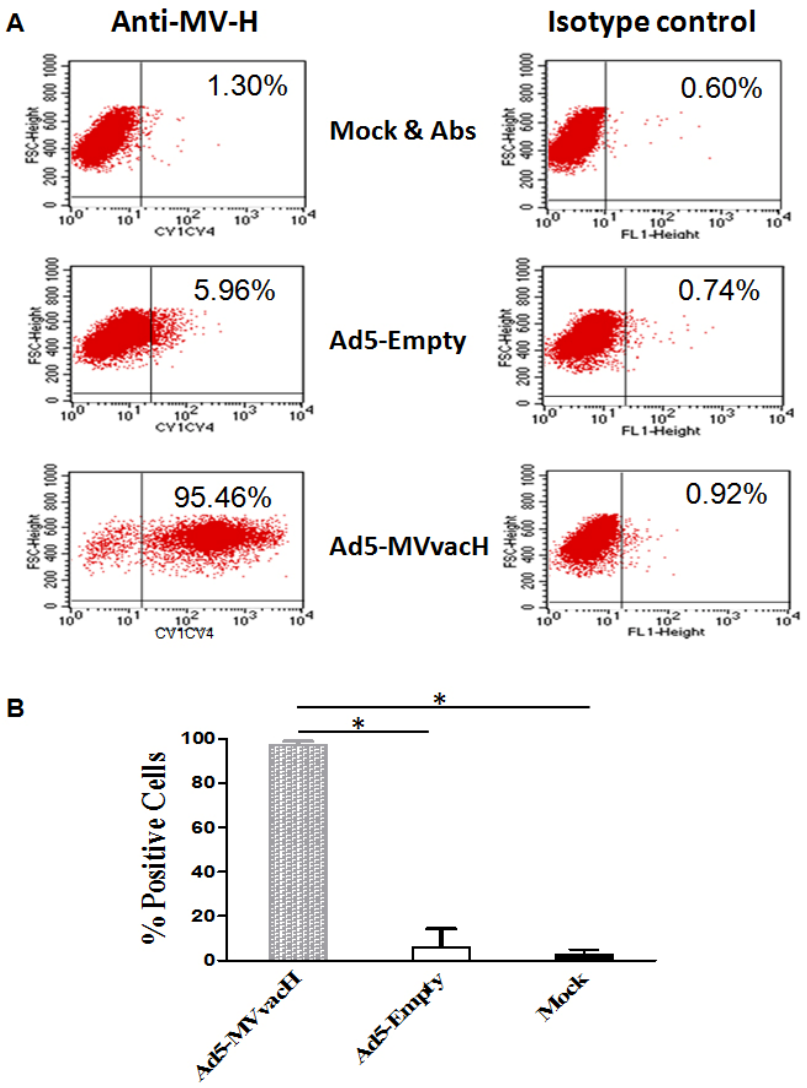

Figure 3.4. Detection of the MV H protein expressed by Ad5-MVvacH on the surface of infected Vero cells by FACS. (A) Representative diagrams of the analysis results. (B) Medians of three independent experiments with range. Non-complementing Vero cells were infected with Ad5-MVvacH, Ad5-Empty or mock-infected. At 48 h post-infection, the cells were collected and incubated on ice with MV H-specific mAb CV_1CV_4 along with goat Cy2-conjugated anti-mouse IgG for 1 h. The percent of Cy2-positive cells was determined by FACS analysis. *, $P < 0.05$.

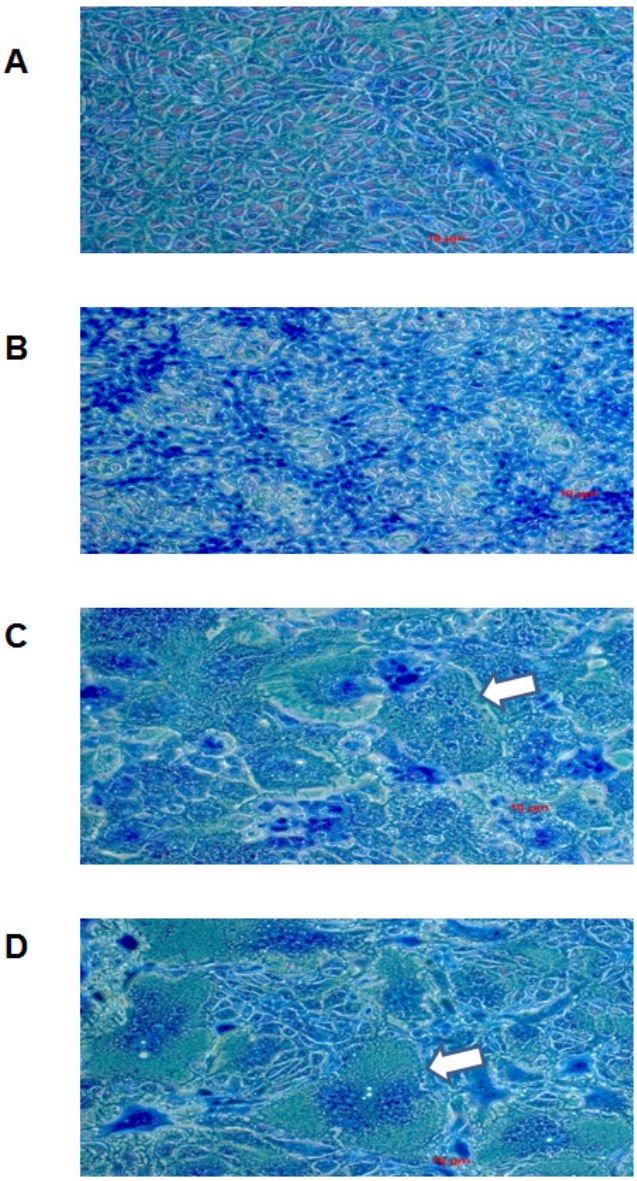

Figure 3.5. Cell fusion assay showing syncytium formation in Vero cells infected with a combination of Ad5-MV-F and Ad5-MVvacH. Vero cells infected for 48 h at MOI of 100 with (B) Ad5-MVvacH, (C) with the combination of Ad5-F/H recombinants (MOI of 100 with Ad5-MV-F and MOI of 100 with Ad5-MVvacH). Uninfected Vero cells (A) and Vero cells infected with MV (D) were taken as a negative and positive control, respectively. Syncytia were visualized by fixation with 75% ethanol/25% glacial acid and stained with Giemsa dye. Syncytia are indicated with arrows.

3.3.3 Measles-specific humoral immune responses in mice immunized with the Ad5 recombinants -

To determine the immunogenicity of the adenovirus-vectored vaccine against MV, two groups of C57BL/6 mice were vaccinated twice IN or IM with a combination of Ad5-MV-F and Ad5-MVvacH with a dose of $5X10^8$ $TCID_{50}$ of each virus per mouse. Humoral immune responses were examined by measuring the MV-specific IgG titers and virus neutralizing titers after the first and second immunization. Significantly higher levels of IgG were elicited after the first inoculation of mice with the Ad5-F/H recombinants compared to the control mice ($P<0.05$) (Fig. 3.6A). Following the second immunization, Ad5-F/H resulted in higher levels of serum IgG production regardless of the route of vaccination, although the difference between IM and IN vaccinated groups was not statistically significant.

To evaluate the biological effectiveness of antibodies produced in the sera, *in vitro* virus neutralizing titers were assessed after each immunization. After the first vaccination, high MV-specific serum-neutralizing antibody titers (Fig. 3.6B) were induced in mice immunized IN or IM compared to the control group ($P<0.01$). Following the second vaccination with Ad5-F/H, neutralizing antibody levels significantly increased ($P<0.05$). However, there was no significant difference in the MV-specific serum-neutralizing antibody titers between the groups of mice vaccinated IM or IN at either week 4 or week 7.

To evaluate the type of immune responses induced by the adenovirus-vectored vaccine, the MV-specific IgG subclass titers were determined after two consecutive immunizations by ELISA. As evident in Figure 3.6C, the mice vaccinated with Ad5-F/H IM or IN experienced a significant increase in IgG1 ($P<0.05$) and IgG2a ($P<0.05$) compared to

the control group. However, IM inoculation of the Ad recombinants resulted in induction of a predominant Th1-type immune response, whereas IN immunization elicited a more balanced Th1/Th2 immune response since there was no statistically significant difference between MV-specific IgG1 and IgG2a titers.

To assess the MV-specific mucosal immune response, the secretion of IgA in BAL samples was measured after two immunizations. As shown in Figure 3.6D, significantly higher MV-specific IgA titers were detected in all immunized mice compared to the control group. In addition, 100-fold increase in IgA titers was found in BAL of mice, vaccinated IN, compared to those observed in BAL samples of mice, vaccinated IM. These results demonstrate advantage of the IN route of vaccination for the induction of mucosal MV-specific immunity after the immunization with the Ad5-F/H recombinants.

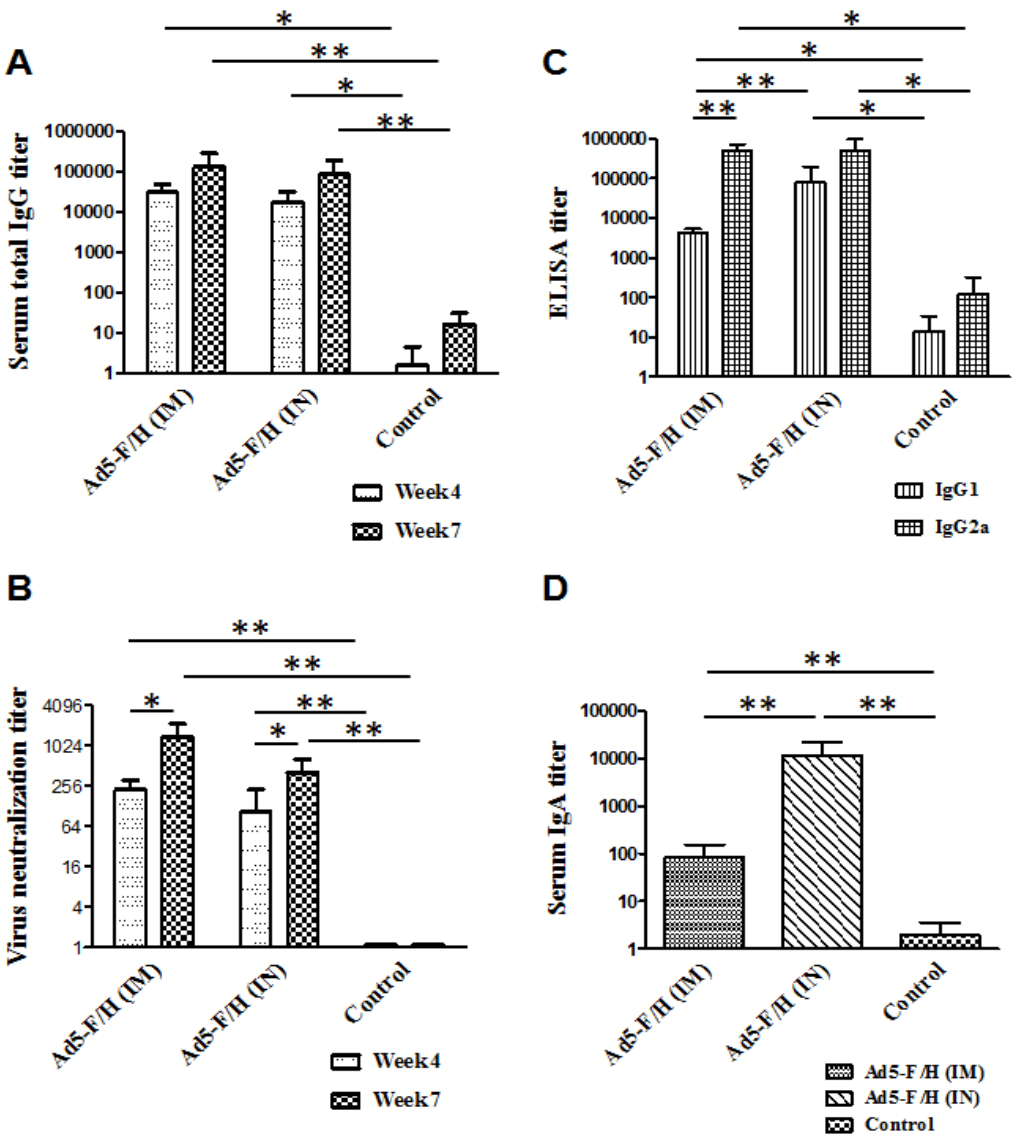

Figure 3.6. MV-specific humoral immune responses in sera of mice immunized with a combination of Ad5-MV-F and Ad5-MVvacH. Two groups of mice (5 mice in each group) were immunized twice at a 4 week interval with Ad5-F/H recombinants ($5X10^8$ $TCID_{50}$ of each virus per mouse). The third group was left as a negative control. One group of mice

received two IM inoculations with Ad5-F/H, whereas the second group received two IN inoculation. Three weeks after the second immunization animals were euthanized. Sera were collected at week 4 (prior to the boost) and week 7, and BALs were collected at week 7. (A) MV-specific IgG titers (mean with SD) in sera of mice after the first and second immunization. (B) Virus neutralizing antibody titers (mean with SD) in sera of mice after the first and second immunization. Virus-neutralizing titers are expressed as the reciprocal of the highest dilution that completely inhibited virus-induced CPE. (C) MV-specific IgG1 and IgG2a (mean with SD) in mouse sera after two consecutive immunizations. (D) MV-specific IgA titers (mean with SD) in BALs of mice at week 7 after the first immunization. ELISA results are expressed as the reciprocal of the highest dilution resulting in a reading of 2 standard deviations above the value of the negative-control serum. *, $P < 0.05$; **, $P < 0.01$.

3.3.4 Measles-specific immune responses in cotton rats immunized with the Ad5 recombinants -

To determine the presence of MV-specific immune responses, virus neutralizing titers were evaluated in sera of cotton rats four weeks after a single vaccination. The cotton rats vaccinated IN or IM with Ad5-F/H displayed high MV-specific serum neutralizing-antibody titers (Fig. 3.7). Importantly, similar serum neutralizing-antibody titers were induced by Ad5-F/H delivered IN or IM. As expected, no virus neutralizing antibody titers were induced by Ad5-Empty regardless of the route of inoculation.

3.3.5 Protection of cotton rats against measles virus respiratory challenge - To evaluate the protective capacity of Ad5-F/H anti-measles vaccine, cotton rats were challenged IN with MV Edmonston four weeks after the vaccination and the viral RNA load in lungs was examined by PT-PCR. The rat β-2 microglobulin mRNA was detected in all samples for the data normalization. The results of RT-PCR analysis revealed that all vaccinated groups showed a decrease in viral replication, compared to the mock-vaccinated, virus-challenged groups (Fig. 3.8A and B). According to the densitometry results, fragments amplified from the lung homogenates of cotton rats immunized IM demonstrated nearly 81% reduction in the normalized band intensity (Fig. 3.8B), whereas MV RNA was undetectable in the lungs of cotton rats immunized IN (Fig. 3.8A and B). These data suggest that IN delivery of the Ad5-F/H vaccine induces a more pronounced suppression of the MV replication in lungs of cotton rats than IM delivery.

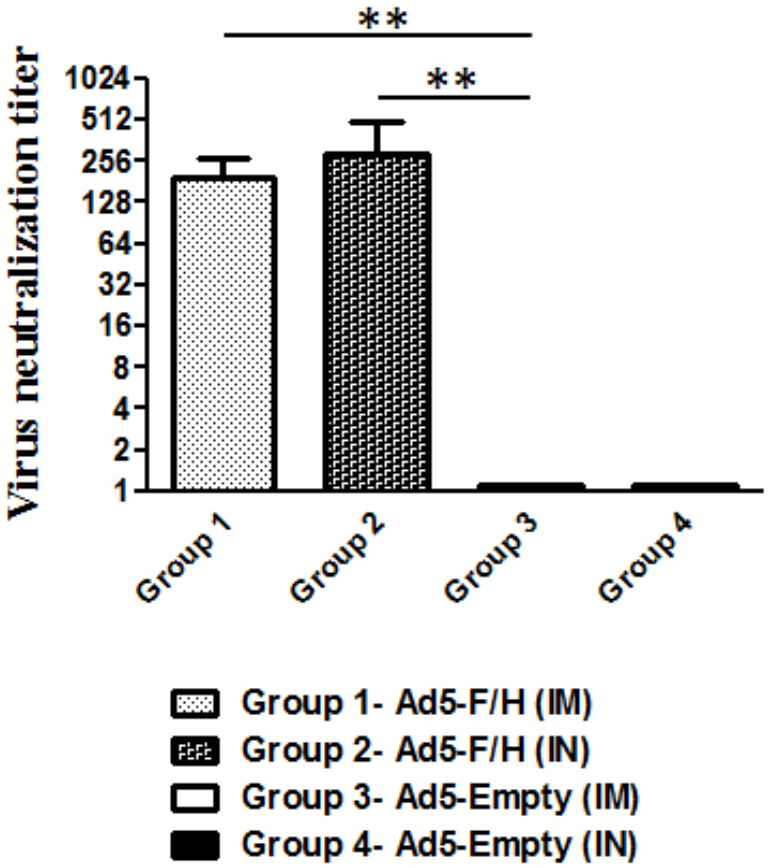

Figure 3.7. MV-specific neutralizing antibody titers (mean with SD) in week 4 sera of cotton rats. Two groups of five rats each were immunized once IM or IN with Ad5-F/H ($5X10^8$ $TCID_{50}$ of each virus per rat). The third and fourth groups (5 mice in each) were immunized once IM or IN with a dose of 10^9 $TCID_{50}$ of Ad5-Empty. Four weeks later, cotton rats were challenged IN with MV Edmonston ($5X10^4$ $TCID_{50}$ per rat). Four days after the challenge, cotton rats were euthanized. Sera were collected at week 4 (prior to the challenge). Virus-neutralizing titers are expressed as the reciprocal of the highest dilution that completely inhibited virus-induced CPE. **, $P < 0.01$.

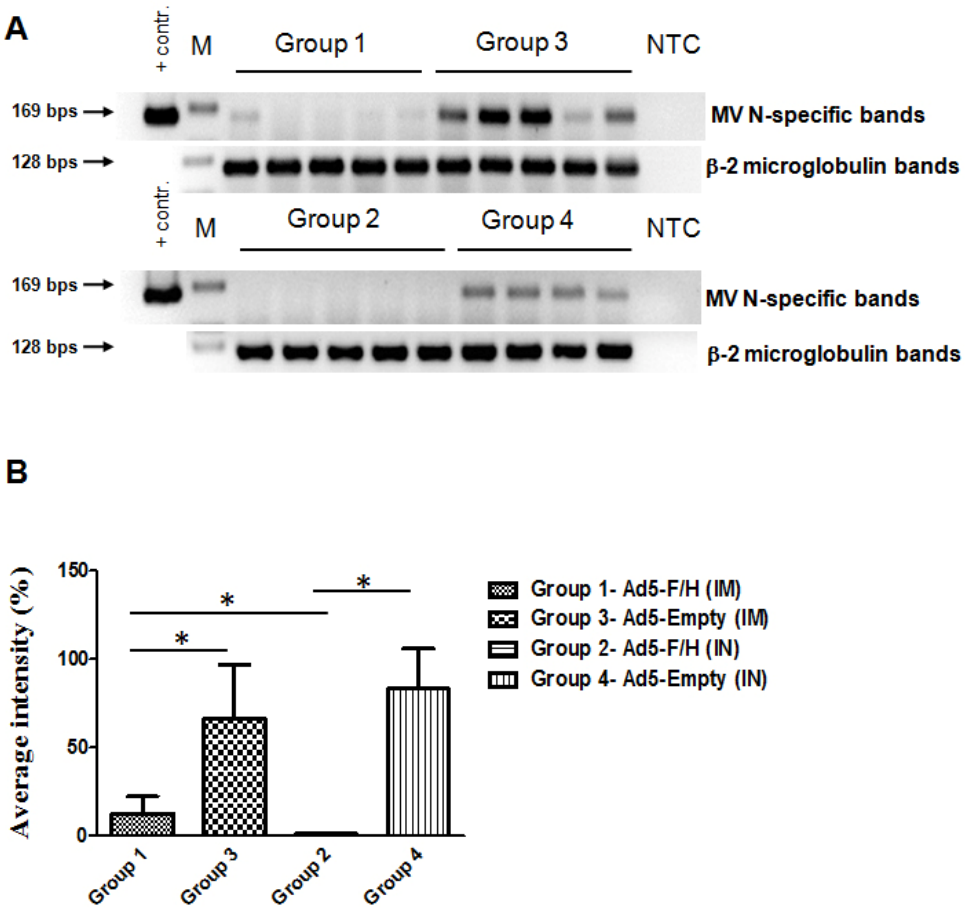

Figure 3.8. Protection of cotton rats against measles virus respiratory challenge. (A) Detection of viral RNA in the lung tissue of cotton rats. Cotton rats were immunized with Ad5-F/H IM (Group 1), Ad5-F/H IN (Group 2), Ad5-Empty IM (Group 3), or Ad5-Empty IN (Group 4). All animals were challenged IN with MV Edmonston strain four weeks after the single immunization. Viral RNA was detected by RT-PCR with primers specific for the N gene of MV. A 169 bp N gene-specific cDNA fragment is depicted on the left of the panels. β-2 microglobulin was chosen as a housekeeping gene. A 128 bp β-2 microglobulin gene-specific cDNA fragment is depicted on the left of the panels. NTC, no template control. +contr, MV RNA control. M, GeneRuler 1 kb Plus DNA Ladder (Fermentas, Burlington, ON). (B) Intensities of bands were measured with the aid of Bio-Rad Quantity One software (version 4.6.3), then normalized to the intensities of the housekeeping gene bands and represented as a graph. *, $P < 0.05$.

3.4 DISCUSSION

Based on the knowledge obtained from studies of LAV and formalin-inactivated vaccines, the correlates of protection against measles have been determined. The induction of both arms of the immune system is necessary. Humoral immune responses play an important role in preventing MV infection and neutralizing antibody titers correlate with protection (Albrecht et al., 1977; Black, 1989; Chen et al., 1990; Halsey et al., 1985; Permar, Griffin, and Letvin, 2006), whereas cell-mediated immunity controls viral replication and is being critical in maintaining long-term recall response (de Vries et al., 2010; Ovsyannikova et al., 2003). In addition, studies of pathogenesis of atypical measles revealed that the induction of a Th1/Th2 balanced immune response is required, as Th2-skewed responses may prime for more severe disease (atypical measles) upon infection with wild-type MV (Polack et al., 1999; Polack et al., 2002). Furthermore, the importance of generating potent immune responses at the site of pathogen entry has been recognized. There is evidence that vaccines administered by mucosal route are superior in eliciting protection from pathogens that initiate their replication at the mucosal site in comparison to other routes of vaccination (Neutra and Kozlowski, 2006). The partial explanation for this is that there are receptor-mediated recognition systems that serve to focus the immune response at the site where antigen or pathogen was initially encountered. Thus, it is believed that greater immune response may be achieved if measles vaccine is administered mucosally via the respiratory tract (Castro et al., 2005; Dilraj et al., 2007; Sepulveda-Amor et al., 2002).

The main goal of my investigation was to evaluate recombinant adenovirus as a vaccine candidate against measles. To this end, two recombinant Ad5 vectors expressing MV H or F proteins were generated and their capability to express recombinant F and H proteins of MV was assessed. Immunogenicity and protective efficacy of the Ad5-based vaccine represented by a combination of Ad5-MV-F and Ad5-MVvacH were examined in C57BL/6 mice and cotton rats.

Two Ad5 recombinants were generated by homologous recombination in HEK293 cells. Ad5-MV-F recombinant contains a codon-optimized cDNA of the gene encoding MV F protein, whereas Ad5-MVvacH contains the native sequence of the MV H gene. The reason for this is that I experienced difficulty in rescuing the Ad5 recombinant containing a codon-optimized MV H gene. This agrees with previously reported studies, where some Ad vaccine vectors were consistently difficult or impossible to rescue due to the toxicity of recombinant transgene expression, especially in the cases when the transgene was codon-optimized and transcribed from a strong promoter (Zhao et al., 2009). In line with the previously published data (Alkhatib and Briedis, 1988; Alkhatib, Richardson, and Shen, 1990; Fooks et al., 1998), this study demonstrated that the recombinant adenoviruses were capable to express the biologically active recombinant F and H proteins that possess characteristics of the wild-type MV proteins.

To evaluate and compare the immunogenicity of Ad recombinants delivered by intramuscular and intranasal routes, isotypic responses in serum were analysed and MV-specific total IgG and neutralizing antibody titers in serum as well as MV-specific IgA titers in BALs from mice immunized with a combination of Ad5 recombinants were measured.

Analysis of MV-specific IgG subclasses in sera from immunized mice revealed that the adenovirus-vectored vaccine inoculated IN elicited a balanced Th1/Th2 immune response, whereas IM immunization induced a predominant Th1-type immune response. Immunization of C57BL/6 mice with the Ad5-based vaccine resulted in induction of similar MV-specific IgG titers regardless of the route of immunization, indicating the ability of mucosal immunization with the Ad5 recombinants to produce systemic antibody responses at levels that were comparable to those induced by parenteral vaccination routes. This is in concordance with previously reported studies, where following intranasal immunization, certain live vectors were able to elicit high systemic IgG titers of the same magnitude as those induced by parenteral vaccination routes (Egan et al., 2004; Enose et al., 2002). Serum neutralizing titers were the key readout for immunogenicity, as these antibodies correlate with protection from measles (Chen et al., 1990). In mice, the Ad5 recombinants delivered IM or IN elicited similar neutralizing titers that were above the protective threshold in humans (titers $\geq$120 mIU/ml measured by PRN, which is ten-fold more sensitive than neutralization assay (Chen et al., 1990), are deemed protective in humans (Samb et al., 1995; WHO, 1993)) following the first and second immunization. Although MV-specific IgA titers were induced by the Ad5 recombinants inoculated IM or IN, nasal administration led to a significant ($P<0.01$) 100-fold increase in MV-specific IgA titers in BALs as compared to those induced by intramuscular immunization. Therefore, this study demonstrated that the mice immunized with the recombinant adenoviruses either intramuscularly or intranasally developed robust MV-specific antibody immune responses even after a single immunization. On the contrary, Fooks et al. showed that mice immunized with one of the adenovirus recombinants orally or intraperitoneally failed to mount a significant neutralizing

antibody response although low levels of MV-specific IgG were detected by ELISA (Fooks et al., 1998). This discrepancy may be due to the use of a different mouse strain or a different route of immunization in the present study. The other contributing factor may be that Fooks et al. (Fooks et al., 1998) immunized rodents with individual viruses, whereas in this study the vaccinations were done with a combination of two recombinant adenoviruses. In addition, the Ad5-MV-F recombinant contains a codon-optimized F gene that may have improved the F protein expression.

The next step of this research was testing the efficacy of IM or IN immunization with the Ad5 recombinants in inducing protection of cotton rats against the intranasal challenge with MV by measuring the levels of neutralizing antibodies and virus replication. In cotton rats, neutralization assay (NT) titers of $\geq$12 are proven to be protective (Schlereth et al., 2000b), similar to the situation in humans (Samb et al., 1995; WHO, 1993). In this study, immunization of cotton rats with the combination of recombinant adenoviruses elicited similar MV-specific serum neutralizing-antibody responses regardless of the delivery route. In addition, the Ad5 recombinants inoculated IM or IN elicited NT titers 29-fold and 34-fold above the protective threshold following a single immunization of cotton rats, respectively. Interestingly, immunization by the intramuscular route with the Ad5 recombinants led to reduced levels of MV replication in the lungs after IN MV challenge, whereas intranasal immunization resulted in undetectable levels of MV RNA, suggesting that mucosal MV-specific IgA antibodies play an important role in protection against MV infection. Therefore, the data presented here may indicate that a single mucosal administration of the recombinant adenoviruses is able to initiate protective systemic and mucosal immune responses that effectively suppressed MV replication in lungs of cotton rats. In contrast,

Fooks et al. demonstrated that intranasal immunization of cotton rats with one of Ad5 recombinants did not result in significant levels of protection (Fooks et al., 1998), which may be explained by the use of individual viruses in the latter study. Similarly to results of this study, a single intranasal, but not parenteral administration of an adenovirus-vectored TB vaccine protected mice from pulmonary tuberculosis (Wang et al., 2004), which is an additional confirmation of capability of replication-deficient adenoviral-based vectors to induce potent mucosal immune protection.

There is evidence that the presence of pre-existing antivector host immunity may hamper the efficacy of an adenovirus-vectored vaccine (McElrath et al., 2008). Furthermore, the attenuated MV vaccine does not induce protection in the presence of anti-measles maternal antibodies in humans (Leuridan and Van Damme, 2007). Therefore, future studies will be set to determine whether intranasal adenovirus-vectored vaccine is efficacious in the presence of MV- and Ad5-specific antibodies. In this regard, different approaches to overcome this limitation are being sought, including engineering of vaccine vectors from viral strains that have not circulated widely in host population (Mastrangeli et al., 1996), employing a non-human adenovirus as a vector (Moffatt et al., 2000) or by mutating viral surface proteins in order to evade host neutralizing antibodies (Roberts et al., 2006). Moreover, the development of Ad5 vectors with additional deletions of their genome may overcome pre-existing Ad5 immunity due to a significant reduction of expression of viral late genes (Gabitzsch et al., 2009). Heterologous prime-boost vaccination strategy (DNA-prime and Ad5-boost) was also shown to be effective in the terms of low levels of antivector immunity (Yang et al., 2003). Another approach to circumvent the problem of pre-existing antivector immunity is to exploit the asymmetry in induction of systemic and

mucosal immune responses. Specifically, mucosal administration of the E1/E3-deleted Ad5 expressing Ebola Zaire glycoprotein, but not intramuscular or oral administrations in mice with pre-existing immunity to Ad 5 (systemic neutralizing antibodies titers of 1:320), induced protection against the lethal challenge (Croyle et al., 2008). In addition, other studies demonstrated that mucosal administration of replication-defective Ad5-derived vaccines has also conferred protection against challenge with other pathogens in the presence of pre-existing immunity to the vector in different models, including humans (Shi et al., 2001; Van Kampen et al., 2005; Xiang et al., 2003). The mucosal route of administration of replication-defective Ad vectors may also allow circumventing maternal pre-existing immunity against measles. For instance, mucosal administration of measles and measles-rubella vaccines was shown to be more efficient than subcutaneous administration in pre-immunized humans (Bennett et al., 2002; Dilraj et al., 2000). Moreover, nasal administration is an easy, fast and painless way of vaccine delivery, which helps to reduce the need for trained healthcare workers, needles, syringes and disposal of hazardous wastes (Kersten and Hirschberg, 2007). In addition, nasal immunization can prime the immune system for both systemic and mucosal immune responses that are critical to provide optimal protection against the disease (Neutra and Kozlowski, 2006).

In summary, this study has demonstrated that a single intranasal immunization of mice and cotton rats with the vaccine, comprising of two recombinant adenoviruses expressing MV H and F proteins, elicited robust MV-specific antibody responses in serum. Furthermore, the results showed that the vaccine given intranasally was more effective than the vaccine given intramuscularly in the induction of MV-specific mucosal IgA in

mouse respiratory tract and in the reduction of viral RNA load in the lungs of immunized cotton rats after intranasal MV challenge.

4.0 RECOMBINANT PROTEIN-BASED VACCINE FOR MEASLES

4.1 INTRODUCTION

Vaccination against measles with the current licensed vaccines has been successful in developed countries. However, in developing countries, measles remains a leading cause of high mortality among young infants (MMWR, 2009). This may in part be attributed to the limitations of the live attenuated measles vaccines, such as low efficacy of LAV in young infants due to the presence of maternal MV-specific neutralizing antibodies and immaturity of infant's immune system. The latter factors hamper successful vaccination with LAV. As a result, many infants under the age of 9 months are left unprotected before they reach the age of vaccination (WHO, 2002). One of the earliest approaches to vaccinate children at an early age (4-6 months) was discontinued due to high mortality rates observed among girls that received the 'high-titer' LAV (Aaby et al., 2006; Seng et al., 1999). Therefore, there is the need for an alternative vaccine that can be administered successfully at an early age and overcomes the negative impact of maternally derived MV-specific neutralizing antibodies and immune immaturity in young infants.

Subunit vaccines formulated with new adjuvants are among the currently considered vaccine candidates (de Vries et al., 2008). The ISCOM and Protollin formulations incorporating MV glycoproteins have been shown to induce both humoral and cellular immune responses, as well as antiviral protection in rodents and macaques (Chabot et al.,

2005; Pasetti et al., 2007; Stittelaar et al., 2002; van Binnendijk et al., 1997; Varsanyi et al., 1987; Wyde et al., 2000). In addition, ISCOM formulations have been shown protective in the presence of passively acquired MV-specific antibodies (van Binnendijk et al., 1997).

H protein of MV plays a vital role in viral tropism, receptor binding, hemagglutinating activity and induction of protective immunity against viral infection (Griffin, 2007). Although two glycoproteins are thought to be important for the induction of effective MV-specific immunity, the majority of neutralizing antibodies are generated against the MV H protein: they neutralize MV *in vitro* and provide protection against MV *in vivo* (Brinckmann et al., 1991; Cardoso et al., 1996; de Swart et al., 2009; Drillien et al., 1988; Giraudon and Wild, 1981; Giraudon and Wild, 1985; Varsanyi et al., 1987). These antibodies are mostly directed against linear epitopes, as well as epitopes dependant on conformation and glycosylation (Griffin, 2007). Glycosylation has been shown to be necessary for the proper folding, dimerization, and antigenicity of the protein (Griffin, 2007). Major conformational epitopes and glycosylation sites are located within the C-terminal globular head domain of MV H (Griffin, 2007). Thus, this protein should be produced in mammalian cells, as its antigenicity and immunogenicity depend on proper conformation and glycosylation. In addition, the strength of a cell line-based technology is its safety, scalability and productivity. Therefore, in this study, the development of a stable human cell line producing a secreted globular head domain of MV H protein was undertaken and the ability of the protein to induce MV-specific immune responses in mice was examined.

4.2 MATERIALS AND METHODS

4.2.1 Construction of the recombinant MV H protein expression plasmid - The pUC-MV-H plasmid (GeneScript Corp., Piscataway, NJ), containing a codon optimized MV H gene (the codon adaptability for human cells and the entire mRNA structure were taken into consideration), served as a template to amplify a part of the MV H gene encoding the globular head domain of the protein (amino acids 156-617) by PCR using the Phusion High-Fidelity PCR kit (New England Biolabs, Pickering, ON). Briefly, PCR was carried out in 50 μl containing 25 pmol of each primer (MV-H-head-FOR) 5'-TTGGCCGGCCAGACGTTGCCGCCGAAGAGTT-3' and (MV-H-head-REV) 5'-ATTTGCGGCCGCTCGGCGATTAGTGCCATCTT-3', and ~0.1 μg of DNA. Reaction parameters were as follows: enzyme activation for 1 min at 95^{o}C; followed by 30 cycles of denaturation for 1 min at 95^{o}C, annealing for 1 min at 56^{o}C, and extension for 1 min at 72^{o}C; and final extension for 1 min at 72^{o}C. The product was then digested with *Fse*I and *Not*I (New England Biolabs, Ipswich, MA) and the resulting fragment was ligated into pIRESpuro (Clontech, Mountain View, CA) in frame with the mammalian transin secretion signal, the protein A purification tag coding sequence and the tobacco etch virus (TEV) protease cleavage site resulting in pProtA-MV-H156/617. Correct sequence of the recombinant gene was confirmed by nucleotide sequencing.

4.2.2 Generation of a stable cell line expressing the globular head domain of MV H protein - For transient transfections, HEK 293T cells (ATCC, CRL-1573) were plated in a six-well plate (10^6 cells/well) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Lonza), 0.1 mM non-essential amino acids (Invitrogen), 10 mM HEPES buffer (Invitrogen) and 50 μg/ml gentamicin (Invitrogen). When the monolayers were 70-80% confluent, the cells were transfected with 6 μg of pProtA-MV-H156/617 using the Calcium Phosphate Transfection Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. To produce stably transfected cell lines, puromycin (Invitrogen) was added one day after transfection to a final concentration of 5 μg/ml. Resistant clones were clearly visible 13 days following transfection. The cell media were collected from wells with expended individual clones and analyzed by Western blotting with the protein A-specific mAb. IgG-sepharose affinity purification of the fusion protein was carried out at the University of Toronto in the laboratory of our collaborator, Dr. James Rini. The purified recombinant protein was released from the tag by TEV protease digestion. The resulting purified MV-H156/617 protein (no protein A tag) was used in the mouse trial and ELISPOT assay.

4.2.3 Western blotting – To detect the expression of recombinant ProtA-MV-H156/617 protein, the collected medium samples were incubated at 96°C for 5 min in 2X loading buffer containing 4% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and separated by SDS-10% PAGE. The separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked overnight with 5% skim milk in TBS/0.05% Tween-20 at 4°C, followed by incubation for 4 h at RT with

the protein A-specific mAb (Sigma-Aldrich, St. Louis, MO) diluted 1:5,000. Bound antibodies were detected by incubating the blot with goat anti-mouse HRP-conjugated secondary antibody (1:3,000) (GE Healthcare, Baie d'Urfe, QC) at RT for 1 h, followed by incubation with the ECL-plus reagent (GE Healthcare).

4.2.4 Mouse trial - The purified recombinant MV-H156/617 protein was used in this study. Six-week-old female C57BL/6 mice were randomly allocated to seven groups of five animals each and vaccinated twice subcutaneously (SC) at a 4-week interval. Three groups of mice were immunized with a total volume of 100 μl at 0.3 μg, 3 μg or 15 μg of MV-H156/617 per immunization. The other three groups were immunized with a volume of 100 μl at 0.3 μg, 3 μg, or 15 μg of the protein formulated with aluminium hydroxide (Brenntag Biosector, Frederikssund, Denmark). Group 7 was left as a negative control. Blood was collected prior to the first immunization as well as at week 4 (prior to the boost) and week 7. Blood samples were allowed to clot at RT for 2 h before centrifuging for 20 min at 2,000 g, and the resulting sera were stored at -80^{o}C. Subsequently, sera were tested for total MV-specific IgG and IgG subclasses by ELISA and for the presence of MV-specific neutralizing antibodies by *in vitro* virus neutralization assay. Mice were euthanized three weeks after the boost for isolation of splenocytes for ELISPOT assays. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council for Animal Care.

4.2.5 Virus neutralization assay - Sera were assayed for MV-specific neutralizing antibodies, as described in section 3.3.13. Virus-neutralizing titers are expressed as the reciprocal of the highest dilution that completely inhibited virus-induced CPE.

4.2.6 ELISA - Sera were assayed for MV-specific IgG, IgG1, and IgG2a, as described in section 3.3.12. Results were expressed as the reciprocal of the highest dilution resulting in a reading of 2 standard deviations above the value of the negative control serum.

4.2.7 IFN-γ and IL-5 enzyme-linked immunospot (ELISPOT) assays - For isolation of splenocytes, mice were euthanized and spleens were removed and placed into tubes containing RPMI medium 1640 supplemented with 10 mM HEPES buffer (Invitrogen, Carlsbad, CA), 0.1 mM non-essential amino-acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 2 mM L-Glutamine, 100 U/ml Penicillin, 100 mg/ml Streptomycin (Invitrogen), and kept on ice. Then spleens were cut into pieces and gently pushed through sterile 100 μl cell strainers (BD Biosciences, Mississauga, ON) into Petri dishes containing RPMI. Splenocytes were centrifuged for 10 min at 1,200 rpm at 4^{o}C and resuspended in 5 ml of RPMI supplemented with 0.1 mM non-essential amino acids (Invitrogen), 10 mM HEPES buffer (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2 mM L-Glutamine, 100 U/ml Penicillin, 100 mg/ml Streptomycin (Invitrogen) and 10% FBS (Lonza). Unifilter plates (GE Healthcare, Piscataway, NJ) were coated overnight at 4°C with rat anti-mouse IFN-γ– or IL-5-specific mAbs (BD Biosciences, Mississauga, ON) at the concentration of 2

μg/ml. Next day plates were washed with RPMI supplemented with 10% FBS and blocked with the same medium for 1 to 2 h at 37°C. Isolated splenocytes were cultured at $5X10^6$ cells per well in triplicate wells (96-well plates) in the presence of 10 μg of recombinant MV-H156/617 protein per ml. Control cells were cultured with medium only. After 48 h of incubation at 37°C in humidified atmosphere with 5% CO_2, plates were washed five times with PBS/0.05% Tween-20 and incubated overnight at 4°C with biotinylated anti-mouse IFN-γ or IL-5 antibodies (BD Biocsiences) at the concentration of 2 μg/ml in PBS/0.05% Tween-20. Subsequently, the plates were incubated for 1.5 h at RT with AP-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA) diluted 1:1,000 in PBS. Bound IFN-γ or IL-5 antibodies were visualized by incubation with 5-bromo-4-chloro- 3-indolylphosphate and nitroblue tetrazolium substrate (Sigma-Aldrich, St. Louis, MO). The plates were washed in distilled water and air dried; spots were then counted using an inverted microscope. The number of IFN-γ or IL-6-secreting cells was expressed as the difference between the number of spots per 10^6 cells from MV-H156/617 stimulated cultures and the number of spots per 10^6 cells from control cultures.

4.2.8 Statistical analysis - All data were analyzed using GraphPad Prism Version 5.03 software. Differences among all groups were examined using the Mann-Whitney U test. Differences were considered significant if $P< 0.05$.

4.3 RESULTS

4.3.1 Characterization of the recombinant MV H protein expression by stable cell lines –

The recombinant MV H protein (762 aa) contains (Fig. 4.1) a transin secretion signal (1-32 aa) which directs the protein through the secretory pathway and induces accumulation of the protein in medium. The secretion signal is followed by a protein A purification tag (33-281 aa) serving for affinity purification. A tobacco etch virus protease cleavage site is located between the protein A purification tag and the H globular head domain (301-762 aa), allowing release of the H globular head domain during purification.

To verify the expression of the fusion protein, transient transfections of HEK 293T cells were carried out and cell culture media collected at indicated time points were subjected to Western blotting. A secreted protein with molecular mass about 95 kDa was detected in the media collected at 24 h, 48 h or 72 h post-transfection (Fig. 4.2), but not in the media of untransfected cells. The amount of the secreted protein in the media of pProtA-MV-H156/617-transfected cells increased from 24 h to 72 h post-transfection. The predicted molecular mass of the nascent ProtA-MV-H156/617 peptide is 85 kDa. However, all active sites of the addition of N-linked oligosaccharide chains are located within the globular head domain of H protein (Hu et al., 1994). Hence, the resulting molecular mass of the secreted fusion protein is expected to be more than 85 kDa. The lower molecular mass bands detected at 48 h and 72 h post-transfection most likely represent the degradation products, whereas high molecular mass protein bands may correspond to the H protein dimers (Hashiguchi et al., 2007).

Transfection of HEK 293T cells with pProtA-MV-H156/617 and selection of stably transfected clones resistant to puromycin resulted in the development of stable cell lines expressing the globular head domain of MV H protein fused with a portion of Protein A of *Staphylococcus aureus* (Fig. 4.3). To ascertain the expression of the fusion protein, samples of the cell media were collected from individual clones and equal volumes of cell media were analyzed by Western blotting with the protein A-specific mAb. Clones 4, 9, 10 and 14 were chosen for further work due to the correct size of the protein expressed and a high level of the protein expression.

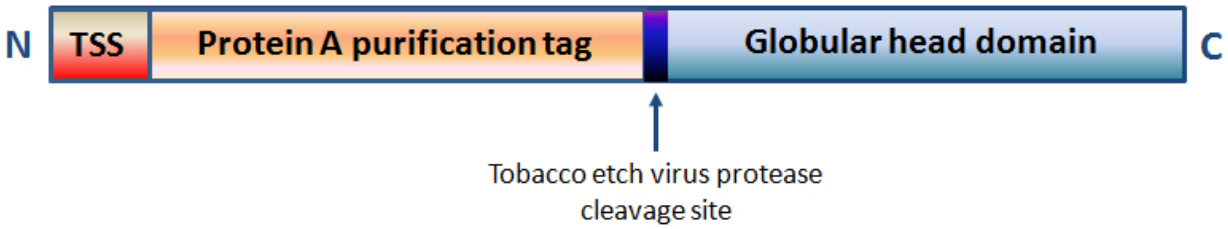

Figure 4.1. Schematic of the recombinant ProtA-MV-H156/617 protein. *TSS*, transin secretion signal; *N* and *C*, N- and C-terminus, respectively.

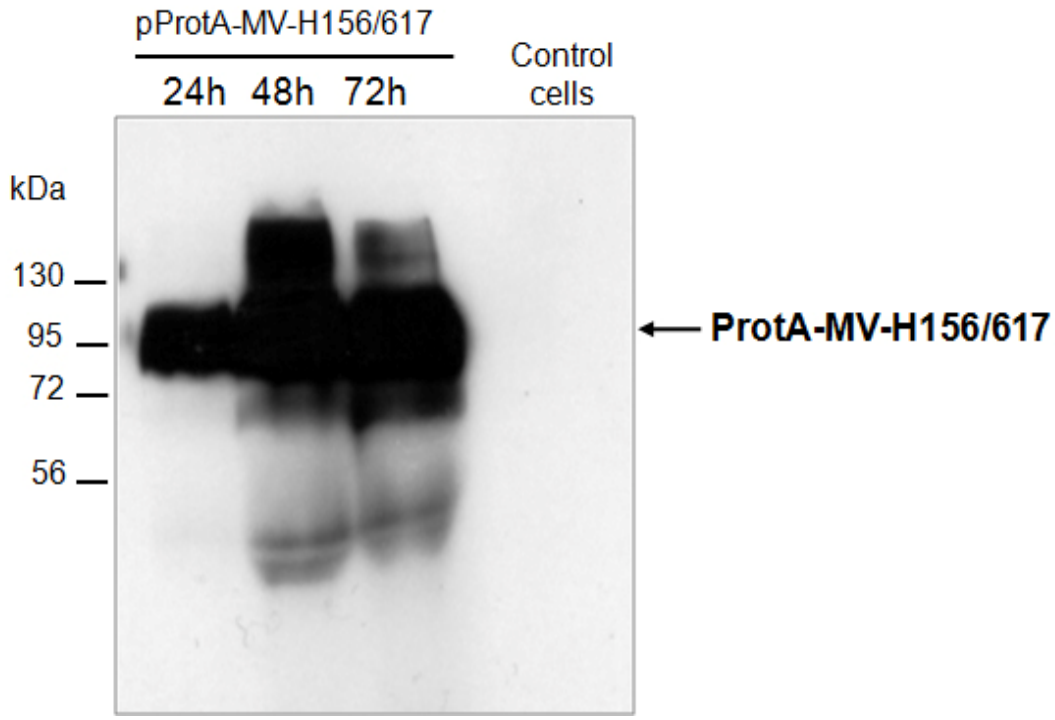

Figure 4.2. Validation of the secretion of the ProtA-MV-H156/617 protein into the media of transfected cells. HEK 293T cells were transfected with the pProtA-MV-H156/617 plasmid using calcium-phosphate method, and the cell culture media was collected at 24 h, 48 h and 72 h post-transfection and subjected to detection of the ProtA-MV-H156/617 fusion protein by immunoblotting with a protein A-specific mAb diluted 1:5,000. Bound antibodies were detected by incubating the blot with goat anti-mouse HRP-conjugated secondary antibody (1:3,000) at RT for 1 h and enhanced chemiluminescence substrate. Values on the left of the panel are molecular masses in kDa.

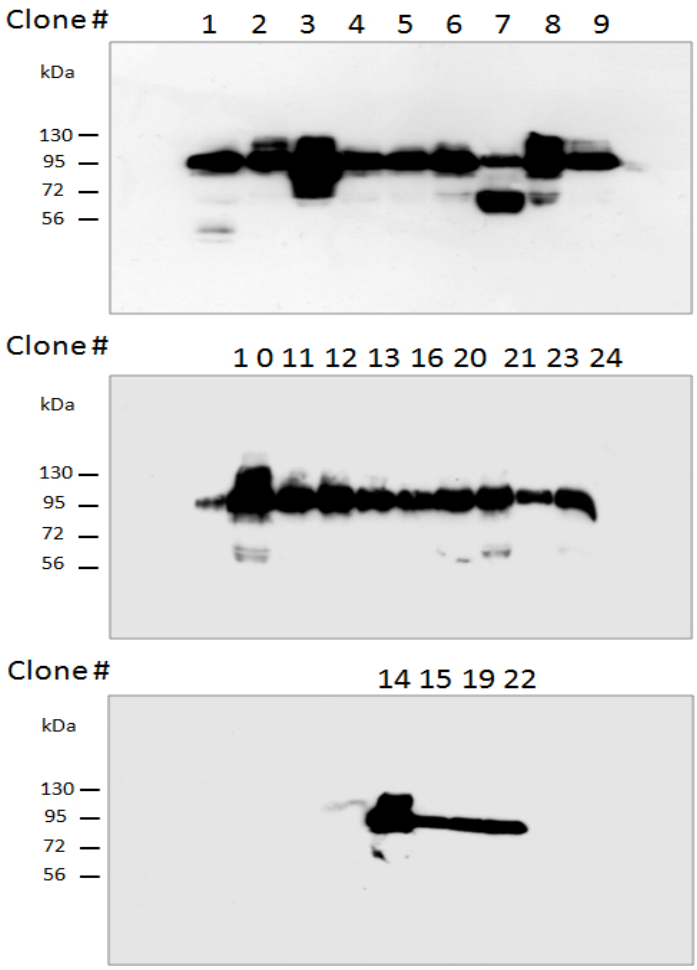

Figure 4.3. Expression of the recombinant ProtA-MV-H156/617 protein by stably transfected cell lines. HEK 293T cells were transfected with pProtA-MV-H156/617 using calcium-phosphate method. Selection of clones resistant to puromycin resulted in the development of stably transfected cell lines expressing the recombinant ProtA-MV-H156/617 protein. Cell culture media were collected and the protein secreted into the media was detected by immunoblotting with the protein A-specific mAb. Values on the left of the panels are molecular masses in kDa.

4.3.2 MV-specific humoral immune responses in mice immunized with the recombinant MV H protein -

Humoral immune responses induced by the purified MV-H156/617 protein alone or the protein formulated with aluminium hydroxide were examined by measuring the MV-specific IgG ELISA titers after two immunizations. Significantly higher IgG production ($P<0.05$) was observed in unadjuvanted groups when 15 μg of the protein were inoculated ($P<0.05$) compared to the 0.3-μg inoculated group (Fig. 4.4A). This response was further increased by the addition of aluminium hydroxide to the formulation. In addition, adjuvanted vaccine groups demonstrated a significant increase of the MV-specific IgG titers compared to the groups vaccinated with MV H protein alone.

To evaluate the biological effectiveness of antibodies produced in the sera, virus neutralizing titers were examined after two vaccinations. Immunization with MV-H156/617 protein/aluminium hydroxide resulted in significantly higher virus neutralizing titers than immunization with the recombinant MV-H156/617 protein alone (Fig. 4.4B), confirming the additive effect of co-formulation with aluminium hydroxide on humoral immunity against MV.

To characterize the type of immune response generated, MV H-specific IgG1 and IgG2 levels in the mouse sera were determined. As shown in Figure 4.4C, immunization of mice with the MV-H156/617 protein alone or MV-H156/617 protein/aluminium hydroxide resulted in the induction of predominantly MV-specific IgG1 titers, which is an indication of Th2-type immune response. Although the IgG2 titers were not significantly different in the MV-H156/617 and MV-H156/617/aluminium hydroxide groups, IgG1 titers were significantly higher ($P<0.01$) in the aluminium hydroxide group after two consecutive

immunization. Correspondingly, IgG1/IgG2 ratio was higher in the MV-H156/617 protein/aluminium hydroxide group after two vaccinations than that in the MV-H156/617 group.

4.3.3 MV-H-specific cell-mediated immune responses in mice immunized with the recombinant protein - To determine the level of MV-specific cellular immunity after two consecutive immunizations, we measured the secretion of IFN-γ and IL-5 by *in vitro* restimulated splenocytes using ELISPOT assay. No IFN-γ secreting cells were induced by mock vaccination, and low numbers of IFN-γ secreting cells were observed after two vaccinations with the MV-H156/617 protein and MV-H156/617 protein/aluminium hydroxide (mean numbers of IFN-γ secreting cells: mice immunized with the protein alone – 3; mice inoculated with the MV-H156/617 protein/aluminium hydroxide – 4). In contrast, high numbers of IL-5 secreting cells were detected in the groups that received MV-H156/617 protein or MV-H156/617 protein/aluminium hydroxide (mean numbers of IL-5 secreting cells: mice immunized with the protein alone – 248; mice inoculated with the MV-H156/617 protein/aluminium hydroxide – 347; control mice – 8) (Fig. 4.5). In addition, there was no significant difference between groups of mice vaccinated with the MV-H156/617 protein or MV-H156/617 protein/aluminium hydroxide in terms of IFN-γ or IL-5 secretion (Fig. 4.5). These results indicate that immunization with the purified MV-H156/617 protein primed for a type 2 skewing of the cytokine response.

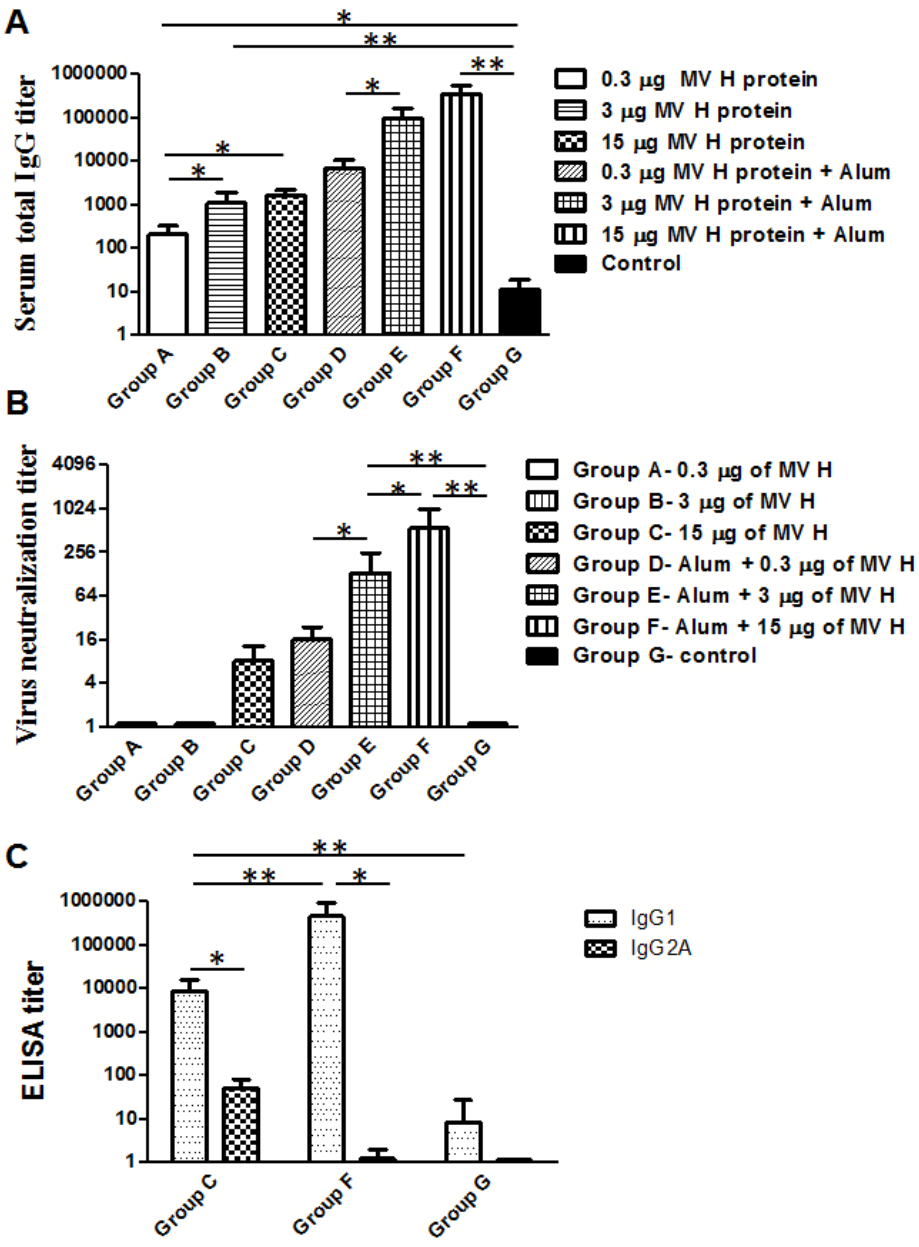

Figure 4.4. MV-specific humoral immune responses in sera of mice immunized with the recombinant globular head domain of MV H protein. Six groups of mice (5 mice in each group) were immunized SC twice at a 4 week interval. Three groups of mice were inoculated with 0.3 µg, 3µg, or 15 µg of the globular head domain of MV H protein. The other three groups were immunized with the same SC doses of the protein formulated with aluminium hydroxide. Group 7 was left as a negative control. Three weeks after the second

immunization animals were euthanized. Sera were collected at week 4 (prior to the boost) and week 7. (A) MV-specific IgG titers (mean with SD) in sera of mice after two consecutive immunizations. (B) MV-specific virus neutralizing titers (mean with SD) in week 7 sera after two immunizations. Virus-neutralizing titers are expressed as the reciprocal of the highest dilution that completely inhibited virus-induced CPE. (C) MV-specific IgG1 and IgG2a (mean with SD) in week 7 sera of mice. ELISA results are expressed as the reciprocal of the highest dilution resulting in a reading of 2 standard deviations above the value of the negative control serum. *, $P < 0.05$; **, $P < 0.01$.

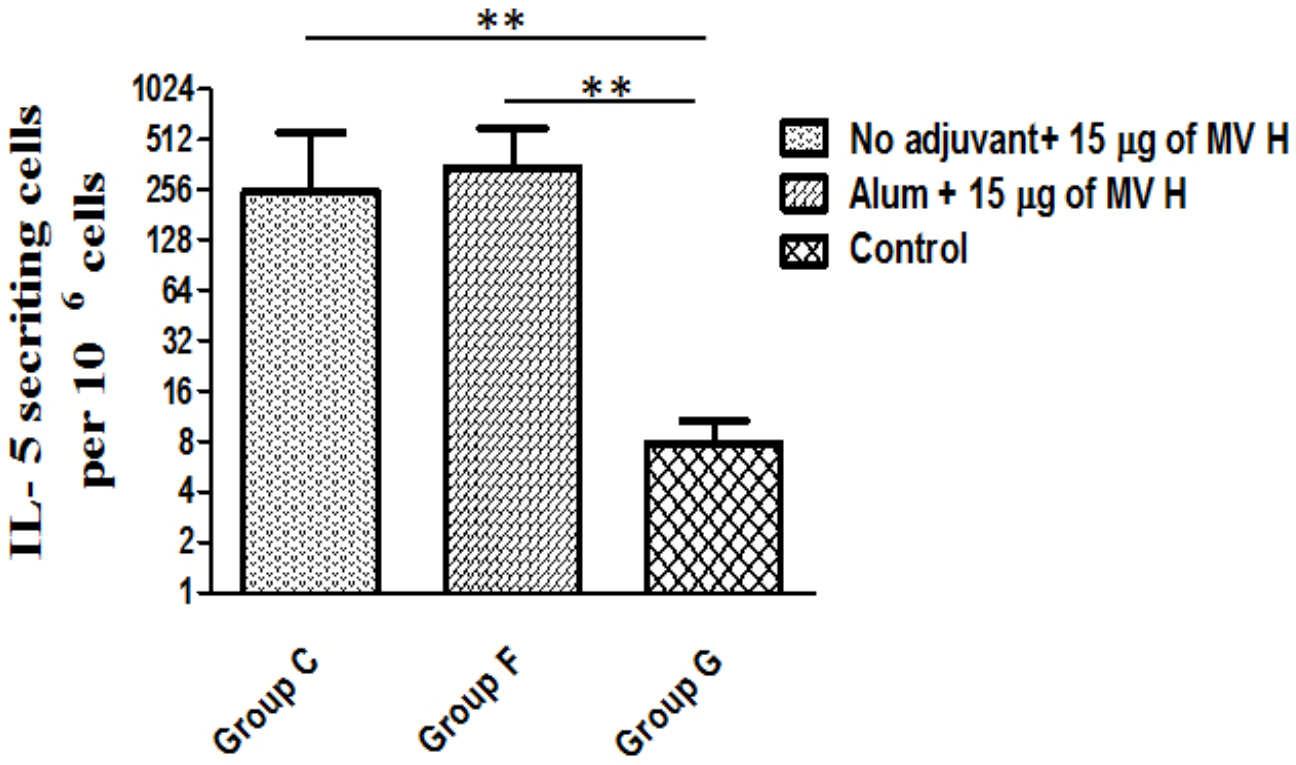

Figure 4.5. Numbers of IL-5 secreting splenocytes (mean with SD) in response to in vitro restimulation with the purified globular head domain of recombinant MV H protein. Group C was immunized twice SC at a 4-week interval with 15 µg of the purified globular head domain of recombinant MV H protein. Group F was inoculated twice SC at a 4-week interval with 15 µg of the purified protein formulated with aluminium hydroxide. Group G was inoculated with saline. Three weeks later after the second immunization animals were euthanized. **, $P < 0.01$.

4.4 DISCUSSION

Surface proteins of MV are the main targets of immune system to generate protective immunity, which makes them ideal antigens for vaccine development. In this regard, several expression systems have been used to express the recombinant MV glycoproteins: mammalian cell expression systems based on vaccinia (Kidokoro et al., 2002), canarypox (Taylor et al., 1992), adenovirus (Alkhatib and Briedis, 1988), Semliki forest vectors (Bouche et al., 1998), insect cell expression system (Sadigh et al., 2008; Takehara et al., 1992; Vialard et al., 1990), and plant-based system (Webster et al., 2005). The lack of proper conformation and post-translational changes in prokaryotes, and to certain extent in insect- and plant-based expression systems, restrict their application for therapeutic protein production (Arya, Bhattacharya, and Saini, 2008; Webster et al., 2005). In contrast, a mammalian cell expression system has been shown to provide proper post-translational modification of the H protein (Gerald et al., 1986). In our study, calcium phosphate transfection of HEK 293T cells with pProtA-MV-H156/617 and selection of clones resistant to puromycin resulted in the development of stable cell lines expressing the recombinant ProtA-MV-H156/617 protein. In Western blotting, the protein migrated under reducing conditions as a strong wide band with an estimated molecular mass of about 95 kDa, which is higher than the predicted molecular mass of the nascent ProtA-MV-H156/617 peptide. This suggests that the protein has undergone some post-translational modifications, perhaps glycosylation, which was shown to play an important role in the processing and antigenicity of the MV H protein (Hu et al., 1994). The secretion signal of

MV H protein is located within the cytoplasmic tail (Griffin, 2007). Even though this domain was deleted from the recombinant ProtA-MV-H156/617 protein, the mammalian transin secretion signal was placed at the N-terminus of the recombinant ProtA-MV-H156/617 protein to direct the protein through the secretory pathway, where glycosylation takes place (Vitale and Denecke, 1999). In addition, the secretion of ProtA-MV-H156/617 into medium and presence of the protein A tag at its N-terminus facilitated functional analysis and subsequent purification of this protein. Immunoblotting revealed high levels of the protein expression as early as 24 h post-transfection, which may be attributed to codon-optimized MV H gene sequence placed under a strong CMV promoter. Furthermore, the advantage of the expression system used in this study over a number of mammalian viral expression systems is the absence of safety issues associated with pathogenicity of viral vectors.

The examination of immunogenicity of purified MV H globular head domain revealed that all groups of mice immunized with the protein alone seroconverted after two SC immunizations. The increases in serum IgG levels were dependent on the concentration of the recombinant protein used for the vaccine formulation. The role of anti-MV neutralizing antibodies in protection against MV infection is well-established, neutralizing titers of $\geq$120 mIU/ml in humans correlate with protection even in the absence of a cellular response (Samb et al., 1995; WHO, 1993). Although antibody responses after two consecutive immunizations with the protein were strong, I found that the purified globular head domain of MV H alone failed to stimulate significant MV-specific neutralizing antibody titers unless the mice were immunized with the protein formulated with aluminium hydroxide. However, the ability of the protein to induce low MV-specific neutralizing titers at the highest dose tested suggests that it possesses a correct conformational structure, which is critical in

induction of antibodies capable of recognizing the pathogen upon infection (protective immune responses). The production of MV-specific IgG and neutralizing antibodies was further increased by the addition of aluminium hydroxide. This was expected, as aluminum hydroxide boosts humoral immunity by providing Th2 cell help to follicular B cells (Lambrecht et al., 2009).

Mice immunized with the globular head domain of MV H protein alone developed a predominantly Th2 immune response. As expected, the addition of aluminium hydroxide further polarized immune response toward a Th2-type response, which agrees with the observation that the major role of aluminium-induced IL-4 in Th-subset stimulation is to downregulate the Th1 response (Lindblad, 2004). However, induction of Th2-skewed responses should be avoided in measles vaccination because they may prime for atypical measles (Polack et al., 1999; Polack et al., 2003a; Polack et al., 2002). A more balanced Th1/Th2-type immune response could be established by inclusion of the F protein (Polack et al., 2003b) and/or Th1-directing adjuvants (e.g., CpG oligodeoxynucleotides) into the vaccine formulation. However, it was shown that induction of a Th2 polarization of immune responses is not sufficient to elicit atypical measles, and other factors, such as the formation and deposition of immune complexes, play an important role in the pathogenesis of atypical measles (Polack et al., 2003b).

The role of cell-mediated immunity in controlling viral replication and maintaining long-term recall response has been extensively studies in non-human primates and humans (de Vries et al., 2010; Permar et al., 2004; Permar et al., 2003). The examination of cellular immune responses revealed that immunization with the globular head domain of

MV H protein elicited high MV-specific IL-5 and low IFN-γ production, indicating priming for a type 2 skewing of the cytokine response. In this study, the Th2 bias may be associated with inability of the globular head domain of MV H protein to induce activation of monocytes via TLR2 and elicit production of IL-12 through this mechanism, as it was shown that the H proteins of UV-inactivated vaccine strains, including Edmonston, do not activate cells via both human and murine TLR2 in comparison to UV-inactivated wild-type MV H protein (Bieback et al., 2002). The data presented here showed that the addition of aluminium hydroxide to 15 μg of MV-H156/617 had little effect on induction of IFN-γ and IL-5 secreting cells. Although the numbers of IFN-γ and IL-5 producing cells were higher in mice immunized with the globular head domain of MV H protein formulated with aluminium hydroxide compared to mice immunized with the protein alone, this difference did not reach statistical significance. These results are in concordance with previously published data, where subunit vaccine based on secreted antigens from *Mycobacterium tuberculosis* and formulated with aluminum hydroxide primed for a cellular response (monitored as antigen-specific proliferation of lymphocytes) with a low production of IFN-γ and IL-5 cytokines in C57BL/6J mice (Lindblad et al., 1997). Although, other aspects of cell-mediated immunity, for instance, antigen-specific lymphocyte proliferation and IL-4 production, remain to be studied, these data provide evidence that the globular head domain of MV H protein is able to induce Th2-skewed cellular responses against MV.

In summary, the results presented here demonstrate that, in mice, the purified MV H protein produced in the mammalian expression system, have displayed certain immunogenic properties. The immunogenicity of the protein is confirmed by its ability to

induce moderate humoral immune responses as well as cell-mediated immunity monitored as antigen-specific production of IL-5 in the ELISPOT assay. Undoubtedly, formulation of the protein with Th1-directing adjuvants is required to enhance its immunogenicity and protective efficacy. In addition, inclusion of the MV F protein into the vaccine formulation may contribute to the optimization of protective immune responses, as generation of MV-specific neutralizing antibodies against both glycoproteins is required for complete protection against measles (de Swart et al., 2009; Varsanyi et al., 1987). The immunogenic properties of the purified globular head domain of MV H protein may be attributed to proper folding and other post-translational modifications conferred by the mammalian expression system.

5.0 GENERAL DISCUSSION AND CONCLUSIONS

Studies investigating MV biology and pathogenesis as well as the efficacy of LAV have advanced our knowledge of the determinants of protective immunity against measles. In numerous studies, the role of MV-specific antibodies in prevention of measles infection following natural infection, vaccination, or passive transfer of antibodies has been shown. Moreover, the neutralizing antibody titers have been defined as correlates of protection (Chen et al., 1990). Evidence for importance of cellular immunity in the control of MV replication and maintenance of long-term recall response was also demonstrated (de Vries et al., 2010; Ovsyannikova et al., 2003). In addition, studies focused on pathogenesis of atypical measles provided insight into mechanisms leading to the exacerbated disease that was observed in individuals vaccinated with FIMV and subsequently exposed to wild type MV. FIMV was licensed in the United States in the 1960s (de Vries et al., 2008). Recipients received this vaccine developed short-lived immunity characterized by moderate levels of hemagglutination inhibiting antibodies and a lack of production of antibodies to F (Merz, Scheid, and Choppin, 1980). In macaques, immunization with FIMV resulted in induction of immunity with no cytotoxic T cell response and nonprotective, low-avidity, complement-fixing antibodies (Polack et al., 1999; Polack et al., 2003a). This leads to immune complex deposition in affected tissues, eosinophilia, pneumonitis and a Th2 polarization of the immune response (Polack et al., 1999). Problems associated with induction of aberrant immune responses and predisposition to the enhanced disease led to the withdrawal of this vaccine in 1967 (de Vries et al., 2008). However, findings of these studies showed the

importance of the quality of immune responses in conferring protection to re-infection. Overall, all these data set the requirements that may lead to improvement of current MV vaccination strategies and/or development of alternative ones.

My work was initially built upon a study done by Fooks et al (Fooks et al., 1998). This research group described the construction and initial characterization of replication-deficient Ad5 recombinants expressing the MV H and F genes under the control of CMV immediate early promoter. In addition, they determined whether these adenovirus recombinants could elicit immune responses protective against MV challenge in mice and cotton rats, when administered by parenteral or mucosal routes. Mice and cotton rats were immunized with the Ad5 recombinants expressing the MV H or F protein in two doses containing 10^8 PFU one week apart. They showed that oral as well as parenteral administration of the H-expressing recombinant adenovirus elicited a significant protective response in mice challenged intracranially with a rodent-adapted MV strain, whereas the F-expressing adenovirus delivered orally or intraperitoneally failed to protect mice. Mice immunized with either recombinant developed low levels of MV-specific IgG. Antibodies elicited in mice following immunization with either recombinant had no *in vitro* neutralizing activity, suggesting involvement of a cell-mediated immune response in protection. In cotton rats, immunization by the intraperitoneal route with the adenoviruses expressing the MV H or F protein resulted in reduced virus titers in the lung and the absence of histological signs of infection, whereas intranasal immunization with the adenoviruses expressing the H or F proteins did not confer significant levels of protection. Based on observations of the previously described study (Fooks et al., 1998) and the fact that antibodies to both glycoproteins of MV are required for virus neutralization to provide

protection (de Swart et al., 2009; Varsanyi et al., 1987), I concluded that a combination of Ad5 recombinants expressing MV H or F proteins should be administered in order to induce protective immune responses. In addition, analyses of syncytium formation assays performed in this study as well as by Fooks et al (Fooks et al., 1998) revealed that the ability of a combination of the Ad5 recombinants expressing MV H or F proteins to cause pronounced cytopathic effects in monolayers of Vero cells could enhance the immunogenicity of this vaccine candidate. This conclusion was based on the observation that cytopathic viruses are extremely immunogenic since they cause massive cell death, which has to be cleared by phagocytes (Chen and Ron, 2006). Therefore, the hypothesis of this study was that the combination of Ad5 recombinants would able to induce potent immune responses to protect animals against MV challenge.

As was done by Fooks et al in mice, I aimed to employ parenteral and mucosal immunization. In contrast to the previously published data (Fooks et al., 1998), this study demonstrated that IM or IN immunization of C57BL/6 mice with two doses of the combination of Ad5 recombinants resulted in induction of a strong humoral immune response characterized by high levels of MV-specific IgG and neutralizing antibodies. In addition, the results revealed that the Ad recombinants administered IN or IM induced balanced Th1/Th2 and predominant Th1-type immune responses, respectively. Furthermore, the data presented here demonstrated that IN immunization with the combination of Ad5 recombinants expressing MV H or F proteins elicited higher levels of IgA in the lung, as well as high MV-specific IgG and neutralizing antibody titers of the same magnitude as those induced by intramuscular immunization. These results suggest potential applicability of the replication-deficient Ad vectors for mucosal immunization

against measles, which are in agreement with previous reports, where adenovirus-based mucosal vaccination was effective in protecting against infections caused by HIV, HSV and *Mycobacterium tuberculosis* in animal models (Santosuosso, McCormick, and Xing, 2005).

The next step was to examine whether the Ad5 recombinants administered IN or IM could confer protection in cotton rats against MV challenge. This study demonstrated that a single intranasal or intramuscular immunization with the combination of Ad5 recombinants resulted in induction of similar MV-specific neutralizing antibody titers, but different levels of suppression of MV replication in the lungs after IN MV challenge. The levels of MV-specific neutralizing antibodies induced by the Ad5 recombinants were well above the protective threshold value, as neutralization assay titers of $\geq$12 are proven to be protective in cotton rats (Schlereth et al., 2000b) and in humans (Samb et al., 1995; WHO, 1993). In contrast to the previous study (Fooks et al., 1998), intranasal immunization of cotton rats was superior in suppression of MV replication compared to parenteral immunization, suggesting an importance of mucosal MV-specific IgA antibodies in protection against MV infection. These data agreed with the studies, where *in vitro* anti-MV IgA functions include the prevention of virus entry and the interruption of virus replication (Yan et al., 2002), as well as another study in which a single intranasal, but not parenteral administration of an adenovirus-vectored TB vaccine protected mice from pulmonary tuberculosis (Wang et al., 2004). Overall, these results agreed with the hypothesis that immunization with the combination of recombinant adenoviruses expressing the MV glycoproteins would induce potent immune responses to protect animals against MV challenge.

The development of a measles subunit vaccine may prevent the limitations associated with LAV and eventually contribute to global measles eradication, as it can be administered safely to young infants at an early age and close the “window of susceptibility” (age 4-9 months) between the decay of maternal antibodies and vaccination with LAV. Several studies proving the immunogenicity, safety and efficacy of measles subunit vaccines had already been completed by others in rodents and macaques. However, to my knowledge, the measles virus antigens used in these studies have been expressed in mammalian cell expression systems based on viral vectors or were derived from a split antigen preparation. Major drawback of these expression systems is safety issues associated with the use of live viruses for production of MV glycoproteins. The development of a stable cell line expressing MV H or F proteins based on the HEK 293 mammalian platform may possess some advantages. This system facilitates a high yield production of recombinant proteins and is suitable for suspension-growing (Pham, Kamen, and Durocher, 2006). In addition, 293 human embryonic cells can confer the most proper post-translational processing of the recombinant MV H protein, which is important for its antigenicity (Hu et al., 1994). Therefore, the goal of this study was to test the feasibility of production of the recombinant MV H protein by stably transfected HEK 293 cells and to examine the ability of this recombinant protein to induce MV-specific immune responses against measles.

In the study presented here, the production of the recombinant globular head domain of MV H protein by the human stable cell lines is characterized by a high yield of the protein with proper post-translational modifications, ease of its functional analysis and purification, and the absence of safety concerns associated with pathogenicity of viral

vectors. Furthermore, this study demonstrated that subcutaneous immunization of C57BL/6 mice with the purified protein alone resulted in induction of a Th2-skewed immune response characterized by the moderate production of MV-specific IgG in the serum, as well as the production of IL-5 by *in vitro* restimulated splenocytes. The addition of aluminium hydroxide increased the magnitude of MV-specific neutralizing titers, which correlate with protection against measles (Chen et al., 1990), but had little effect on cellular immune responses. Thus, these results demonstrated that the purified globular head domain of MV H protein was able to induce moderate humoral immune responses as well as cell-mediated immunity monitored as the antigen-specific production of IL-5 in the ELISPOT assay.

Ultimately, it was demonstrated here that parenteral and mucosal immunization with the combination of Ad5 recombinants expressing the MV glycoproteins resulted in the induction of predominant Th1-type and balanced Th1/Th2 immune responses in mice, respectively. Intranasal immunization of mice confirmed mucosal applicability of the replication-deficient adenoviruses, as they induced mucosal MV-specific IgA antibodies, as well as the production of MV-specific IgG and neutralizing antibodies in the magnitude comparable to IM immunization. In addition, IN delivery of the Ad5 recombinants was superior to IM delivery, in terms of reducing viral replication in the lungs of cotton rats. Thus, the intranasal delivery of the combination of Ad5 recombinants is superior for inducing mucosal immunity, as well as protection against MV challenge in cotton rats. Furthermore, this study demonstrated the feasibility of production of the globular head domain of MV H protein by the stable HEK 293 cell line and the immunogenicity of the purified protein in mice characterized by the induction of Th2-skewed immune responses.

There are essentially several problems that will need to be addressed in future studies with respect to the immunization against MV and to the vaccine candidates characterized in this study. The MV immunization-related problems include the induction of protective immune responses in the presence of maternal MV-specific antibodies and risks of vaccine-enhanced disease (atypical measles) upon subsequent infection. A problem attributed to the immunization with Ad5 recombinants is induction of protective immunity in humans in the presence of pre-existing Ad5-specific antibodies.

There are many studies that documented the role of pre-existing MV-specific antibodies in suppression of subsequent immune responses against MV in infants (Albrecht et al., 1977; Leuridan and Van Damme, 2007), primates (Premenko-Lanier et al., 2003; Premenko-Lanier et al., 2004; van Binnendijk et al., 1997; Zhu et al., 2000) , cotton rats (Pueschel et al., 2007; Schlereth et al., 2000a; Schlereth et al., 2000b; Weidinger et al., 2001), and mice (Galletti, Beauverger, and Wild, 1995). In infants, it was shown that preexisting MV-specific maternal antibodies interfere with vaccine-induced seroconversion, and do not protect against wild-type MV (Leuridan and Van Damme, 2007). In cotton rats, inoculation with high titers of human MV-specific antiserum blocked completely the neutralizing antibody immune response and reduced the T cell response after the immunization with a MV vaccine virus (Pueschel et al., 2007). Although T cell responses were detectable, the absence of neutralizing antibodies resulted in no protection. In macaques, the presence of relatively low levels of MV-specific antibodies at the time of vaccination interfered with MV-specific antibody responses induced by vaccination with MV-Schwarz (a vaccine strain), but did not affect the induction of MV-specific proliferative T cell and CTL responses (van Binnendijk et al., 1997). As a result, only partial protection

was demonstrated in monkeys vaccinated with MV-Schwarz, suggesting the role of T cell-mediated immunity in protection in the presence of low levels of specific neutralizing antibodies (van Binnendijk et al., 1997). However, some studies revealed that the suppressive effects of the pre-existing MV-specific antibodies can be overcome by a two-dose IM/IN delivery of a modified vaccinia virus Ankara expressing the MV glycoproteins, or IN immunization with a single dose of VSV expressing MV H (Schlereth et al., 2003; Schlereth et al., 2000b). Furthermore, aerosol vaccination of children with LAVs has been proven to be more effective in the presence of pre-existing MV-specific antibodies than vaccination by parenteral route (Bennett et al., 2002; Dilraj et al., 2000; Sepulveda-Amor et al., 2002). Thus, these results indicate that mucosal delivery of MV vaccines may circumvent the suppressive effect of maternal antibodies on the induction of MV-specific immune responses. In addition, mucosal immunization also offers other advantages, such as the induction of both systemic and mucosal immune responses that provide optimal protection against the disease (Neutra and Kozlowski, 2006), avoidance of pre-existing Ad5-immunity in humans (Croyle et al., 2008; Shi et al., 2001; Van Kampen et al., 2005; Xiang et al., 2003), ease of vaccine administration, and minimization of the risk associated with transmission of infectious diseases through unsafe injection.

With regard to atypical measles, it seems that the replication-deficient Ad5-based vaccine candidate is not likely to predispose for this type of immunopathology. This assumption is supported by the fact that, in general, replication-deficient Ad5 vectors possess type 1 adjuvant properties and can induce potent long-term humoral and cellular immune responses directed to the expressed product (Juillard et al., 1995; Seder and Hill, 2000). The results of this study confirmed the induction of a Th1-type immune response by

the combination of Ad5 recombinants expressing MV H and F proteins. However, the immunogenicity and protective efficacy of the subunit vaccine candidate may benefit from the inclusion of Th1-directing adjuvants and MV F protein into the vaccine formulation.

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