A Vaccine to Close the Window of Opportunity for Measles Infection

by

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ABSTRACT

Despite the safe and effective use of attenuated vaccines for over fifty years, measles virus (MV) remains an insidious threat to global health. Problematically, infants less than one year of age, who are the most prone to severe infection and death by measles, cannot be immunized using current MV vaccines. For this dissertation, I generated and performed preclinical evaluation of two novel MV vaccine candidates. Based on data from clinical trials that showed increasing the dosage of current MV vaccines improved antibody responses in six-month-old recipients, I hypothesized that increasing the relevant antigenic stimulus of a standard titer dose would allow safe and effective immunization at a younger age. I generated two modified MVs with increased expression of the hemagglutinin (H) protein, the most important viral antigen for inducing protective neutralizing immunity, in the background of a current vaccine-equivalent. One virus, MVvac2-H2, expressed higher levels of full-length H, resulting in a three-fold increase in H incorporation into virions, while the second, MVvac2-Hsol, expressed and secreted truncated, soluble H protein to its extracellular environment. The alteration to the virion envelope of MVvac2-H2 conferred upon that virus a measurable resistance to *in vitro* neutralization. In initial screening in adult mouse models of vaccination, both modified MVs proved more immunogenic than their parental strain in outbred mice, while MVvac2-H2 additionally proved more immunogenic in the gold standard MV-susceptible mouse model. Remarkably, MVvac2-H2 better induced protective immunity in the presence of low levels of artificially introduced passive immunity that mimic the passive maternal immunity that currently limits vaccination of young infants, and that strongly inhibited responses to the current vaccine-equivalent.

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Finally, I developed a more physiological infant-like mouse model for MV vaccine testing, in which MV-susceptible dams vaccinated with the current vaccine-equivalent transfer passive immunity to their pups. This model will allow additional preclinical evaluation of the performance of MVvac2-H2 in pups of immune dams. Altogether, in this dissertation I identify a promising candidate, MVvac2-H2, for a next generation measles vaccine.

Dedicated to my parents, Beth and Pat.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

Measles, Still a Threat

The unqualified success of decades of attenuated measles vaccination has drastically reduced incidence of this highly infectious viral disease. Ironically, the disappearance of endemic measles transmission from many areas of the world has resulted in the secondary effect that measles infection is not always regarded as a serious threat, and subsequent refusal of vaccination by eligible individuals (1). Apart from allowing the eruption of measles outbreaks after importation to areas of local elimination, as for a recent highly publicized outbreak (2) that culminated in the first death of a patient from measles in the U.S. in nearly a decade (3), this social phenomenon obscures the persistent global health impact of this pathogen.

Measles remains a leading cause of death and disability around the world, infecting 20 million people and killing 114,900 in 2014 (4), which equates to 300-400 deaths around the world every day due to a virus that we have good vaccines against (5). As recently as 2008, measles ranked 10th among the leading causes of death for children under the age of 5 globally (6). Even in areas of control, where the first measles virus (MV) vaccine dose is received at one year of age, failure to maintain high vaccine coverage allows outbreak transmission after MV importation (7); in recent years, this problem has been of particular importance in the U.S. and European Union, where vaccine coverage has faltered and measles has subsequently resurfaced (8). The impact of measles persists despite the availability of attenuated vaccines with proven effectiveness in adults and older children.

Nonetheless, measles virus can and should be eradicated, according to biological criteria that have been suggested for eligibility for eradication (9). MV is of a single serotype, persistent infections are rare, and no animal or environmental reservoir exists, apart from humans. Generally accurate diagnostic tests exist and global surveillance systems are in place. Most significantly, effective intervention measures exist in the form of MV vaccines, and these vaccines have already allowed local elimination of transmission in large geographical areas, including all of the Americas. The 2011 eradication of rinderpest virus, the evolutionary sister of measles, sets the stage for eradication of biologically similar MV (10).

How, then, can we promote eradication of measles? Increasing delivery of the MV vaccine to all eligible individuals will certainly increase herd immunity. Apart from increasing delivery, though, opportunities remain to perfect the already effective measles vaccines. Current vaccines are dependent upon cold-chain maintenance and are delivered by the subcutaneous or intramuscular route; a next generation of measles vaccine would ideally be less heat labile and delivered without a needle. We may also suggest that, although MV vaccines are generally considered among the most effective vaccines available, inducing protective immunity in approximately 95% of recipients, an even more effective vaccine would further expand herd immunity against this highly infectious pathogen. And finally, an MV vaccine that is more effective in young infants, and that could thus be delivered at a younger age, has been highly sought after. This dissertation explores strategies to address these latter two points.

Biology of Measles: The Hemagglutinin Protein

Measles is an enveloped, pleiomorphic virus with a non-segmented, negative sense RNA genome of 15,894 ribonucleotides in length. The genome contains six cistrons encoding eight viral proteins (11).

Of special importance to this dissertation is the hemagglutinin, or H, protein encoded by the second-to-last cistron of the viral genome. H is a 617 amino acids-long type II transmembrane glycoprotein composed of an N-terminal cytoplasmic tail, a transmembrane region, a membrane-proximal stalk domain, and a large C-terminal globular ectodomain. H serves as the receptor attachment protein of measles. The crystal structure of the H ectodomain has been solved; it is composed of a six-bladed β-propeller fold (12). Much of this propeller-like structure is masked by N-linked sugars, but residues to the side of the propeller are unshielded. In virions, two H proteins form a disulfide-linked dimer in which the two proteins tilt significantly away from one another, orienting the unshielded "side" residues of each propeller upward and away from the viral envelope, and toward host cells. The unshielded region contains residues that are highly conserved and have been demonstrated to be important for binding to the signaling lymphocyte activation molecule (SLAM, also known as CD150), a measles receptor, both by mutagenesis (13) and by further crystallization of H in complex with SLAM (14). Importantly, the epitopes of neutralizing antibodies against the H protein also map to this unshielded, conserved, SLAM-binding region of the H protein. This finding helps to explain why measles is of a single serotype, despite the high mutation rate of such an RNA virus: SLAM exerts strong selective pressure on the region of H responsible for binding this cellular receptor, such that neutralizing immunity may easily confer

protection against the virus by targeting this unshielded and conserved region of the protein.

The H protein also helps to explain the tropism and pathogenesis of MV (15). MV is spread by respiratory droplets and infects susceptible individuals via the respiratory route. The virus initially infects SLAM-expressing cells in the respiratory epithelia, likely dendritic cells, and is trafficked to regional lymph nodes. Here the virus encounters a significant SLAM+ cell population, allow rapid primary replication and dissemination to diverse body sites. The SLAM-directed lymphotropism of MV explains the main pathological consequence of measles infection, a profound immunosuppression that renders the patient susceptible to secondary infections for weeks, months, and perhaps even years after measles infection and is the primary cause of measles-related morbidity and mortality (11). The tropism of MV H also helps to explain the extreme infectiousness of the virus. It has been recognized for decades that MV must make use of a host receptor expressed on epithelial cells, but the identity of that receptor remained a mystery until recently, when two independent groups simultaneously defined it to be nectin-4 (16, 17). Nectin-4 is expressed on the basolateral surface of epithelial cells. MV is thus able to enter epithelial cells, replicate prodigiously, and bud from the apical epithelial cell surface. Accumulation of infectious virus budding from epithelial cells in the respiratory tract in combination with the cough and sneezing induced by the virus ensure effective respiratory transmission to the next susceptible host. Morbilliviruses like MV are thus among the most infectious agents in existence. The basic reproduction number (R_0) of measles is estimated to be 15 to 20 secondary infections produced by every typical infective person in an entirely susceptible population (18).

The function of H has two important implications for development of new measles vaccines. First, while neutralizing antibodies are directed against the two surface glycoproteins of MV, which include the fusion (F) protein in addition to the H protein, the great majority of neutralizing antibodies are directed against H (19). H has thus been recognized as the most relevant MV antigenic stimulus for induction of neutralizing immunity. Second, the high R_0 of MV, conferred at least in part by the tropism of the H protein, necessitates that similarly high population-level immunity is required to interrupt transmission of the virus, estimated at 95% immunity (20). Measles eradication, thus requires more than the current 83-84% global vaccination coverage to attain this goal (21) and, indeed, the requisite level immunity is difficult to envision achieving with imperfect adherence to a current vaccine regimen of the same approximate effectiveness (9).

Predicting Protection

Neutralizing immunity against MV is the correlate of protection against infection, and therefore the target to achieve for a successful experimental vaccine. It is documented that measles rash is the clinical manifestation of the T cell-mediated response peak, which is important for viral clearance and recovery from MV infection. For immunity to reinfection, however, T cell responses in the absence of humoral immunity are not consistently protective (22). On the other hand, neutralizing antibody titers in excess of a defined threshold (200 mIU/mL using the 1st International Standard for anti-MV serum or 120 mIU/mL for the 2nd International Standard) (23) measured by plaque reduction neutralization (PRN) assay consistently provide protection (24, 25). Neutralizing antibody titers measured by PRN appear to slightly underestimate

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protection, as some children testing seronegative against MV are still protected from infection (25, 26). Protection in these instances might result from low levels of neutralizing immunity, from cellular immune responses, or from a combination thereof. Nonetheless, the power of neutralizing immunity for predicting protection is well established and is used in the field of MV vaccinology, including in this dissertation, as the measure of vaccine efficacy.

Vaccines Against Measles

Before MV vaccines were introduced in 1963, measles infected an estimated 135 million people and killed more than 6 million people globally each year, most of them children (5). Despite opportunities for improvement, measles vaccination has thus proven to be one of the most successful public health interventions of all time.

The derivation of measles vaccines has been extensively described elsewhere by Rota and colleagues (27) and Strebel and colleagues (28). In brief, measles virus was first isolated from an infected child, David Edmonston, by John Enders and Thomas Peebles (29). Subsequent passage of the wild-type Edmonston virus in human kidney and amnion cells resulted in the Edmonston-Enders stain, and further passage in the intraamniotic cavity of chick embryos and in chick embryo fibroblasts resulted in the derivation of an attenuated strain, Edmonston B, which was licensed as a measles vaccine in 1963. The vaccine was immunogenic and induced protection in recipients, but also proved reactogenic, causing fever and rash. To eliminate this reactogenicity, two independent groups further passaged Edmonston B at low temperature (32°C) in chick embryo fibroblasts, resulting in the Moraten ("More attenuated") vaccine developed by Maurice Hilleman (30), which is currently used in the United States, and the Schwarz vaccine

developed by Anton Schwarz (31), which is used in many other countries. Despite the separate passage histories of the Moraten and Schwarz vaccines, the two attenuated viruses are, surprisingly, genomically identical (32). The Edmonston-Enders strain was also separately attenuated to derive two additional vaccine strains, the Edmonston-Zagreb and AIK-C vaccine strains. Additionally, attenuation of three other individual wild-type isolates has resulted in the production of the Changchun-47, Shanghai-191, and CAM-70 vaccine strains. Germane to this dissertation is the Moraten vaccine strain, which has identical protein-coding capacity to the parental, control vaccine MVvac2 first presented in Chapter 2 that served as the genomic background for my development of modified vaccines; by extension, MVvac2 also has identical protein coding capacity to the Schwarz vaccine. The Schwarz and Edmonston-Zagreb vaccines are also important to this dissertation for their use at high titer doses in young infants, discussed in the next section.

Barriers to Immunization of Young Infants

Problematically, current vaccines are much less effective in infants under one year of age than they are in older children and adults (33, 34). Young infants are a greater risk than any other age group of death and disability from measles infection. During epidemic outbreaks infants younger than one year of age experience the highest fatality rates of any demographic group due to measles infection; case fatality rates two and nearly eight times higher than in three- and four-year-old children, respectively, have been recorded (35). Ironically, as measles vaccination coverage increases, a greater proportion of young infants are susceptible to MV infection. For example, in recent outbreaks of measles in Europe, infants too young to vaccinate constituted between 0.25-83% of case incidence in

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individual outbreaks, or approximately 10% of case incidence in the region in total (36)).

In recent outbreaks in the United States, one in four of all cases identified occurred in an

infant less than one year of age (37).

Current MV vaccines' inefficiency at ages less than one year stems primarily

from the variable presence of maternal antibodies (38). In humans, a mother passes her

own IgG antibodies across the placenta to the bloodstream of her developing fetus,

especially in the last month of pregnancy; this transfer is proposed to occur at least in part

by a neonatal Fc receptor (FcRn)-mediated mechanism (39). A mother also transmits IgA

antibodies to the gastrointestinal tract of her child in breast milk. Maternal immunity is

crucial to protecting young infants from severe neonatal infection; over time, however,

passively transferred maternal immunity will decline to a level that may no longer protect

against wild-type pathogen challenges but can still suppress vaccine-induced immunity

(40-43). Consequently, only approximately 85% of nine-month- old (44) and 65% of six-

month-old infants (45) will mount antibody responses to vaccination. Vaccination is

therefore generally not indicated before nine months of age due to poor responses, but

many infants become susceptible to infection before nine months due to the loss

of maternal protection (36). Infants thus enter a window of susceptibility to severe MV

infection defined by two related and opposing trends: the decline of protective maternal

antibody and the simultaneous increase of successful vaccination as they age (Fig. 1.1). A

vaccination strategy that closes this window is highly desirable.

It has also been suggested that young infants may be immunologically

incompetent to respond successfully to measles vaccination. Indeed, six-month-old

infants even in the absence of passive immunity have been observed to seroconvert at

lower rates and to lower PRN titers than infants first immunized at a later age (33, 41-43, 46). At the same time, however, infants immunized at six months of age can mount detectable, protective-level neutralizing responses (33, 41-43, 46), which are boosted after receipt of the second recommended MV vaccine dose to match those of infants first immunized at a later age (41, 42, 46). Clinical data also show that maternal antibodies are the primary barrier to overcome (42). For example, infants immunized in the presence of detectable maternal immunity failed to mount protective neutralizing responses after a first dose of MMR-II, a vaccine cocktail containing attenuated rubella and mumps viruses in addition to the Moraten measles vaccine strain, at six months of age (average 39 mIU/mL, 95% confidence interval 16-95 mIU/mL). On the other hand, six-month-old infants lacking detectable maternal immunity mounted protective responses after a single dose of MMR-II (average 516 mIU/mL, 95% confidence interval 209-1274 mIU/mL). Consequently, following two doses of MMR-II, the proportion of six-month-old infants lacking maternal antibodies at time of first vaccine dose who were considered protected from infection matched that of infants who received their first dose at nine months of age (93% vs. 94%, respectively), as well as population level estimates for the protectiveness of two doses of MV vaccine. Significantly, this demonstrates that, if the barrier of maternal immunity can be overcome, the outcomes of vaccination of even young infants match those of individuals first vaccinated at an older age.

High Titer Measles Vaccines in Infants

Alternative measles vaccination strategies that would allow immunization of infants at a younger age have been strongly sought after. The most extensively tested MV vaccination strategy tailored to younger ages made use of higher titer doses of current

vaccines in infants as young as four months of age. In clinical trails, six-month-old infants administered a standard ($10^{3.7}$-$10^{3.8}$ PFU), medium ($10^{4.5}$-$10^{4.6}$ PFU), or high ($10^{5.3}$-$10^{5.6}$ PFU) titer dose of the Edmonston-Zagreb or Schwarz MV vaccines demonstrated a statistically significant, dose- dependent increase in response to vaccination so that seroconversion rates of six-month-old recipients of the high titer vaccine improved to match those of nine-month-old recipients of the standard titer vaccine (Fig. 1.2) (47). Two other studies demonstrated that four-month-old recipients of the medium titer dose were at least as well protected from wild-type MV as nine- month-old infants administered the standard titer dose (48, 49). Despite the increase in seroconversion against and protection from measles, however, female recipients of the high titer vaccines experienced higher rates of mortality from non-measles causes in the years after vaccination (50-54). Measles was the first viral pathogen associated with immunosuppression, and opportunistic infections during and early after measles rash are the major cause of measles-associated mortality (55). It has been proposed that high titer vaccine-induced immunosuppression, similar to that caused by wild-type measles infection, is the cause of the high mortality observed mainly in African females. The strategy was withdrawn, but these findings demonstrate that current MV vaccines are sufficient to effectively immunize even young infants if their stimulus is increased; the challenge is to present this increased stimulus within the safe context of the current vaccine dosage.

Central Hypothesis

Based on the clinical performance of high titer doses of MV vaccines, which improved infant seroresponses but were unsafe, as well as the demonstrated primacy of

the H protein in inducing protective neutralizing immunity against MV, we explored an alternative pediatric vaccine strategy based on increasing the antigenic dosage of H protein in a standard titer MV vaccine. We specifically hypothesized that selective increase of H expression in the background of the current MV vaccine would safely improve the vaccine's immunogenicity and potentially evade inhibition by maternal immunity to allow infant responses to vaccination (Fig. 1.3). The goal of this dissertation has been to develop modified MVs with increased H expression and test their immunogenicity in mouse models incorporating barriers that prevent successful immunization of young infants, with the expectation that this research would lay the foundation for future experimentation in more relevant primate models.

Chapter Organization

Chapter 2 of my dissertation, entitled "Generation of a more immunogenic measles vaccine by increasing its hemagglutinin expression," describes my development of two MVs with increased expression of envelope-bound or soluble H protein by inserting another full-length or truncated copy of the H-coding cistron in a high expression locus in a Moraten-equivalent genome. The two vectors replicate with equal efficiency to their parental strain in two cell lines, and form plaques not suggestive of over-attenuation. I show that the vector encoding a truncated additional copy of H secretes measurable levels of the soluble protein to its extracellular environment, and that the insertion of another full-length copy of H in the other vector results in an approximately three-fold relative increase of its incorporation into viral particles. I provide evidence that the modification to the latter virus's envelope confers upon it a degree of resistance to neutralization by polyclonal immune serum *in vitro* without

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impacting its thermostability. I finally provide evidence that both vectors are more immunogenic than their parental strain in a non-susceptible mouse model, and that the virus with increased expression of envelope-bound H is additionally more immunogenic in the gold standard MV-susceptible small animal model for MV vaccination.

Chapter 3 of my dissertation, entitled "A measles vaccine with increased hemagglutinin expression outperforms its parental Moraten-equivalent in infant-like mouse models," describes further evaluation of the two modified MVs introduced in Chapter 2 in mouse models that replicate barriers that prevent immunization of young infants. Here, I show that neither use of particulate inocula nor inclusion of a boosting dose rescue the immunogenicity of the vector expressing soluble H protein in MV-susceptible mice, but that the virus expressing additional full-length H is more immunogenic than its parental strain even in young animals. Most significantly, I show that the virus that incorporates higher levels of full-length H induces stronger immune responses than its parental current vaccine-equivalent in a mouse model of passive immunity, where inoculation of homologous polyclonal anti-MV serum was able to significantly inhibit responses of MV-susceptible mice to the parental strain. Finally, I show that dam-to-pup transfer of maternal immunity against MV occurs in the gold standard MV-susceptible mouse model, and suggest the utility of this model for preclinical assessment of alternative pediatric MV vaccines.

Chapter 4 of my dissertation, entitled "Summary and future directions," discusses the implications of the findings presented in this dissertation as well as avenues for future research. Much work remains to be done, but this dissertation has importantly identified a likely candidate for a next generation pediatric measles vaccine.

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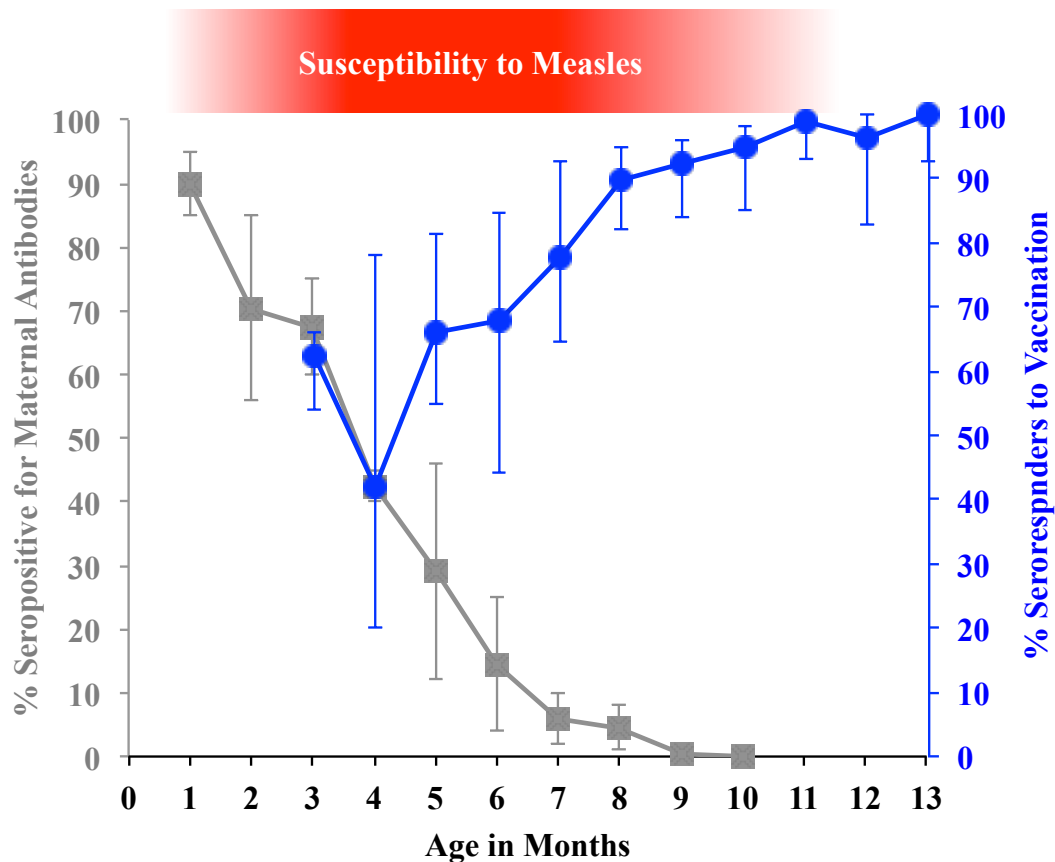
Figure 1.1 The window of opportunity for measles infection. In young infants, susceptibility to measles infection (indicated by red shading) is defined by two related and opposing trends: the decline of maternal antibodies (gray squares) and increase in responsiveness to vaccination (blue circles). The youngest infants are protected from infection by passively transferred maternal antibodies, but over time maternal immunity declines to intermediate levels (approximately four to eight months of age) where it interferes with vaccination, but no longer protects against wild-type infection, thus creating a window of opportunity for measles infection where infants cannot be vaccinated but are prone to severe infection. Percentage of infants seropositive for maternal antibodies are derived from Caceres and colleagues (56) and percentage of infants seroresponsive to vaccination are derived from Moss and Scott (45).

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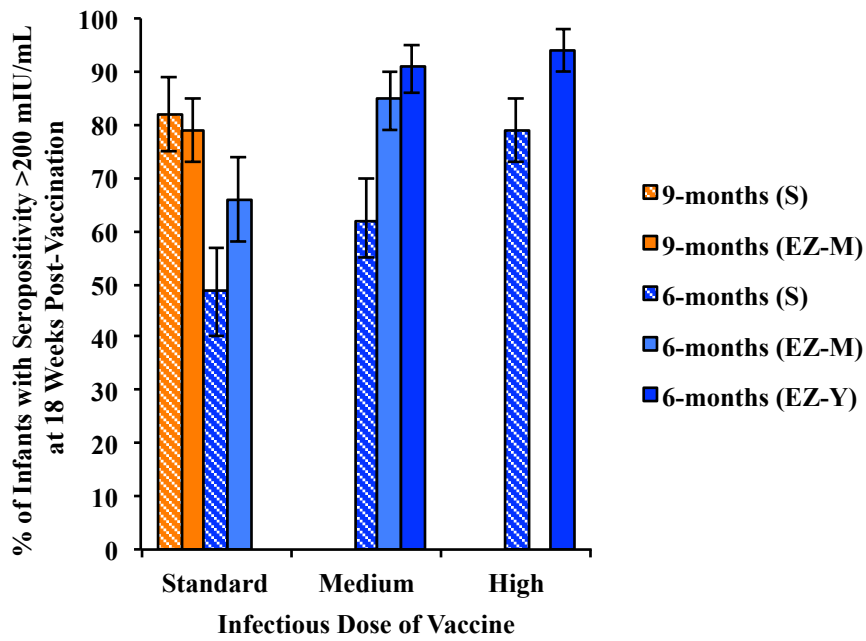
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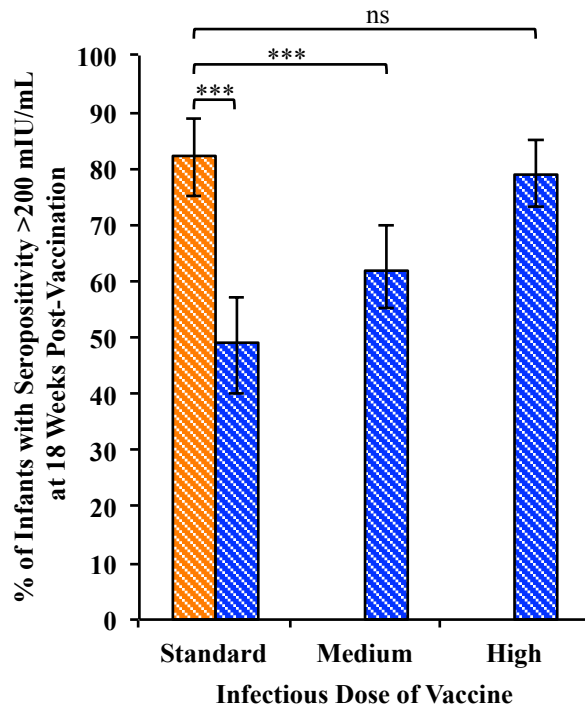
Figure 1.2 Increasing the dose of measles vaccine improved antibody responses of six-month-old infants. (A) Six-month-old infants (blue bars) were administered a standard ($10^{3.7}$ PFU), medium ($10^{4.5}$-$10^{4.6}$ PFU), or high ($10^{5.3}$-$10^{5.6}$ PFU) titer infectious dose of the Schwarz (S, striped bars) or Edmonston-Zagreb (EZ, solid bars) measles vaccine. For the Edmonston-Zagreb vaccine, two separate batches produced in Mexico and Yugoslavia were used, indicated by –M and –Y respectively. Vaccine efficacy is indicated as the percentage of infants with seropositivity greater than 200 mIU/mL, the protective threshold for neutralizing immunity against measles, at 18 weeks after vaccination. (B) Outset are the results from (A) for the Schwarz vaccine, which has identical protein coding capacity to the vaccine strain background used for the development of the modified MVs described in this dissertation. Statistical significance was assessed by Mantel-Haenszel chi-square test, using responsiveness to the standard dose of Schwarz vaccine in nine-month-old infants as reference. Statistical comparisons are omitted from (A) for clarity, but with the exception of the two significant differences indicated in (B), the only other difference to reach significance was between the reference and six-month-old recipients of the high titer EZ vaccine ($P < 0.01$). ***, $P < 0.001$. Data shown here are derived from Markowitz and colleagues (47).
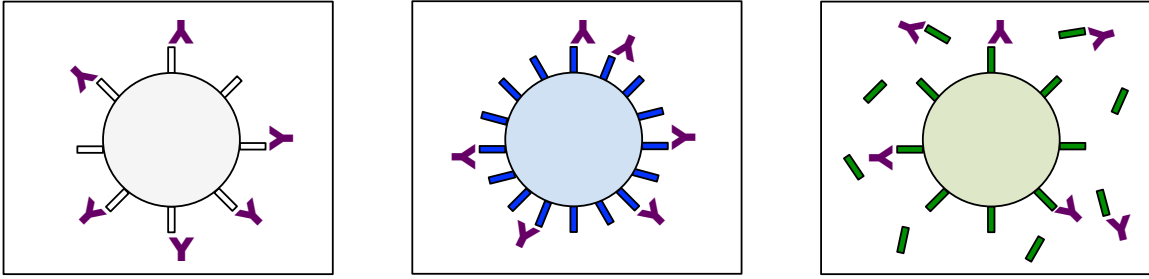
Figure 1.3 Central hypothesis. The left panel depicts a cartoon representation of a current MV vaccine, in gray, to which infant immune responses are inhibited in the presence of residual maternal antibodies (purple). In the central panel, a modified MV (blue) incorporates additional H protein (rectangles) into its viral envelope, quenching the inhibitory effect of residual maternal immunity to allow induction of active infant neutralizing responses. The right panel illustrates a second modified MV (green) that induces release of high levels of soluble H protein from infected cells. Here, soluble H proteins may serve as antigenic decoys to divert the inhibitory effect of maternal immunity and again allow active immune responses from young infants.

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CHAPTER 2

GENERATION OF A MORE IMMUNOGENIC MEASLES VACCINE BY

INCREASING ITS HEMAGGLUTININ EXPRESSION

ABSTRACT

Imported measles virus (MV) outbreaks are maintained by poor vaccine responders and unvaccinated people. A convenient but more immunogenic vaccination strategy would enhance vaccine performance contributing to measles eradication efforts. We report here the generation of alternative pediatric vaccines against MV with increased expression of the H protein in the background of the current MV vaccine strain. We generated two recombinants: MVvac2-H2, with increased full-length H expression resulting in a three-fold increase in H incorporation into virions, and MVvac2-Hsol, vectoring a truncated, soluble form of H protein that is secreted to the supernatants of infected cells. Replication fitness was conserved despite the duplication of the H cistron for both vectors. The modification to the envelope of MVvac2-H2 conferred upon that virus a measurable level of resistance to *in vitro* neutralization by MV polyclonal immune sera, without altering its thermostability. Most interestingly, both recombinant MVs with enhanced H expression were significantly more immunogenic than their parental strain in outbred mice, while MVvac2-H2 additionally proved more immunogenic after a single, human-range dose in genetically modified MV-susceptible mice.

IMPORTANCE

Measles incidence reduced drastically following introduction of attenuated vaccines, but progress toward eradication of this virus has stalled and MV still threatens unvaccinated populations. Due to the contribution of primary vaccine failures and too-young-to-be-vaccinated infants to this problem, more immunogenic measles vaccines are highly desirable. We generated two experimental MV vaccines based on a current vaccine's genome but with enriched production of H protein, the main MV antigen in provoking immunity. One incorporated H to higher rates in the viral envelope, and the other secreted a soluble H protein from infected cells. Increased expression of H by these vectors improved neutralizing responses induced in two small animal models of MV immunogenicity. The enhanced immunogenicity of these vectors, mainly from the MV that incorporates additional H, suggests their value as potential alternative pediatric MV vaccines.

INTRODUCTION

Despite the existence of an effective vaccine, measles virus (MV) infections remain an insidious threat to global health. After decades of reduction in measles-related mortality due to vaccination, this has stalled between 110,000-120,000/year since 2012. Approximately 20 million people are infected by measles each year with significant morbidity and up to 1% mortality (1). In 2014, the United States saw the highest number of annual measles cases since native transmission was declared locally eliminated in 2000 (2) and experienced a large multi-state outbreak in the first months of 2015 (3).

Protecting young infants against measles is an important goal to attain to improve the current MV vaccine (4). The highest fatality rates due to MV infection occur in infants younger than one year of age (5). Ironically, with the current vaccine and schedule, the higher the coverage, the greater proportion of young infants is susceptible to MV infection. Infants retain antibodies passed from their mothers across the placenta and in breast milk for months after birth and these protect against early-life infection, but interfere with early vaccination (6). Only approximately 85% of nine-month-olds (7) and 65% of six-month-olds mount antibody responses to the vaccine (8). This level of efficiency proves prohibitive for a public health intervention that must balance cost against protection; infants are therefore vaccinated at nine months of age in areas at great risk of measles transmission, or at twelve to fifteen months of age where there is not significant risk of infection.

Young infants can however, mount protective immune responses to current MV vaccines. Clinical trials revealed a dose-dependent, statistically significant increase in seroconversion with increasing measles vaccine titer (9), thus demonstrating that the

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increased immunogenic stimulus of the higher titer vaccine was sufficient to overcome barriers to MV immunization in young infants. Although efficient, the use of high-titer MV vaccines was withdrawn following observation of increased mortality rates not related to wild-type MV infection in female recipients (10-14) reviewed in (15). Here, we show a strategy to present an enhanced immunogenic stimulus within the safe context of the current vaccine dosage.

For the *Paramyxoviridae*, the progressive transcriptional attenuation from the 3' end leader region of the negative-sense genome, determines the expression level of viral proteins. Cistrons encoding the structural envelope proteins, which are the principal targets of protective neutralizing immunity, are located in the second- or third-to-last positions of the genomes of paramyxoviruses, resulting in relatively low expression levels (16). Envelope protein expression can be augmented by increasing the copy number of their coding cistrons and/or by moving these cistrons proximally within the viral genome (17-27). To develop an MV vaccine containing a greater immunogenic stimulus within the standard WHO-approved dosage, we constructed two recombinant MVs that express an additional copy of the hemagglutinin (H) protein in membrane-bound or soluble form from the second position of a Moraten strain-equivalent genome, resulting in increased expression and incorporation or secretion of H while maintaining replicative fitness and stability. We show here that both viruses induce significantly stronger neutralizing antibody responses in non-MV-susceptible mice. The vector expressing additional envelope-bound H also proved more immunogenic in an MV-susceptible mouse model and shows resistance to marginal levels of neutralizing antibodies *in vitro*.

MATERIALS & METHODS

Cells and viruses. Vero/hSLAM cells (28, 29) were maintained in Dulbecco's modified

Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal

bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 1% penicillin-

streptomycin (Sigma-Aldrich, St. Louis, MO) and 0.5 mg/mL G418 (Enzo Life Sciences,

Farmingdale, NY). Helper 293-3-46 cells (30) were maintained in DMEM with 10%

FBS, 1% PS, and 1.2 mg/mL G418.

Recombinant MVs were rescued using the method of Radecke et al. (30) modified

by Parks et al. (31). After detecting cytopathic effect in mixed cultures of Helper 293-3-

46 and Vero/hSLAM cells, individual syncytia were transferred to and propagated in

Vero/hSLAM cells. To prepare stocks of the viral clones thus generated, Vero/hSLAM

cells were infected at a multiplicity of infection (MOI) of 0.03 and incubated at 37°C.

When approximately 80% cytopathic effect was observed, cells were scraped in Opti-

MEM (Life Technologies, Grand Island, NY) and viral particles were released by two

freeze-thaw cycles.

Multi-step growth kinetics of the viruses were measured by infecting

Vero/hSLAM cells at an MOI of 0.03 and incubating them at 37°C. Supernatants and

infected cells were collected and lysed by a single freeze-thaw cycle at prescribed times

post-infection, and the 50% tissue culture infectious dose ($TCID_{50}$) was assessed in

Vero/hSLAM using the Spearman-Kärber end-point dilution method (32).

Construction and recovery of recombinant MVs. The recombinant MVs were

constructed in the background of pB(+)MVvac2(HBsAg)N (33). The MV genome

coding capacity in this plasmid is identical to those of the Moraten and Schwartz vaccine

strains (34) with an additional transcription unit (ATU) inserted downstream of the

nucleocapsid (N) cistron to direct the expression of an inserted foreign gene. To obtain

the recombinant MVs with higher H expression levels, *Mlu*I and *Aat*II digestion swapped

the HBsAg insert with a modified H cistron obtained by PCR. To generate the insertion

for pB(+)MVvac2-H2, the H coding sequence was amplified by PCR from

pB(+)MVvac2(HBsAg)N using forward primer 5'-

ACTGACGCGTCAGGGTGCAAGATCATCGA-3' and reverse primer 5'-

ACTGGACGTCAATCTAGTGATGGTGATGGTGGTGTCTGCGATTGGTTCCATCT

TCCCGGGTGAC-3' to amplify the entire transcribed region of H with 18 added

nucleotides encoding a carboxy-terminal tag composed of six histidines (His tag). To

generate the insertion for pB(+)MVvac2-Hsol, the same reverse primer was used in

conjunction with forward primer 5'-

ACTGACGCGTATTATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTC

TTGCACTTGTCACAAATAGCCGACTTCATCGGGCAG-3' to amplify the portion of

the H cistron corresponding to the H stalk and ectodomain with additional sequence

encoding a carboxy-terminal His tag and an amino-terminal signal sequence from

interleukin-2. *Mlu*I and *Aat*II restriction sites are underlined. Sanger sequencing

confirmed correct junctions and coding sequence within the ATUs.

We constructed in the background of pB(+)MVvac2(ATU)N two additional

viruses used as controls here, each encoding a fluorescent protein at the same locus as the

insertions in pB(+)MVvac2-H2 and pB(+)MVvac2-Hsol. We inserted an enhanced green

fluorescent protein (EGFP) as well as a red fluorescent protein (RFP, tandem dimer

Tomato, obtained initially by PCR from pCS2-tdTO) coding sequence into the ATU

downstream of the N cistron. Expression of both proteins in cells infected with the corresponding virus was confirmed by epifluorescence microscopy.

Western blot analysis. For protein expression analysis, 1 x 10^6 Vero/hSLAM cells were seeded in 100-mm-diameter dishes and infected at an MOI of 0.3 with MVvac2-H2 or MVvac2(EGFP)N, or were mock-infected. Forty h after infection, cells were washed three times with phosphate-buffered saline (PBS) then lysed with RSB-NP40 buffer (1.5 mM MgCl2, 10 mM Tris-HCl, 10 mM NaCl, and 1% Nonidet P-40, Sigma-Aldrich, St. Louis, MO) plus protease inhibitors (c*O*mplete Mini Protease Inhibitor Tablets, Roche Diagnostics, Manheim, Germany). The protein extracts were mixed with Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) containing β-mercaptoethanol and denatured at 96°C for 10 min.

To purify viral particles for protein incorporation analysis, the supernatants of 5 x 10^6 cells infected with the parental strain MVvac2, MVvac2-H2, or MVvac2(RFP)N were initially clarified by centrifugation at 5,000 x *g* for 15 min in an SX4750 rotor. The clarified supernatants were layered over a 2 mL 20% sucrose cushion and viral particles were pelleted by ultracentrifugation at 104,000 x *g* for 2 h in an SW 28 rotor. The pellets were resuspended in TNE buffer (10 mM Tris [pH 7.8], 100 mM NaCl, 1 mM EDTA) and the infectivity of each particle preparation was determined by end-point dilution assay. Purified particles were mixed with 6X SDS-PAGE sample buffer (0.35 M Tris [pH 6.8], 30% glycerol, 10.28% sodium dodecyl sulfate [SDS], 0.6 M dithiolthreitol, 0.012% bromphenol blue) and denatured at 96°C for 10 min.

Protein extracts or purified virion lysates were separated by SDS polyacrylamide gel electrophoresis (PAGE) in a 4-15% or 10% acrylamide gel, respectively. Protein

extracts were loaded in volumes that achieved equalized expression of F and N, while for the viral particles 2.5 x 10^3 TCID$_{50}$ were loaded for each sample. Following electrophoretic separation, proteins were transferred to nitrocellulose for immunoblotting using a 1:20,000 dilution of rabbit polyclonal anti-MV N, a 1:7,500 dilution of rabbit polyclonal anti-MV-F, a 1:10,000 dilution of rabbit polyclonal anti-MV-H (35), or a 1:10,000 dilution of mouse anti-actin (Sigma-Aldrich, St. Louis, MO), followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare, Little Chalfont, United Kingdom). An additional immunoblot was performed using a 1:5,000 dilution of anti-His (C-term)-HRP (Invitrogen, Carlsbad, CA). Reactions were developed using a chemiluminescence kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL).

Immobilized cobalt ion affinity chromatography. To isolate 6x His-tagged soluble H protein from the supernatants of MVvac2-Hsol-infected cells, 100-mm-diameter dishes were seeded with 1 x 10^6 Vero/hSLAM cells and infected with MVvac2 or MVvac2-Hsol at an MOI of 0.1. Supernatants and cells were collected at 24 h intervals and lysed by one freeze-thaw cycle, and supernatants were clarified by centrifugation at 3,000 x g for 5 min in an SX4750 rotor. TALON Superflow Metal Affinity Resin (Clontech Laboratories, Mountain View, CA) equilibrated in interaction buffer (50 mM NaH$_2$PO$_4$ pH 8, 200 mM NaCl, 1% Triton X-100) was added to the clarified supernatants for 2 h of batch-interaction. The resin was subsequently washed four times in washing buffer (50 mM NaH$_2$PO$_4$ pH 8, 500 mM NaCl, 5 mM imidazole, 1% Triton X-100), followed by a final wash in 0.5X PBS. The resin was resuspended in 6X SDS-PAGE sample buffer and incubated at 96°C for 10 min, then separated by SDS-PAGE in a 10% acrylamide gel.

The electrophoretically separated proteins were visualized by staining with Coomassie Brilliant Blue G-250. A replicate gel was transferred to nitrocellulose and subjected to immunoblot for the 6 x His tag as described above to visualize soluble H protein.

Preparation of viral particles for thermostability assay and animal experiments. To generate high titer preparations of purified viral particles, viral stocks were layered over 1 mL 20% sucrose cushions through which viral particles were pelleted by ultracentrifugation at 147,000 x g for 2 h in an MLS-50 rotor. Pellets were resuspended in TNE buffer and titrated by end-point dilution assay.

Thermostability assay. To test the thermostability of recombinant MVs, particles of MVvac2 and MVvac2-H2 were diluted in Opti-MEM to generate aliquots of approximately 1 x 10^5 $TCID_{50}$/mL. Aliquots of each virus were treated by incubation at 4°C, 24°C, 37°, 45°C, or 96°C for one hour. Additionally, one aliquot of each virus was titrated immediately to determine the baseline titer. Titers were determined by measurement of plaque-forming units (PFU) generated in plaque assays. Briefly, serial 10-fold dilutions of the untreated or treated viruses were inoculated in duplicate onto Vero/hSLAM cells in 24-well plates. After allowing 2 h for infection, the plates were overlaid with carboxymethylcellulose (CMC) in DMEM to bring the final concentration of CMC in the plate to 0.75%. Five days later, titer was determined by counting the plaques per mL virus applied in the first well with discrete countable plaques, averaging the titers from the two tests. Infectivity was calculated as the PFU/mL in the treated sample as a percentage of the PFU/mL in the untreated sample for that virus.

In vitro **neutralization of viruses with MV-immune sera.** To test the susceptibility of our recombinant MVs to neutralization by anti-MV antibodies, we performed a modified

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logarithmic neutralization index using sera of defined MV neutralization titer from mice

that had been immunized with MVvac2. In a 96-well plate, 1:100 dilutions of sera (MV

neutralization titers of 1:33, 1:80, 1:133, 1:213, 1:427, 1:640, and 1:1066) in Opti-MEM

were incubated with 1 x 10^4 $TCID_{50}$ of MVvac2, MVvac2-H2, or MVvac2-Hsol for one

hour at 37°C. Following incubation, serial ten-fold dilutions of the serum-virus mixture

were generated, then transferred onto Vero/hSLAM cells and incubated at 37°C for three

days, at which time infectivity in the samples was assessed as $TCID_{50}$.

Mouse inoculations. All experimental procedures were performed according to a

protocol approved by the Arizona State University Institutional Animal Care and Use

Committee. To determine the immunogenicity of recombinant MVs in a non-susceptible

small animal host, groups of eight CD1 mice (Jackson Laboratories, Maine) were

inoculated initially by the intraperitoneal (i.p.) route with 10^5 $TCID_{50}$ of purified viral

particles in 100 μL of Opti-MEM. Three weeks after the initial inoculation, the mice

received a second i.p. dose of 10^5 $TCID_{50}$ of purified particles in Opti-MEM. Two weeks

after the second dose, mice were bled. Sera were separated and heat-inactivated at 56°C

for 1 h. To determine the immunogenicity of recombinant viruses in an MV-susceptible

model, groups of six HuCD46Ge-IFNar^{KO} mice (36) were inoculated by the i.p. route

with 10^3 $TCID_{50}$ of the viruses in 100 μL of Opti-MEM. Mice were bled 28 days post-

inoculation and sera were separated and heat-inactivated.

MV microneutralization assay. Serial two-fold dilutions of heat-inactivated sera in

Opti-MEM were incubated with 100 $TCID_{50}$ of MVvac2 for 1 h at 37°C. Vero/hSLAM

cells were added to the serum-virus mixtures and the plates were then incubated at 37°C

for three days, at which time neutralization titer was assessed as the highest dilution of

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serum capable of complete neutralization of infectivity, defined as the absence of MV cytopathic effect. Neutralization titers were reported as the average of a determination made in triplicate.

MV ELISA. Overall anti-MV IgG reactivity from available individual samples was determined with a mouse-adapted semi-quantitative ELISA kit (Alpha Diagnostic International, San Antonio, TX) and expressed as arbitrary ELISA units per milliliter by comparison with internal standards supplied by the manufacturer**.**

RESULTS

Generation of measles vaccine vectors encoding additional H protein in the second position of the genome. We hypothesized that H expression could be augmented by inserting an additional copy of the H-encoding sequence at a high-expression locus in the viral genome. To generate recombinant MV vaccines with an increased expression of H, we made use of a previously described MV cDNA identical in amino acid sequence to the Moraten vaccine strain with an inserted additional transcriptional unit (ATU) downstream from the N cistron (34). Initially, an additional copy of the full-length Moraten H-encoding sequence was cloned into this ATU, yielding pB(+)MVvac2-H2 (Fig. 2.1A). To distinguish expression at the ATU from expression of H at its native locus in the viral genome, we tagged the extracellular carboxy-terminus of H with six histidines (His tag), increasing the length of the additional H copy to 623 amino acids. By this approach, we expected pB(+)MVvac2-H2 to generate recombinant MVs incorporating additional H into the viral envelope. We then generated a second vector encoding a modified copy of H from the same ATU locus. Here we exchanged the amino-terminal cytoplasmic tail and transmembrane region of the additional H (58 amino

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acids) with 20 amino acids corresponding to the signal sequence of interleukin-2. The modified copy of H was again marked with a carboxy-terminal His tag for a total length of 565 amino acids, following cleavage of the signal sequence. We expected this recombinant genome to direct secretion of soluble forms of H from infected cells, and thus named it pB(+)MVvac2-Hsol (Fig. 2.1A).

We rescued the two recombinant viruses using the system of Radecke et al. (30) and assessed their replication fitness *in vitro* by multi-step growth kinetics using as hosts Vero/hSLAM cells and primary chick embryo fibroblasts grown at 37^0C and 32^0C, respectively. The replication of parental MVvac2 was determined as reference. As shown in Fig. 1B, in Vero/hSLAM cells recombinant vectors with an additional H copy reached maximal titers of approximately $10^{6.5}$ to $10^{6.25}$ $TCID_{50}$/mL for cell-associated virus and of 10^4 to 10^5 $TCID_{50}$/mL for cell-free virus at 36 and 48h post-infection, respectively. Viral replication in chick embryo fibroblasts did not show a different trend (Fig 2.1C). Recombinants and the parental strain MVvac2 reached titers around 10^4 $TCID_{50}$/mL for the intracellular and an order of magnitude lower for the secreted virus at 6 days post-infection. Thus, our recombinant viruses with duplicated H genes thus replicated as efficiently as the parental strain using two different cell substrates. Finally, we determined viral plaque size in Vero/hSLAM cells. While the parental strain MVvac2 and MVvac2-H2 had indistinguishable, homogeneous plaques with a diameter of 1.09 mm in average, MVvac2-Hsol showed heterogeneous plaques of 0.51 to 1.2 mm in diameter (Fig 2.2D).

Recombinant MVs express H at higher rates. We initially tested whether our incorporation of an additional H-encoding cistron in the genome of MVvac2-H2 resulted

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in increased H expression by Western blot of infected cell extracts, 48 h post-infection. Non-infected cells and cells infected with the reference MVvac2(eGFP)N were used as controls. With approximately equivalent levels of expression of actin and two viral proteins, N and F, expression of H was greatly increased in cells infected with MVvac2-H2 (Fig. 2.2A). We also documented that the expression of the immature form of the F protein (an approximately 60kDa protein band) was slightly increased in MVvac2-H2-infected cell extracts.

To test if the increased cellular expression of H resulted in increased incorporation of H into virions, we performed a similar analysis using purified particles of MVvac2-H2, with MVvac2 and MVvac2(RFP)N as controls. Western blot detection of 2.5 x 10^3 TCID$_{50}$ of purified particles of each virus demonstrated increased levels of H in MVvac2-H2 particles (Fig. 2.2B). While expression of N and F is also slightly higher in MVvac2-H2 particles, densitometric analysis of the blots revealed an approximately 1.5- to 3-fold increase in the ratio of H to the other viral proteins in MVvac2-H2 relative to MVvac2, supporting increased incorporation of H into MVvac2-H2 virions. The *bona fide* nature of the analyzed material is supported by the absence of the immature form of F, as seen in lysates of infected cells. Additionally, immunoblot using an anti-His tag monoclonal antibody showed a band of approximately 78 kD, the expected size of the H protein, in MVvac2-H2 particles. Taken together, these results indicate that the expression of additional His-tagged H protein from an ATU downstream of N in MVvac2-H2 results in increased cellular expression of H and enhanced incorporation of H into MVvac2-H2 particles.

To test whether the enhanced H expression by MVvac2-Hsol induces the

secretion of soluble H forms upon infection and to purify this protein using affinity

chromatography, we collected supernatants of cells infected at an MOI of 0.1 at 24 h

intervals and interacted their clarified supernatants with immobilized cobalt ion resin

using a batch approach to isolate His-tagged proteins. We used supernatant from

MVvac2-infected cells as a control. We were able to isolate by affinity chromatography

a protein doublet with electrophoretic migration of 73 and 70 kDa specifically in

supernatants of MVvac2-Hsol-infected cells from 48 to 96 h post-infection (Fig. 2.2C,

left panel). Quantitatively, according to our Coomassie stain analysis of the purified

material, we estimated a yield of approximately 250ng/mL at 96h post-infection

(intracellular virus replication peaked at 48-72h post-infection for this experiment).

Furthermore, an immunoblot using anti-His tag antibodies to probe the affinity

chromatography-purified material documented that the 73 kDa band protein results from

the H secretion driven by MVvac2-Hsol infection from 48 to 96 h PI (Fig. 2.2C, right

panel). These results indicated that soluble His-tagged H protein is expressed from the

ATU of MVvac2-Hsol and secreted from cells; it is possible that the His tag addition

altered its electrophoretic migration as observed in the Coomassie stained gel.

Alternatively, immature unglycosilated His-tagged H was secreted from infected cells.

Recombinant MV with enhanced H incorporation is as thermostable as

parental MV and possesses a different kinetic of neutralization by measles-immune

sera. To test the stability of MVvac2-H2 at temperatures ranging from 4 to 96°C,

aliquots of purified viral particles, each with an infectivity of approximately 10^5 TCID$_{50}$,

were incubated at increasing temperatures for 1 h, using MVvac2 as a control. Following

treatment, the remaining infectivity in each sample was determined by plaque assay and compared to the pre-treatment titer. As shown in Fig. 3, MVvac2-H2 responds to heat treatment in a similar manner as MVvac2, maintaining most of its infectivity at 20°C for 1 h, while the infectivity was reduced to 21% (MVvac2) or 25% (MVvac2-H2) of the pre-treatment titer by heating the viral particles at 37°C for the same period of time. MVvac2-H2 thus possesses equivalent thermostability to MVvac2, despite the enhanced H incorporation in its envelope.

MV H is the major target of neutralizing antibodies against MV (37). We therefore aimed to test whether the susceptibility to neutralization of our recombinant MVs expressing additional H differed from that of their parental strain. We hypothesized that in the presence of marginal levels of anti-MV antibodies, additional envelope-bound H in MVvac2-H2 could remain free to bind receptors or to alter the ratio of H antigen to neutralizing antibodies, increasing the amount of antibodies required to prevent the virus-cell interaction that triggers entry. We assessed this by comparing the sensitivities of MVvac2, MVvac2-H2, and MVvac2-Hsol to neutralization *in vitro* by polyclonal mouse sera of varying neutralizing capacities generated by immunization with MVvac2. Aliquots of recombinant MVs or the parental strain, each with a defined equalized infectivity, were mixed with 1:100 dilutions of seven anti-MV serum samples, ranging in microneutralization titer from 1:33 to 1:1067, and incubated at 37°C for one hour. Residual infectivity was then determined by end-point dilution assay.

As shown in Fig. 2.4, for serum dilutions with relatively lower capacity to neutralize MV (neutralizing titer ≤1:213), all three viruses maintained their titer in an approximately equivalent manner. When incubated with the more strongly neutralizing

preparations obtained from dilutions of neutralizing sera with titers >1:427, however, MVvac2 precipitously lost most of its infectivity so that the aliquot incubated with the most strongly neutralizing sera retained less than 0.1% of its original titer, while MVvac2-H2 retained approximately 10% of its original titer. Indeed, while MVvac2 infectivity declined almost three logs in titer over the assayed range of neutralization, MVvac2-H2 lost only one log of titer over the same series. This suggests that the presence of additional H protein on the envelope of MVvac2-H2 renders this virus relatively more resistant than MVvac2 to neutralizing antibodies. Since MVvac2-Hsol and MVvac2 preparations had a theoretically equivalent viral surface, they showed a similar pattern of reduction in infectivity in response to serum dilutions of increased neutralization potential.

MVs expressing additional H are more immunogenic. Since H is the major antigenic target of neutralizing immunity, we hypothesized that the enhanced expression of H protein by our recombinant MVs correlated with an increased immunogenicity relative to that of the parental vaccine strain. To test this hypothesis, we initially inoculated non-MV-susceptible CD1 mice with the three viruses. This animal host does not replicate measles to the best of our knowledge, but we reasoned that their immune system would detect differences in viral structure such as the enhanced display of H epitopes in the absence of the potentially confounding effects of viral replication. In this host, we expected MVvac2-H2 to generate stronger neutralizing immunity, given the enhanced display of envelope-bound H by particles of this virus. Three experimental groups of eight mice (MVvac2, MVvac2-2H, or MVvac2-Hsol) received two intraperitoneal doses of purified particles with an infectivity of 10^5 TCID$_{50}$ on a 28 days

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time interval. Two weeks after the second dose, we assessed the neutralizing immunity

of individual mice against MV by microneutralization assay. In support of our

hypothesis, animals that received MVvac2-H2 developed significantly higher

neutralizing titers (1:733 in average) than those immunized with MVvac2 (1:297 in

average Fig. 2.5A). This 2.46-fold difference reached statistical significance ($P = 0.0229$,

unpaired two-tailed T test). Unexpectedly, MVvac2-Hsol also induced greater

neutralizing titers in average than did MVvac2 in these mice, also statistically significant

(1:861 vs. 1:297, respectively with $P = 0.0473$). Furthermore, serous IgG reactivity

against MV was determined using a quantitative ELISA. As shown in Figure 2.5B,

animals from the three experimental groups were indistinctively reactive against MV

with readings from 181 to 468 arbitrary ELISA units per ml (AEU/ml). To corroborate

the antigenic nature of the inocula in terms of viral proteins composition, we analyzed the

presence of N, F and H proteins in viral particles with an infectivity of 2.5 x 10^3 TCID$_{50}$

by immunoblots (Fig. 2.5C). We used the same viral particle preparations for

immunoblots as were used for animal experiments. As expected, MV H was

incorporated to higher rate in MVvac2-H2 than MVvac2, while the viral surface of MV-

Hsol more closely resembled the virion surface composition of MVvac2, although with a

relatively lower signal for MV N. Since we were not able to detect the His-Tag signal in

MVvacHsol virion preparations, the secreted Hsol may not have been passively adsorbed

in virions. Thus, by increasing the incorporation of the MV H glycoprotein or inducing its

secretion, we demonstrated an increased neutralizing immune response in CD1 mice.

To further validate the enhanced immunogenic potential of our recombinant MVs,

we inoculated HuCD46Ge-IFNar^{KO} mice with the viruses. These mice express the

human CD46 receptor for vaccine strains of MV with human-like specificity in a type I interferon knockout background and are therefore susceptible to measles (36), allowing evaluation of our vaccine candidates' immunogenicity in a more human-like model than the CD1 mouse. In consequence, we inoculated animals with a single dose of 10^3 TCID$_{50}$. Four weeks after inoculation, we documented neutralizing titers that were significantly higher in mice immunized with MVvac2-H2 than in those immunized with MVvac2 (1:79 vs. 1:214 in average respectively, $P = 0.0436$, Fig. 2.5D). Contrary to our expectations, mice immunized with a single dose of MVvac2-Hsol generated neutralizing titers almost three times lower but not significantly different from those documented in mice immunized with MVvac2 (1:79 vs. 1:27 in average respectively, $P = 0.1117$); MVvac2-Hsol provoked detectable neutralizing immunity in five out of six recipients while the other two viruses elicited measurable titers in all of the animals tested. As expected, Ig reactivity against MV did not differ among the three experimental groups. Quantitative readings from 7 to 23 AEU/ml were documented (Fig. 2.5E). Again, the relative content of MV H, F and N was documented by immunoblot of viral preps used in the animal experiment and with the same infective potential (10^3 TCID$_{50}$) as inoculated to the mice (Fig. 2.5F). These demonstrated that the increase of measles immunogenicity per infective unit was not caused merely by the initial antigenic input. In sum, the 2.79-fold enhanced immunogenicity documented for MVvac2-H2 confirms our hypothesis that increased display of H targets on the virion surface improves stimulation of neutralizing immunity against MV in two small animal models of measles vaccination, with similar rates of increased immunogenicity compared to the parental strain in both hosts.

DISCUSSION

Eradication of MV remains a theoretically feasible but elusive goal. The variable presence of maternal antibodies is the hurdle to overcome to reduce measles mortality among the very young. The use of DNA vaccines (38-50), non-MV vectors expressing H, F, and/or N (51-57), immune-stimulating complexes incorporating the H and F proteins (57) or a combination of these strategies (58) have been proposed. Vaccination by these methods presumably avoids inhibition by maternal antibodies, and some have indeed proven protective when administered to infant macaques with circulating maternal antibodies. Still, for the most part, the strength, durability, and safety of the immunity induced by the current MV vaccine has been difficult to recapitulate in these experimental vaccines. Our approach is appealing because it takes advantage of the time-tested efficiency of the current vaccine, industrial production and distribution operative procedures are already in place and safety studies would be arguably more straightforward and experience-based. More appealing is the fact that our optimization of the current vaccine by genetically modifying its antigenic potency is cost-effective, if safety and efficacy as assessed in the macaque model support its further development.

We show here that recombinant MV can express and incorporate additional copies of its hemagglutinin into its viral envelope by doubling the H cistron. *In vitro,* we have corroborated that passage six of MVvac2-H2 still expresses the additional copy of H. The genetic stability of the insertion of an additional copy of H gene in MV after *in vivo* passage remains to be explored. But in other studies, the stability of genetic modification in the MVvac2 vector platform has been documented after an *in vivo* passage in non-human primates. Inactivation of the interferon-control viral proteins V

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and C or expression of HBsAg (34, 59) was genetically stable. The genetic stability of MVvac2-H2 could be promoted, if necessary, by deletion of the native H cistron, forcing selection on the added copy of H at the high expression locus.

Despite a careful clonal selection process, our MV-Hsol vector showed a heterogeneous array of plaque size in Vero/hSLAM cells. This observation might be caused by subtle interference of the soluble H protein with viral fusion. The two recombinants showed a conserved replicative profile in both Vero/hSLAM cells and primary chick embryo fibroblasts, grown at 32^0C, the latter of which are suitable for vaccine production. For our vector expressing an additional full-length copy of the H cistron, western blots demonstrated a much greater increase in the cellular expression of H than in its incorporation into virions. The excess of H expression relative to its incorporation suggests a structural limitation preventing further inclusion of H into virions. This result is interesting given the generally accepted pleiomorphic structure of measles virions (60-62). Others have proposed the notion of a more highly structured particle for members of the *Mononegavirales*, such as Newcastle disease virus (NDV (63). Such a rigid model of envelope structure for MV would seem not permissive of the up to three-fold incorporation enhancement of H per infectious unit documented here. Nonetheless, even the presumably more rigidly structured RV can incorporate additional glycoprotein units in its bullet-shaped envelope (19). It remains to be tested if further H expression intensifies H incorporation in a functional viral vector.

The enhancement of H immunogenic stimulus increased neutralizing responses in both non-susceptible and MV-susceptible mice. Inoculation of viral particles purified by ultracentrifugation resulted in improved induction of neutralizing immunity by our

modified H-expression vectors in non-susceptible, outbred mice. We expected these

hosts to provide only a limited number of replicative cycles, so that the antigenic stimulus

these animals received would be majorly represented by the composition of the viral

envelope. Purified viral particles were used to eliminate the presence of unincorporated H

or soluble H present in crude viral preparations as a confounding factor, and as shown in

the corresponding immunoblot. The MVvac-Hsol viral envelope did not differ majorly

from the parental strain's, indicating that the initial antigenic stimulus presented to the

CD1 mice was the same for animals receiving either MVvac2 or MVvac2-Hsol. As

expected, MVvac2-H2 was significantly more immunogenic than the parental vaccine

MVvac2 in this host. Surprisingly, MVvac2-Hsol also induced significantly higher

neutralizing antibody responses than did MVvac2 in the CD1 mice. In contrast,

HuCD46Ge-IFNar^{KO} mice represent a more human-like model of measles virus infection

than CD1 mice; their interferon signaling-deficient background is permissive of viral

replication. These mice are susceptible to MV infection and considered the gold-standard

small animal model for MV vaccine immunogenicity. Indeed, recent results from the first

clinical trial of a recombinant MV vaccine vectoring Chikungunya glycoproteins

document the relevance of immunogenicity results obtained using HuCD46Ge-IFNar^{KO}

mice as host in preclinical developments (64). A single IP dose of 10^3 TCID$_{50}$ allowed a

significant separation of vaccine performance. This dosage is equivalent to the minimal

WHO-mandated dosage. As in the CD1 mice, MVvac2-H2 was significantly more

immunogenic than the parental strain, further supporting the notion that increased H

dosage improves MV immunogenicity. Contrary to our findings in CD1 mice, MVvac2-

Hsol provoked immune responses from HuCD46Ge-IFNar^{KO} mice that were not

statistically different from those observed in animals immunized with parental MVvac2, and in fact showed a downward trend. It is possible that production of high levels of soluble H subverted the immune response as observed in other viral systems (65). The possibility that genetic differences between the outbred CD1 and our genetically modified HuCD46Ge-IFNar^{KO} mouse strain account for the documented differences in immunogenicity is impossible to discard experimentally. The fact that serous anti-MV IgG reactivity determined by ELISA did not show any difference between experimental groups supports the idea that we have specifically increased neutralizing immunity with our approach while maintaining the overall anti-MV humoral immune response largely directed against MV-N.

A more immunogenic MV vaccine might further support measles control. More robust neutralizing immune responses would positively impact the proportion of first-dose responders and would prolong measles immunity after a second optimized dose (66). With the current 83-84% coverage levels (67), a more immunogenic vaccine will more efficiently reach herd immunity. MVvac2-H2 might more reliably elicit efficacious neutralizing titers in very young infants where initiation of vaccination at six months of age has been successfully implemented in epidemic settings (68, 69). Some have promoted the adoption of early (<9 months) vaccination against MV in endemic areas to reduce child mortality (70). We hypothesize that since our MVvac2-H2 vector has a different dynamic neutralization profile *in vitro* (Fig 4), it may maintain infective stimulus and immunogenicity better than the current vaccine in the presence of maternal antibodies, reducing infants' window of susceptibility to measles. In the future, the potential of MVvac2-H2 in particular as an alternative pediatric vaccine can be more

definitely assessed in infant-like HuCD46Ge-IFNar^{KO} models that incorporate features such as artificial or natural passive immunity and relative immunological immaturity.

ACKNOWLEDGMENTS

A

MVvac2 (15,894 nts)

N P/C/V M F H L

MVvac2-H2 (17, 874 nts)

N H P/C/V M F H L

6x His-COOH

MVvac2-Hsol (17,742 nts)

N H P/C/V M F H L

IL2 ss 6x His-COOH

B

C

Time Post infection (h)

D

MVvac2   MVvac2-H2   MVvac2-Hsol

45
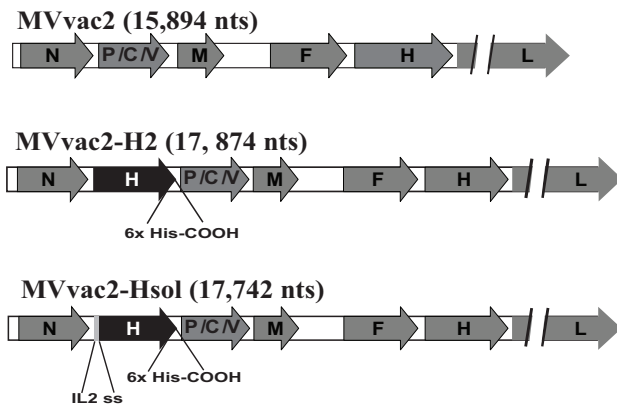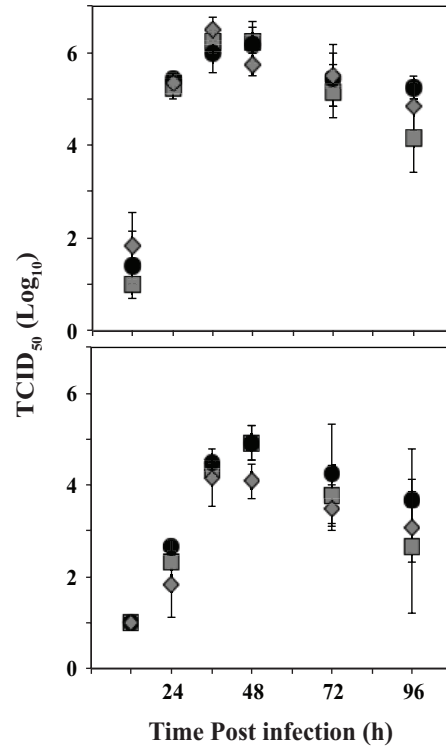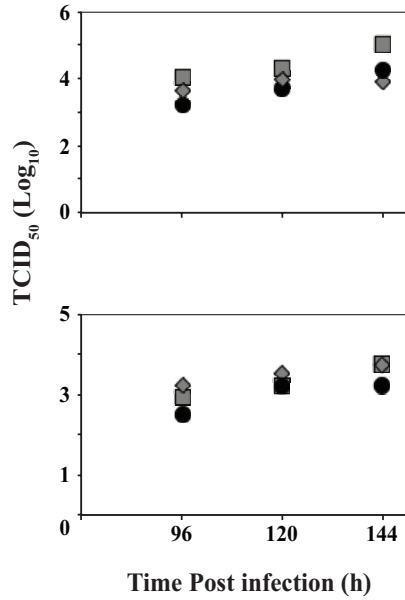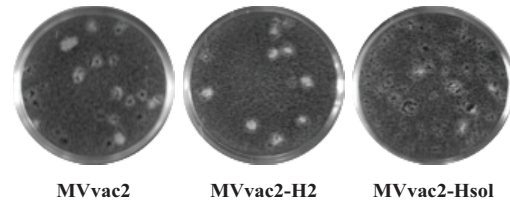
Figure 2.1 Generation and multi-step growth kinetics of recombinant MVs expressing additional H protein. (A) Diagram of MVvac2, MVvac2-H2 and MVvac2-Hsol. In the second and third viruses an additional, engineered H coding sequence was inserted as an ATU downstream of the N cistron. MV genes are indicated in gray with the added copies of H in black. A lighter grey bar represents the signal sequence for interleukin-2 (IL2 ss), replacing the transmembrane region and cytoplasmic tail coding sequence of the added H cistron in MVvac2-Hsol to direct secretion of a truncated, soluble form of the H protein. Each added copy of H was modified to encode a six histidine tag (6X-His COOH) at its carboxy terminus, as indicated. (B) Time-course of cell-associated (upper panel) and cell-free (lower panel) virus production at the indicated time points after Vero/hSLAM cells grown at 37^0Cwere infected at an MOI of 0.03 with either the parental strain MVvac2 (black circles) MVvac2-H2 (grey squares), or MVvac2-Hsol (grey diamonds). Averages and standard deviations of three independent experiments are shown. (C) Growth kinetic profile of cell-associated (upper panel) and cell-free (lower panel) MVs obtained from primary chick embryo fibroblasts grown at 32^0C and infected at an MOI of 0.03, same symbols as the previous graphics. (D) Viral plaque appearance in Vero/hSLAM cells infected with the indicated MV. Plaques were visualized five days post-infection when ethanol-fixed cells were stained with naphthol blue black.
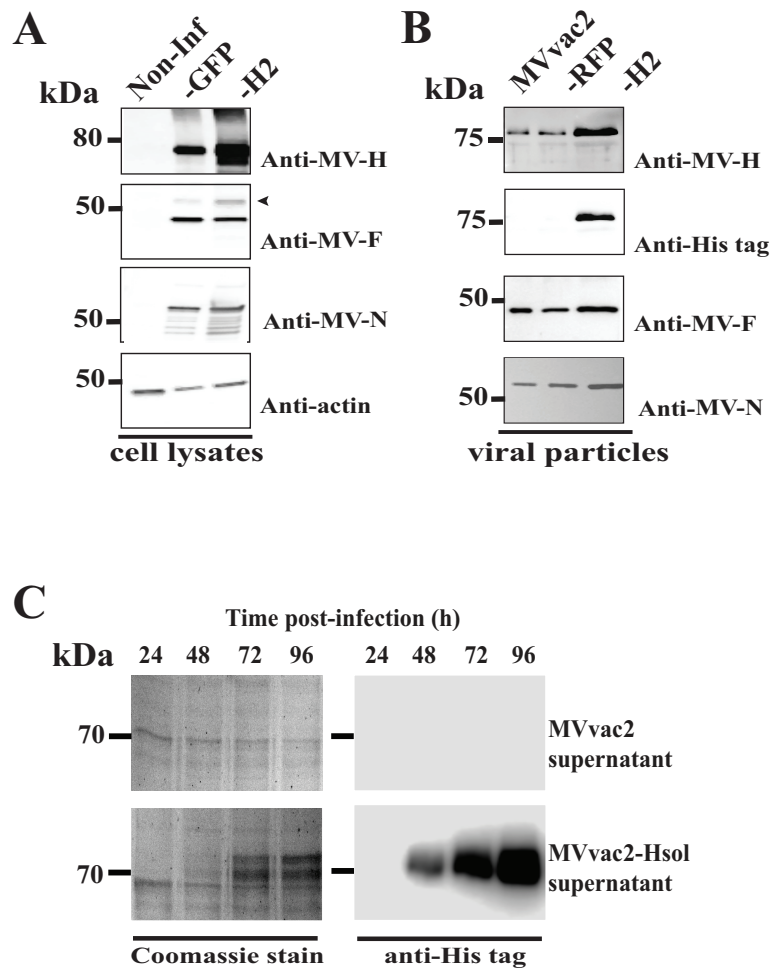
Figure 2.2 Characterization of additional H expression by recombinant viruses. (A) Expression of H by MVvac2-H2 in infected cells. Total protein extracts from non-infected cells or cells infected with MVvac2(eGFP)N (-GFP, a control virus expressing a fluorescent reporter from the same locus as the additional H) and MVvac2-H2 (-H2) were probed with antibodies against MV-H, F, N, and actin. Migration of molecular weight standards are indicated to the left. Detection of the immature form of MV F is indicated by an arrowhead. (B) Incorporation of the additional H in viral particles (right panel). Two thousand and five hundred TCID$_{50}$ of sucrose-gradient purified particles of MVvac2, MVvac2(RFP)N (-RFP, a control virus similar to previous) and MVvac2-H2 served as antigenic material in immunoblots using antibodies against H, the His tag, F, and N. (C) Detection of H sol in supernatant of cells infected with MVvac2Hsol. Coomassie stain (left panel) and anti His tag immunoblot (right panel) of immobilized cobalt ion affinity chromatography-purified supernatants of cells infected with MVvac2 or MVvac2-Hsol. Supernatants were collected at the indicated time points after infection. Molecular weight standard positions are indicated to the left.

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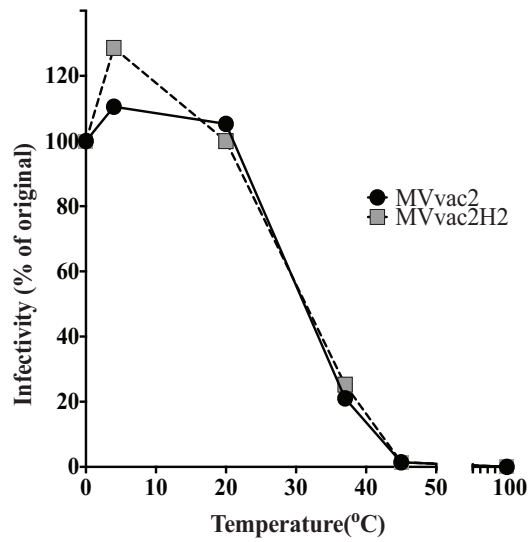
Figure 2.3 Thermostability of MVvac2-H2. Purified particles of MVvac2 or MVvac2-H2 recombinant viruses were incubated at 4, 20, 37, 45, or 96 °C for 1 hour, then infectivity in each sample was determined by end-point dilution assay. Remaining infectivity is expressed as a percentage of the titer of pre-treatment particles. A representative experiment is shown.
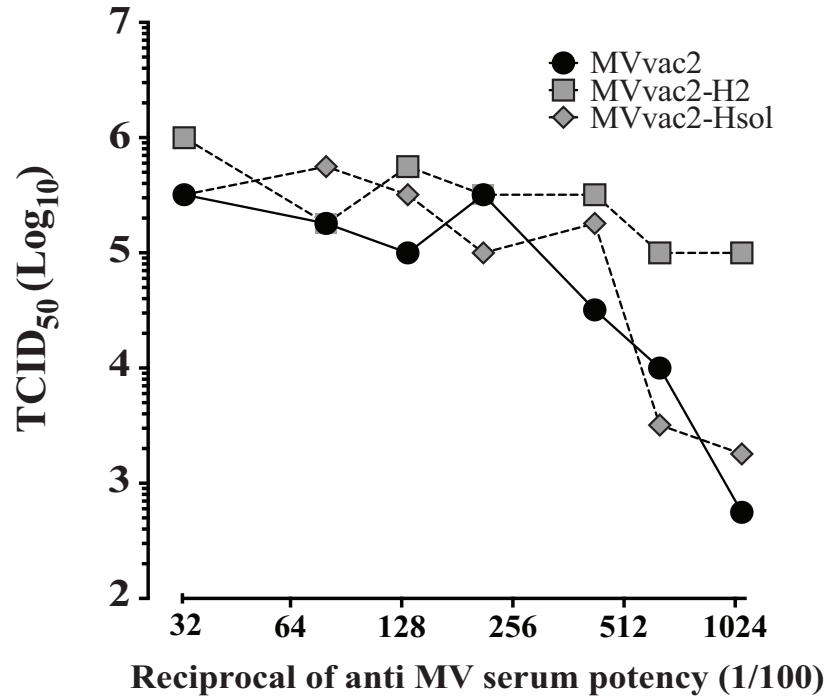
Figure 2.4 Kinetic of neutralization of recombinant MVs by anti-MV polyclonal sera. Standardized titers of MVvac2, MVvac2-H2, or MVvac2-Hsol were incubated at 37 °C for 1 hour with 100-fold dilutions of immune sera of known anti-MV titer from HuCD46Ge-IFNar[KO] mice vaccinated with MVvac2. Anti-MV neutralizing potency ranged in titer from 1:33 to 1:1067 as indicated. Following incubation, the remaining infectivity in each sample was determined in triplicate by end-point dilution assay, and graphed.

A

B

C

D

E

F
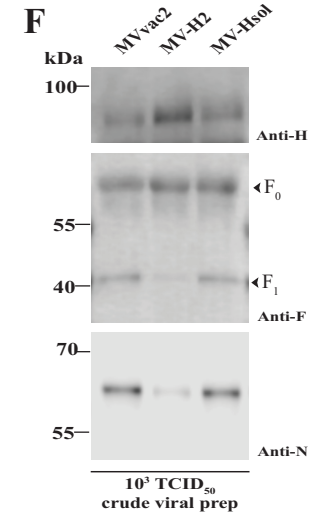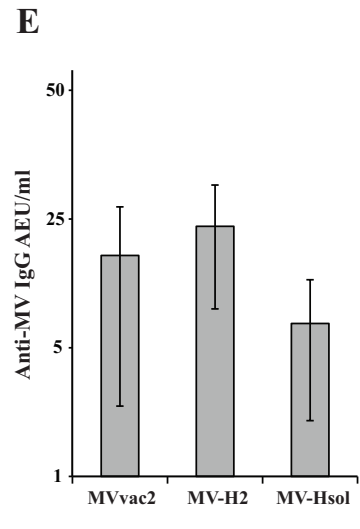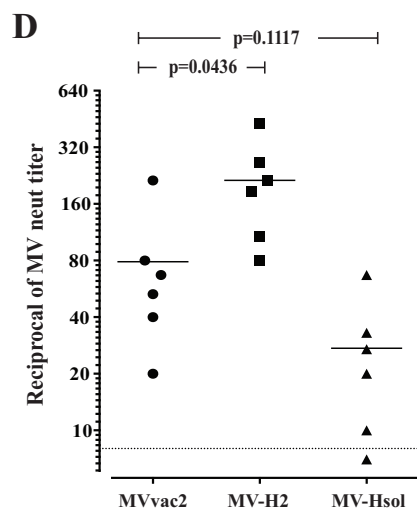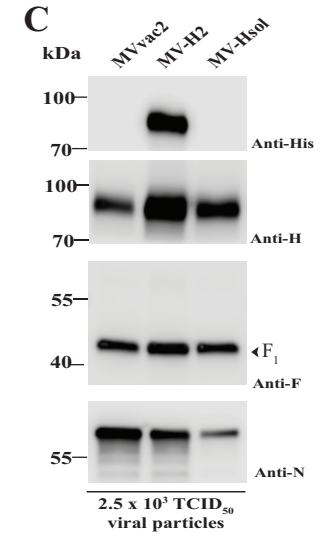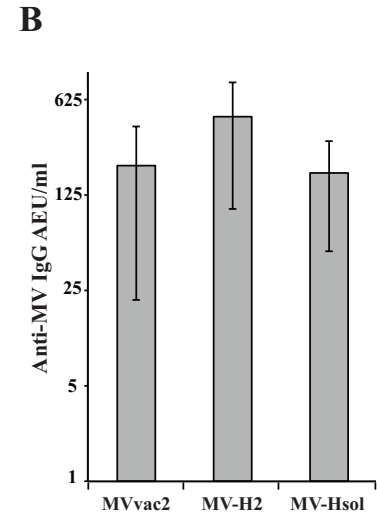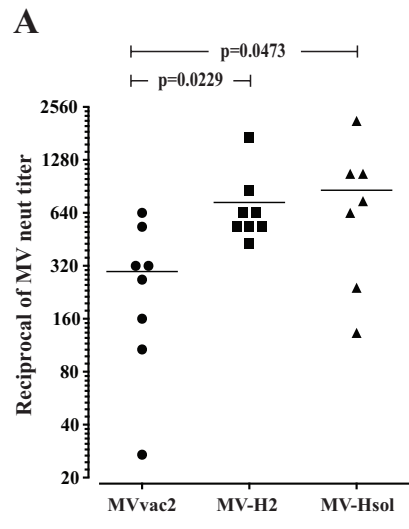
Figure 2.5 Humoral immune responses in outbred and genetically modified mice vaccinated with MVs expressing additional H. (A) Groups of eight CD1 mice received two doses of 10^5 TCID$_{50}$ of purified particles of MVvac2, MVvac2-H2, or MVvac2-Hsol on a 28-day interval. Sera obtained 14 days after the boosting dose were assayed for anti-MV neutralizing immunity. (B) Anti-MV IgG reactivity measured by ELISA, readings were obtained from individual mice; group average and standard deviation from each animal reading are shown. (C) Analysis of the antigenic composition of the viral inocula used for the previous experiment. Viral particles with the indicated infectivity were subjected to SDS-PAGE and analyzed by immunoblot using antibodies with the indicated specificity (D) Groups of six HuCD46Ge-IFNar^{KO} mice received a single dose of 10^3 TCID$_{50}$ MVvac2, MVvac2-H2, or MVvac2-Hsol. Anti-MV microneutralization titers were assessed in sera obtained 28 days post-immunization. (D) Reactivity against MV determined by ELISA as before. (F) Analysis of expression of key MV proteins in viral preparations used for the previous animal experiment. Antigenic material of the same titer used in the animal experiment was subjected to SDS-PAGE, membrane-transferred and probed against the indicated antibody. In (A) and (D), statistical significance of differences in neutralizing immunity was assessed by two-tailed, unpaired t-test. In (B) and (E) AEU, arbitrary ELISA units were quantitatively determined by comparison with an internal MV standard.

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CHAPTER 3

A MEASLES VIRUS WITH INCREASED HEMAGGLUTININ EXPRESSION OUTPERFORMS THE IMMUNOGENICITY OF A MORATEN-EQUIVALENT IN AN INFANT-LIKE MOUSE MODEL.

ABSTRACT

A more immunogenic measles vaccine that could be administered at a younger age would contribute strongly to the stalled effort toward measles eradication. This contribution builds upon previous work from our group reporting the development of a more immunogenic modified measles virus (MV) vaccine, MVvac2-H2, with increased hemagglutinin (H) expression and incorporation in the background of the current vaccine strain. Given that our previous work documented the immunogenicity of MVvac2-H2 in a mature mouse model, we aimed here to test its potential in more infant-like conditions. We show that MVvac2-H2 retains its enhanced immunogenicity when administered in purified particulate form to very young mice. Most interestingly, we show that MVvac2-H2 resists low levels of neutralizing immunity *in vivo*, such that it is not inhibited at concentrations that prove significantly inhibitory for its parental Moraten vaccine-equivalent, and subsequently induces significantly stronger neutralizing antibody titers than that parental vaccine. Finally, toward a more physiological model of maternal anti-measles immunity transfer, we measure vertical acquisition of passive anti-MV immunity in the clinically predictive genetically modified MV-susceptible mouse model.

IMPORTANCE

Despite the use of effective attenuated vaccines, measles still threatens unvaccinated populations, the most numerically significant of which is infants too young to be vaccinated by current means. We expand here on previous work from our group documenting the generation of two MV vaccines in the background of the current vaccine but with enriched production of H. Here, we show that our alternative MV vaccine incorporating additional H is more immunogenic than the current vaccine-equivalent in a very young small animal model and in the presence of artificial passive immunity, the primary limiting factor preventing vaccination of young infants. Our results support our central hypothesis that this virus will prove to be a more effective measles vaccine for young infants, a hypothesis that we are currently testing in a small animal model that combines both relative youth and the presence of natural passive immunity.

INTRODUCTION

Infection by measles virus (MV) continues to have a weighty effect on global health, and particularly on the health of the very young. Despite the use of safe and effective attenuated vaccines against MV for more than 50 years, this extremely infectious virus still killed 114,900 people in 2014, the great majority of them young children. Furthermore, though measles incidence declined drastically over the first four decades of attenuated vaccine use, measles incidence stabilized over the last decade at approximately 20 million cases and 100,000 to 150,000 deaths each year. Part of the continuing impact of MV results from the interplay of the extreme infectiousness of this pathogen, with a basic reproduction number of 15-20 (1), the imperfect effectiveness of current vaccines, and the low reliability of current vaccines in young infants. Indeed, up to 2-10% of recipients may lack protective immunity after vaccination (2). In a further limitation to herd immunity, apart from those individuals who fail to mount a successful response to vaccination, several significant groups are considered ineligible for vaccination by   current methods. Among these, the most numerous is the very young.

Infants below a certain age are considered ineligible for current MV vaccination methods due to their poor rates of seroresponsiveness. In countries with high risk of MV transmission, the vaccine is administered at 9 months of age, when it induces protective immunity in approximately 85% of recipients, while vaccination is delayed until 12 to 15 months of age in countries at low risk of MV transmission, when it reaches its maximal efficiency of approximately 95% responsiveness in a single dose (3, 4). But the highest fatality and complication rates from MV infection occur in infants less than one year of

age (5). A next-generation measles vaccine with a higher rate of responsiveness and that can be administered to younger infants is thus needed.

Previously, our group developed two Moraten-equivalent background modified MVs with added copies of the full-length (MVvac2-H2) or truncated (MVvac2-Hsol) hemagglutinin (H) cistron (6). For MVvac2-H2 in particular, its enhanced immunogenicity in two mouse models suggested that it might improve responses to vaccination, and its marginal resistance to *in vitro* neutralization by low levels of polyclonal immune serum suggested another intriguing possibility. The primary factor that limits successful vaccination of young infants is their variable retention of passive maternal immunity. This humoral immunity, passed across the placenta and in breast milk, protects against early life infection (7), but soon declines to subneutralizing levels that may interfere with vaccination but not with wild-type MV infection (8-11). The maintenance of infectivity by MVvac2-H2 in the presence of low level neutralizing immunity suggested another potential benefit of this modified virus as a new measles vaccine—it might retain immunogenicity in the presence of the maternal immunity that proves limiting for current vaccines.

We show here that MVvac2-H2 is more immunogenic than its parental Moraten-equivalent, MVvac2, in young mice, while the immunogenicity of its counterpart, MVvac2-Hsol, could not be rescued in MV-susceptible mice. Compellingly, in the presence of artificially introduced sub-neutralizing anti-MV titers, mimicking what might be observed in a human infant, MVvac2-H2 induced substantially stronger neutralizing antibody responses than MVvac2, which was significantly inhibited. Finally, we document the transfer of passive anti-MV immunity in the gold standard MV-susceptible

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mouse strain, and suggest its utility for evaluating next-generation infant-targeted MV vaccine candidates.

MATERIALS & METHODS

Cells and viruses. Vero/hSLAM cells served as hosts for growth of viral stocks and for microneutralization assays. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) and also 0.5 mg/mL G418 (Enzo Life Sciences, Farmingdale, NY) to maintain expression of the hSLAM receptor.

Three MVs recombinant strains were used in the experiments presented here. The first, MVvac2 (12), has identical amino acid coding capacity to the Moraten and Schwarz vaccine strains and so serves as a current vaccine-equivalent control. The second, MVvac2-H2, a modified MV with increased expression of the H protein, was constructed by inserting an additional, His-tagged copy of the full-length H coding cistron into an additional transcription unit downstream of the nucleocapsid (N) cistron in the MVvac2 genomic background. The third, MVvac2-Hsol, directs the secretion of a His-tagged, soluble form of the H protein. It is genomically identical to MVvac2-H2 except that its cytoplasmic tail and transmembrane region were swapped by a signal sequence of interleukin-2. The recovery of those strains by reverse genetics and their initial characterization has been described in detail elsewhere (6).

Viruses were amplified by passage at a multiplicity of infection (MOI) of 0.03 in Vero/hSLAM cells incubated at 37°C. When approximately 80% cytopathic effect was observed, cells were scraped in Hank's Balanced Salt Solution (HBSS, Mediatech,

Manassas, VA) with 1% FBS, and viral particles were released by two freeze-thaw cycles. Viral titer was determined as 50% tissue culture infectious dose ($TCID_{50}$) in Vero/hSLAM by the Spearman-Kärber end-point dilution method (13).

Mouse inoculations and sera transfers. All experimental procedures were performed according to a protocol approved by the Arizona State University Institutional Animal Care and Use Committee.

Immunogenicity of purified particles in young mice. To determine the immunogenicity of viral particles after a single human-standard titer dose in young MV-susceptible mice, groups of three-week-old HuCD46Ge-IFNar[KO] mice (14) were inoculated by the intraperitoneal (i.p.) route with a viral particle dose of 10^3 $TCID_{50}$. Mice were bled 28 days after inoculation and sera were separated and heat-inactivated. To test whether a second standard titer dose of viral particles boosted responses, groups of approximately five-week-old HuCD46Ge-IFNar[KO] mice were initially inoculated by the i.p. route with 10^3 $TCID_{50}$ of purified viral particles in HBSS with 1% serum. Four weeks after the initial inoculation, mice received a similar booster dose. Two weeks after the second dose, mice were bled, and sera were separated and heat-inactivated.

Immunogenicity in the presence of artificial passive immunity. To artificially model the passive anti-MV immunity that hinders vaccination of young infants, we introduced dilute MV-immune serum to young HuCD46Ge-IFNar[KO] mice and subsequently inoculated these mice with MVs. Strongly neutralizing serum, with an anti-MV neutralization titer of 1:3413, was obtained from a HuCD46Ge-IFNar[KO] mouse immunized with two 10^5 $TCID_{50}$ MVvac2 vaccine vector doses. This strongly neutralizing serum was diluted 1:300 in HBSS with 1% FBS for a final neutralization

potency of approximately 1:10. As a control, non-immune serum derived from a naïve HuCD46Ge-IFNar^{KO} mouse was diluted and inoculated by the same procedure. Five-week-old mice received 500 μL of the diluted anti-MV or the control serum via the i.p. route. One day after artificial introduction of passive immunity, the mice that received anti-MV serum were bled to measure anti-MV passive immunity present at the time of vaccination. Mice were inoculated by the i.p. route with a 10^5 TCID$_{50}$ dose of MVvac2 or MVvac2-H2, or with vehicle alone. All mice were terminally bled 28 days after inoculation of viruses or mock to determine the immunogenicity of the viruses in the presence of artificial passive immunity. Sera were separated and decomplemented at 56°C for 1 h.

Kinetic of anti-MV maternal passive immunity transfer. The kinetic of anti-MV maternal passive immunity transfer and waning in HuCD46Ge-IFNar^{KO} mice was determined by comparing serous anti-MV neutralization titers of MV-immune dams to those of their two-, three-, and four-week-old pups. Initially, a single six-week-old female was inoculated with a 10^5 TCID$_{50}$ i.p. dose of an MVvac2 vaccine vector and mated. She bore two litters. Anti-MV titers in pup sera were assessed for the first litter when they reached four weeks of age and for the second litter when they reached two weeks of age. For this group, pup titers were expressed both as their absolute value of reciprocal neutralization titer, and as a ratio compared to the immunity in their dam. To obtain an additional time point, 10^5 TCID$_{50}$ of MVvac2 were inoculated by the subcutaneous route to three additional six-week-old females, which were subsequently mated. Neutralizing immunity was assessed at three weeks of age in pups of these immune dams and expressed as a ratio of their dam's titer.

MV microneutralization assay. In 96-well plates, serial two fold dilutions of heat-inactivated sera in HBSS with 1% FBS were incubated with 100 $TCID_{50}$ of MVvac2 in a volume of 100 μL for 1 h at 37°C. An equal volume of DMEM with 5% FBS containing 1 x 10^4 Vero/hSLAM cells was added to the serum-virus mixture in each well and the plates were incubated at 37°C for three days. Microneutralization titer was determined as the highest dilution of sera affording elimination of infectivity, defined as the absence of syncytia. Titers were reported as the average of a determination made in triplicate.

RESULTS

MV incorporating additional H is more immunogenic in young mice. Previously, a modified MV with increased expression and incorporation of the full-length H protein, termed MVvac2-H2, proved significantly more immunogenic than the parental vaccine MVvac2 in mature (6-8 week old) outbred, non-susceptible CD1 mice and in mature genetically modified MV-susceptible HuCD46Ge-IFNar[KO] mice, eliciting approximately 2.5-fold higher neutralization titers than those elicited by MVvac2 in both animal models (6). On the other hand, MVvac2-Hsol, a modified MV that vectors a soluble form of H from a high expression locus, was significantly more immunogenic than MVvac2 in non-susceptible mice but slightly less immunogenic in MV-susceptible mice. We initially hypothesized that differences in the vaccination schedule used for each animal model accounted for the observed difference in the immunogenicity of MVvac2-Hsol in the two mouse models; MV-susceptible mice received a single standard titer dose (10^3 $TCID_{50}$) of viral preparation, while non-susceptible mice received two high titer doses (10^5 $TCID_{50}$) of purified viral particles.

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To assess whether the immunogenicity of MVvac2-Hsol could be rescued in the more relevant MV-susceptible model by the use of purified viral particles rather than a crude viral preparation, we inoculated three-week-old HuCD46Ge-IFNar^{KO} mice with a single dose of 10^3 TCID$_{50}$ of purified viral particles. Four weeks later, we documented neutralizing immunity in sera of individual animals by MV neutralization assay. Titers followed the same trend as observed previously using the same immunization schedule but with crude viral preparations, with MVvac2-H2 inducing neutralization titers 2.13-fold higher than those induced by MVvac2 (1:117 for MVvac2 vs. 1:249 for MVvac2-H2 in average, Fig. 3.1) and MVvac2-Hsol inducing neutralization titers almost three times lower than those induced by MVvac2 (1:117 for MVvac2 vs. 1:40 for MVvac2-Hsol). One animal with an undetectable neutralization titer (<1:10) was observed in both the MVvac2-H2 and MVvac2-Hsol groups. At this point, it is unclear whether these outliers represent non-takers of the vaccine or whether the viruses were indeed able to replicate, but the animals failed to mount a neutralizing response. If the outlier is excluded from the MVvac2-H2 group, the difference in induction of neutralizing immunity between this virus and MVvac2 increases to 2.55-fold (now 1:299 for MVvac2-H2 in average) and reaches statistical significance ($P = 0.0354$, two-tailed, unpaired t test), while the difference is not significant if the outlier is included ($P = 0.1248$). For the MVvac2-Hsol group, exclusion of the outlier results in a non-significant difference in immunogenicity compared to MVvac2 (now 1:50 for MVvac2-Hsol in average, $P = 0.0599$), while this animal's inclusion results in a significant difference ($P = 0.0383$). Though the statistical importance of these results thus remains undefined, this nonetheless documents approximately equivalent differences in immunogenicity using either inocula prepared

from purified viral particles or from cell lysates in MV-susceptible mice, with induction

of neutralizing antibody responses more than 2-fold higher by MVvac2-H2 and nearly 3-

fold lower by MVvac2-Hsol than those induced by parental strain. We thus rejected our

hypothesis that use of purified viral particles could alone improve the immunogenicity of

MVvac2-Hsol in a single standard titer dose in susceptible mice. Furthermore, the

difference in age between animals with neutralizing titers documented here (three weeks)

and in previous experiments (six weeks) (6) demonstrates that even relatively young mice

develop higher neutralization titers in response to MVvac2-H2 than in response to

MVvac2.

　　　　We next assessed whether the use of two doses of purified particles could boost

responses against MVvac2-Hsol. Groups of mice received two doses of 10^3 TCID$_{50}$ of

purified particles of MVvac2, MVvac2-H2, or MVvac2-Hsol on an interval of 28 days.

Two weeks after the boosting dose, mice inoculated with MVvac2 or MVvac2-Hsol

developed comparable levels of neutralizing immunity (1:173 vs. 1:152, respectively, $P =$

0.7874). The use of two doses of particles in MV-susceptible mice thus improved

neutralizing responses induced by MVvac2-Hsol in most mice to match those induced by

MVvac2, but failed to improve the immunogenicity of MVvac2-Hsol to the level

observed in non-susceptible mice. Mice that received MVvac2-H2 developed

neutralization titers 2.53-fold higher (1:437 in average) than those documented in

MVvac2 recipients, with the notable caveat that neutralizing responses from two animals

in the MVvac2-H2 group were undetectable by our neutralization assay. As above, it is

currently not clear whether these animals were non-takers or failed to develop

neutralizing immunity, but the presence of non-neutralizing immunity could be assessed by anti-MV ELISA.

Taken together, these results suggested that the difference in induction of neutralizing immunity by MVvac2-Hsol in non-susceptible versus MV-susceptible mice did not result from differences in the vaccine schedules and inocula used in each animal model. Given that we could not reproduce the enhanced immunogenicity of MVvac2-Hsol observed in non-susceptible mice in the MV-susceptible model, we focused further testing in this latter, more relevant model on MVvac2-H2, which demonstrated consistent enhanced immunogenicity.

MV incorporating additional H is more immunogenic in the presence of artificially introduced, low level, anti-MV passive immunity. In previous work from our group, MVvac2-H2 proved more resistant than did MVvac2 to diluted strongly neutralizing serum *in vitro*. For example, when both viruses were incubated with a 100-fold dilution of polyclonal mouse serum with a microneutralization titer of 1:1066, thus generating a solution with approximate anti-MV titer of 1:10, MVvac2 retained less than 0.1% of its infectivity, while MVvac2-H2 retained approximately 10% of its original infectivity (6). Based on these results and its performance in mouse models, as discussed above, we hypothesized that MVvac2-H2 would better induce humoral immunity at protective levels than would MVvac2 in the presence of sub-protective, low level neutralizing immunity. Specifically, we predicted that MVvac2-H2 would induce higher neutralizing titers than MVvac2 would in the presence of passive immunity. To initially test this, we sought to artificially introduce *in vivo* enough neutralizing serum potential to allow us to discern between the infectivity retention of MVvac2 and MVvac2-H2. We

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aimed to introduce an approximate titer of 1:10 anti-MV in HuCD46IFNar^{KO} mice by inoculating diluted MV-immune serum to the animals. The day after administration of passive immunity, three experimental groups consisting of two (mock), seven (MVvac2), or eight (MVvac2-H2) mice were bled and then received a single intraperitoneal dose of 10^5 TCID$_{50}$ virus or vehicle alone. A control group of mice received diluted non-immune sera the day prior to inoculation with either MVvac2 (seven animals) or MVvac2-H2 (seven animals).

As expected, the passive anti-MV serum became further diluted within the mouse system so that anti-MV titers in sera obtained from mice on the day of viral inoculation fell below the limit of detection by our microneutralization assay (<1:4). Though only sub-neutralizing anti-MV passive immunity was thus introduced, this passive immunity nonetheless interfered with vaccination by MVvac2. Mice vaccinated with MVvac2 in the presence of anti-MV artificial passive immunity developed 17-fold lower neutralizing titers on average (1:43) than those vaccinated with MVvac2 in the presence of control non-immune artificial passive immunity (1:731), a difference that was highly statistically significant ($P = 0.0005$, Fig. 1). On the other hand, while mice that received MVvac2-H2 in the presence of anti-MV artificial passive immunity developed 1.86-fold lower titers on average (1:242) than those that received MVvac2-H2 in the presence of the irrelevant sera (1:449), the difference did not reach statistical significance ($P = 0.0749$). Furthermore, in the presence of anti-MV immunity, MVvac2-H2 induced significantly stronger, 5.63-fold higher neutralizing titers than MVvac2 induced (1:242 vs. 1:43, respectively, $P = 0.0375$). Taken together, these results show that even very low levels of passive anti-MV immunity strongly inhibit the induction of active humoral immunity by

MVvac2. This low-level passive anti-MV immunity proved insufficient, however, to significantly interfere with vaccination by MVvac2-H2, though neutralization titers of animals inoculated with MVvac2-H2 in the presence of passive immunity trended slightly downward from those inoculated in the presence of dilute non-immune serum. MVvac2-H2 thus elicits significantly higher neutralizing antibody titers than MVvac2 does in the presence of artificial passive anti-MV immunity. Interestingly, in the presence of the irrelevant non-immune sera, MVvac2 induced slightly higher neutralization titers than MVvac2-H2 (averages of 1:731 compared to 1:449, respectively, p=0.0500). We have also observed this trend, not reaching statistical significance, in another MV-susceptible mouse model simply immunized with 10^5 $TCID_{50}$ virus without prior introduction of homologous sera and bled 28 days after inoculation (averages of 1:516 and 1:354 for MVvac2 and MVvac2-H2, respectively, p=0.3949).

MV-susceptible mice transfer maternal anti-MV passive immunity from dam to pup. While the increased performance of MVvac2-H2 in the presence of artificially introduced passive immunity signals the promise of this candidate as an alternative pediatric MV vaccine, we seek to evaluate the modified virus in a more physiologically relevant mouse model. Specifically, we seek to test the immunogenicity of MVvac2-H2 in the presence of natural passive anti-MV immunity, transferred from dam to pup. Though we had every expectation that this transfer should occur in HuCD46Ge-IFNar^{KO} mice, it has not been documented before, to our knowledge. We thus aimed to detect and quantify maternally-derived humoral immunity in pups of MV-immune HuCD46Ge-IFNar^{KO} dams.

Initially, a single female mouse received 10^5 TCID$_{50}$ of an MVvac2 derivative and was co-housed with a male of the same strain. The female became pregnant and gave birth to two successive litters, which were analyzed at four and two weeks of age, respectively, to assess anti-MV passive immunity. Neutralizing immunity was also assessed in the dam at the time of collection of sera from the second, two-week-old litter. At two weeks of age, we documented neutralization titers in pups an average of 1.98-fold higher than those documented in their dam (1:133 on average vs. 1:67, respectively) (Fig. 3.3A, B). By four weeks of age, neutralizing immunity in pups declined to a level similar to that in their dam (1:61 on average vs. 1:67, respectively, 0.91-fold difference).

To obtain another, intermediate time point for measuring the waning of maternal passive immunity in this animal model, three additional female mice received 10^5 TCID$_{50}$ of MVvac2 and were co-housed with males of the same strain. The females became pregnant and each gave birth to a single litter. Neutralizing titers against MV were measured at three weeks of age in two pups from each litter, and anti-MV titers were also determined for their dams at the same time. Given that dams displayed a range of neutralization titers against MV (1:160, 1:267, and 1:320), pup versus dam titers for this second group were expressed only as the ratio of pup over dam titer (Fig. 3.3B). On average, neutralization titers of three-week-old pups were 1.21-fold higher than those of their dams.

Altogether, MV-immune HuCD46Ge-IFNar^{KO} dams thus passively transmit their own anti-MV humoral immunity to their pups. The efficiency of this transfer is such that passive immunity accumulates to levels almost twice as high as in dams when pups reach two weeks of age. Over the next two weeks of life, passive maternal immunity in pups

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declines so that it approximates the level of immunity in their dams when pups reach four weeks of age.

DISCUSSION

We show here that, consistent with our previous findings in older mice, young mice respond more strongly to MVvac2-H2 than to its parental current vaccine-equivalent, MVvac2. Remarkably, in the presence of low levels of artificially introduced passive immunity, responses of MV-susceptible mice to MVvac2 are significantly inhibited, while those to MVvac2-H2 are not, and MVvac2-H2 thus induces significantly stronger neutralizing antibody responses than MVvac2 in the presence of this passive immunity. Finally, we show that female MV-susceptible HuCD46Ge-IFNar[KO] mice efficiently transfer anti-MV passive immunity to their pups. These mice are already considered the gold-standard small animal model for MV vaccine immunogenicity. Remarkably, data obtained from this mouse model of MV immunogenicity was recently corroborated in Phase I clinical trials (15, 16). Our results presented here further suggest the utility of HuCD46Ge-IFNar[KO] mice as a model for preclinical evaluation of experimental pediatric vaccines against measles.

In this and our previous work, we have shown that MVvac2-H2 can induce titers of neutralizing antibodies more than 2-fold higher than those induced by MVvac2 in both non-susceptible and MV-susceptible mice, at young (3 weeks) or older (6-8 weeks) ages, delivered in purified particulate or crude preparation forms, in one or two doses. Intriguingly, thus far the apparent single exception to the relatively enhanced immunogenicity of MVvac2-H2 is its administration in a high titer dose (10^5 TCID$_{50}$) to MV-susceptible mice. Recipients of high titer MVvac2-H2 developed neutralizing

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responses approximately 1.5-fold lower than those of MVvac2 recipients in this (Fig. 3.2, Non immune serum groups) and another MV-susceptible mouse model (Julik and Reyes-del Valle, unpublished observation). Furthermore, while increasing the dosage of MVvac2 inoculated to HuCD46Ge-IFNar[KO] mice drastically increased the neutralization titers elicited, the increase is much more modest for MVvac2-H2. Use of 10^5 rather than 10^3 TCID$_{50}$ of MVvac2 increased neutralization responses nearly 10-fold from an average of 1:79 (my paper) to an average of 1:731 (Fig. 3.2, Non immune serum groups), while the same increase of viral dosage for MVvac2-H2 increased responses by only approximately 2-fold from an average of 1:214 (my paper) to one of 1:449 (Fig. 3.2, Non immune serum groups). While this difference in increase results partially from the enhanced immunogenicity of 10^3 TCID$_{50}$ of MVvac2-H2 compared to MVvac2, it nonetheless points to a different dynamic range of induction of neutralizing immunity between MVvac2 and MVvac2-H2, where a single high titer dose of an MV vector with increased H expression paradoxically is less immunogenic. As mentioned, this effect disappears when the single dose is reduced by two orders of magnitude. It remains of great interest to us to test MVvac2-H2 at a medium titer dose, such as 10^4 TCID$_{50}$. Furthermore, the presence of multiple animals with undetectable neutralizing titer in the groups of mice that received MVvac2-H2 and MVvac2-Hsol in the experiments presented in Fig. 3.1 suggests that the genetic stability of these viruses should be defined by sequencing viral genomes isolated after *in vivo* passage.

Previously, we documented a perplexing difference in the immunogenicity of MVvac2-Hsol in outbred CD1 mice, where this virus induced significantly stronger neutralizing antibodies than did MVvac2, as compared to in HuCD46Ge-IFNar[KO] mice,

where MVvac2-Hsol induced antibody titers on a downward trend from those induced by MVvac2 (6). The difference in vaccination schema for non-susceptible CD1 mice and MV-susceptible HuCD46Ge-IFNar^{KO} could have precipitated this difference; non-susceptible mice received two doses of 10^5 TCID$_{50}$ of purified particles, while susceptible mice received a single dose of 10^3 TCID$_{50}$ of a crude viral preparation. To test this, we initially changed the nature of the inoculated material, measuring neutralizing responses of MV-susceptible mice to 10^3 TCID$_{50}$ of purified particles. We documented the same trend in immunogenicity as was observed when crude viral preparations of the same dose were inoculated to these mice, with MVvac2-H2 inducing stronger antibody responses and MVvac2-Hsol inducing slightly weaker neutralizing responses than MVvac2, and thus discarded the use of purified particulate material as the origin of the enhanced immunogenicity of MVvac2-Hsol in non-susceptible mice. Similarly, the administration of two doses of 10^3 TCID$_{50}$ of purified viral particles to MV-susceptible mice boosted responses to MVvac2-Hsol to approximate those to MVvac2, on average, but could not recapitulate the enhanced immunogenicity of the modified virus that was observed in non-susceptible mice. These doses, of course, were lower than those administered to the non-susceptible animals. Even inoculation of 10^5 TCID$_{50}$ of a crude preparation of MVvac2-Hsol to susceptible mice, however, could not improve their responses to match those mounted in response to MVvac2 (1:208 vs. 1:725, respectively, Julik and Reyes-del Valle, unpublished observation). We found these data sufficient to trust that MVvac2-Hsol is slightly less immunogenic than its parental strain in HuCD46Ge-IFNar^{KO} mice and, given that results in this mouse model have proven predictive for vaccine performance in clinical trials, we would expect MVvac2-Hsol to also induce slightly

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lower neutralizing antibody titers in humans in the absence of passive immunity. It is important to note that observations in these animals do not necessarily predict vaccine performance in animals with passive immunity. For example, recombinant modified vaccinia virus vectoring the MV F and H proteins was less effective than a control in older macaques, but performed better than that control in infant macaques with passive immunity, where responses to the current vaccine were significantly inhibited (17). We nonetheless focused our efforts on MVvac2-H2, whose enhanced immunogenicity made it a stronger next-generation vaccine candidate.

Remarkably, in the presence of artificially introduced anti-MV passive immunity in the form of dilute homologous polyclonal immune serum, MVvac2-H2 elicits significantly stronger neutralizing immunity than MVvac2 elicits. The level of anti-MV passive immunity in mice on the day of viral inoculation fell below the limit of detection of our neutralization assay; we are working to more precisely define this level of immunity by a more sensitive logarithmic neutralization index approach. Nonetheless, passive immunity to MV was clearly successfully introduced, as neutralization responses to MVvac2 in the presence of anti-MV passive immunity were strongly significantly inhibited compared to responses in the presence of irrelevant artificial passive immunity. Responses to MVvac2-H2 were also slightly lower in the presence of anti-MV passive immunity compared to in the presence of non-immune serum, but the reductive effect on MVvac2-H2 was more than ten times less than the effect on MVvac2 and did not reach statistical significance. The slight downward trend of the immunogenicity of MVvac2-H2 in the presence of anti-MV passive immunity suggests that this modified virus is not without limitation in its performance in the presence of neutralizing passive immunity.

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Similarly, however, the much higher neutralization titers elicited by MVvac2-H2 than by MVvac2 suggest that the modified virus has also not reached its limit in terms of resistance to neutralizing immunity. It remains of interest to us to test the range of resistance of MVvac2-H2 to passive immunity *in vivo* by repeating this experiment in mice with different levels of artificially introduced neutralizing immunity. In this era of vaccination, however, when only approximately 40% (18) of infants have detectable maternal immunity by 4 months of age, and the neutralization titer of such immunity is of a low level correspondent to its waning, we may argue that vaccine performance in the presence of faded immunity is the most clinically relevant indicator of potential success in young infants.

The passive transfer of maternal immunity has been described in the cotton rat model of measles infection (19). To our knowledge, however, we present here the first report of the dam-to-pup transfer of passive maternal immunity to MV in HuCD46Ge-IFNar[KO] mice. While unsurprising, this result is significant nonetheless, given the proven preclinical utility of this mouse model for testing MV vector vaccine responses. These results suggest the further utility of this model for testing responses to experimental infant-targeted MV vaccines. Certainly, there are known differences in the transfer of passive maternal immunity in mice as compared to humans. For example, human maternal immunity is transferred primarily across the placenta, and subisotype IgG1 is transferred most efficiently, while for mice, maternal antibodies are transferred primarily in breast milk (20, 21). Maternal immunity also accumulates and fades on a slightly different kinetic in humans compared to mice. Studies of the fading of human maternal immunity measured by neutralization titer have suggested an average half-life of

approximately 47 days (4). Our data suggest a more rapid half-life for the waning of neutralizing maternal immunity in these mice of approximately 14 days. Notably, in the cotton rat model where maternal antibody titers were also measured by neutralization assay (19), maternal immunity also appears to fade on a more rapid kinetic, similar to our observations. In these mice, neutralizing passive maternal immunity accumulated in pups to levels at least 1.98-fold higher than in their dams. While it is not clear whether this represents the absolute maximal accumulation of maternal antibodies in MV-susceptible mice, it nonetheless reflects observations in certain human populations, where maternal antibodies accumulate in newborns to levels 1.8-fold higher than in their mothers (18). Though the maximal accumulation of maternal immunity is not yet defined in mice, to our knowledge, the transfer of maternal immunity against hemorrhagic fever with renal syndrome virus from dam to pup was observed to peak at two weeks of age and had entirely disappeared by eight weeks of age in rats (22). In preliminary experiments, we also observed that maternal immunity can significantly inhibit pup responses to MVvac2 (Julik and Reyes-del Valle, unpublished observations).

In sum, these data show that MVvac2-H2 maintains its enhanced immunogenicity in young MV-susceptible mice and in the presence of low levels of passively transferred anti-MV neutralizing antibodies. These two key observations support our central hypothesis that MVvac2-H2 will perform better than current MV vaccines in young infants. We expect this to prove true both in young infants retaining passive maternal immunity, where MVvac2-H2 may overcome the inhibitory effect of this immunity on vaccine responses, and in young infants lacking maternal immunity, where the enhanced immunogenicity of this vaccine at a standard titer dose will boost protective

neutralization responses. We also present a new twist on a proven mouse model for MV vaccination. The passage of neutralizing maternal antibodies in MV-susceptible mice will allow preclinical assessment of the performance of MVvac2-H2 in the presence of passive immunity. We anticipate that experiments presented here and ongoing experiments in our mouse model of natural passive antibody transfer will lay additional foundation for future evaluation of MVvac2-H2 in the most relevant animal model for MV, the macaque, where features like potential reactogenicity and protection from challenge can be assessed in addition to measuring immunogenicity.

ACKNOWLEDGMENTS
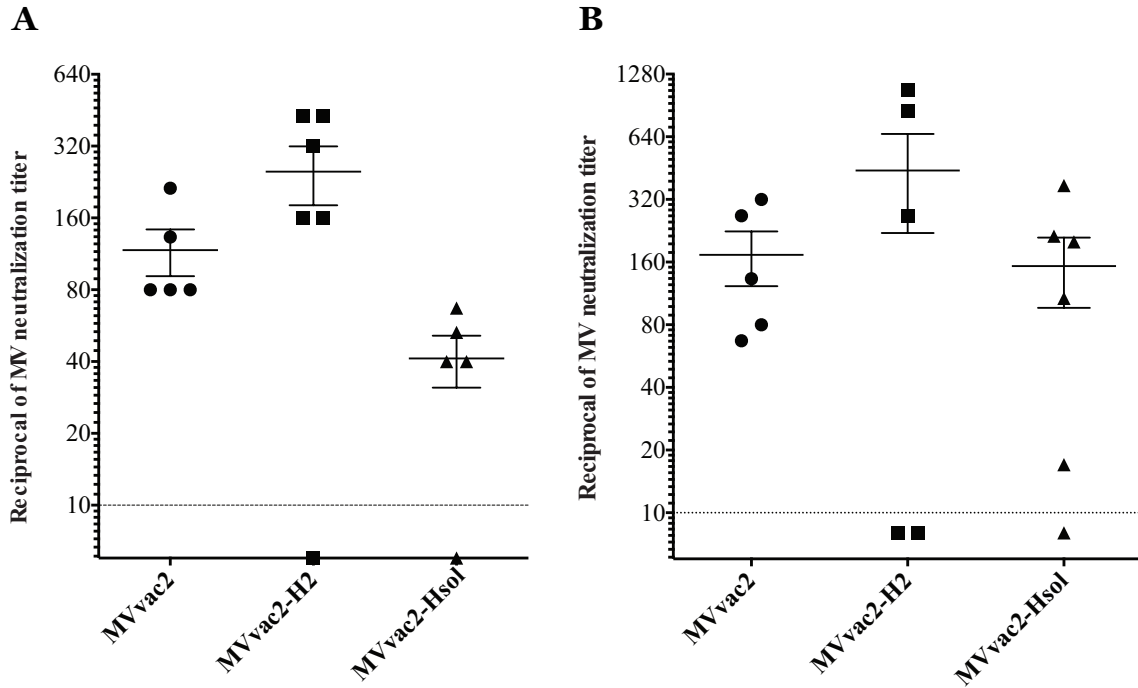
Figure 3.1 Humoral responses of genetically modified mice vaccinated with one or two standard doses of purified particles. (A) Groups of five to six HuCD46Ge-IFNar[KO] mice received a single dose of 10^3 TCID$_{50}$ of purified particles of MVvac2, MVvac2-H2, or MVvac2-Hsol and sera were assayed for anti-MV neutralizing immunity 28 days later. (B) Groups of five to six HuCD46Ge-IFNar[KO] mice received two doses of 10^3 TCID$_{50}$ of purified particles of MVvac2, MVvac2-H2, or MVvac2-Hsol on a 28-day interval. Sera were obtained and assayed for anti-MV neutralizing immunity 14 days after the boost. Dashed line indicates limit of detection.
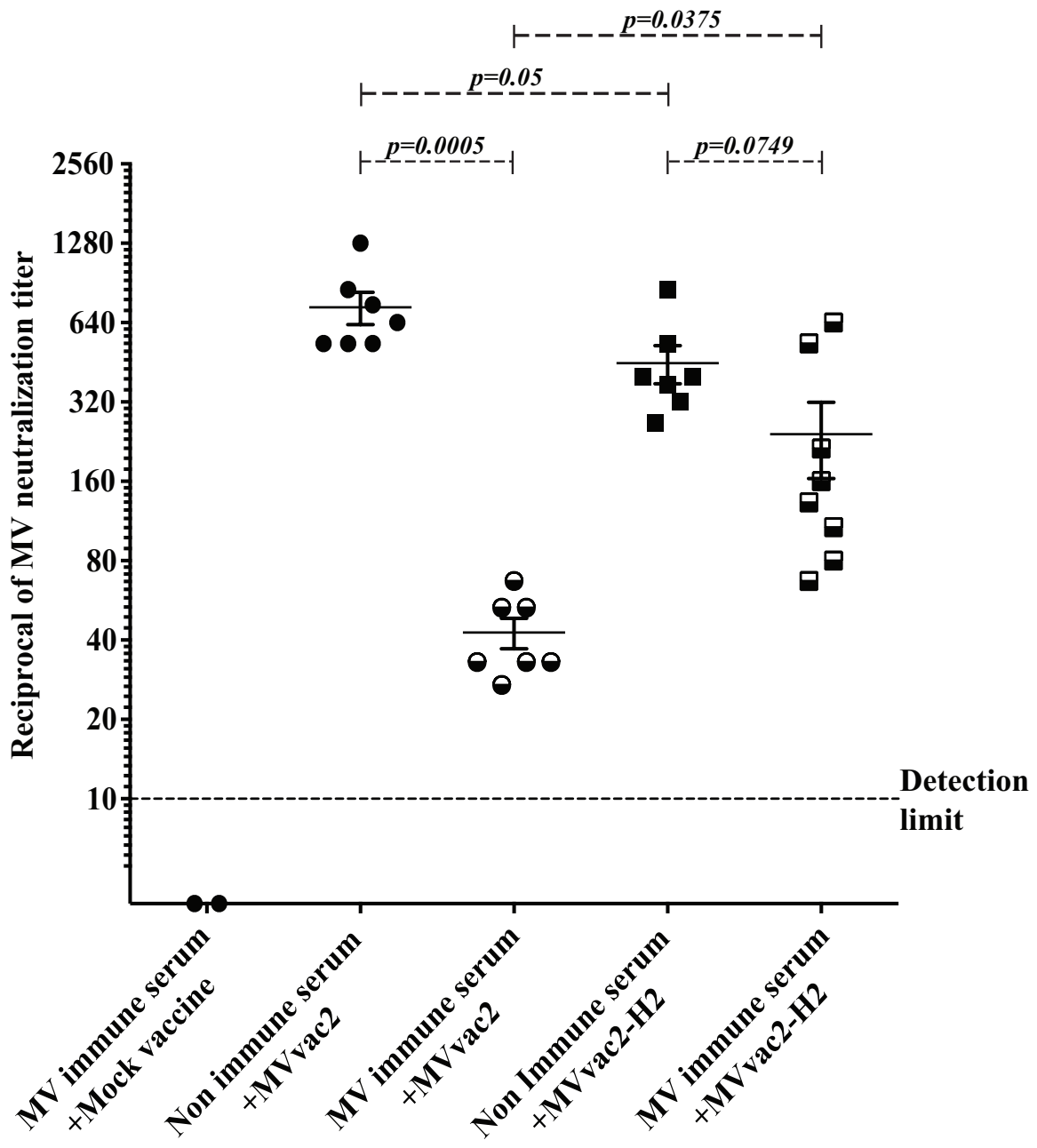
Figure 3.2 Humoral responses of genetically modified mice vaccinated with MV expressing additional H in the presence of artificially introduced passive anti-MV immunity. Groups of seven to eight HuCD46IFNar^{KO} mice received 0.5 mL of diluted sera obtained from an MV-naïve mouse of the same strain (Non immune serum) or from a strongly MV-immune mouse of the same strain (MV immune serum). One day later, the mice received a single dose of 10^5 TCID$_{50}$ MVvac2 or MVvac2-H2; an additional control group consisting of two mice that received MV immune serum the previous day were inoculated with vehicle alone (Mock vaccine). Twenty-eight days after the viral or mock vaccine inoculations, sera were obtained and assayed for anti-MV neutralizing immunity. Anti-MV microneutralization titers were assayed in the sera obtained on the day of viral inoculation and also in sera obtained 28 days later. Microneutralization titers obtained on the day of inoculation fell below the limit of detection by our test (<1:4). Dashed line indicates limit of detection.
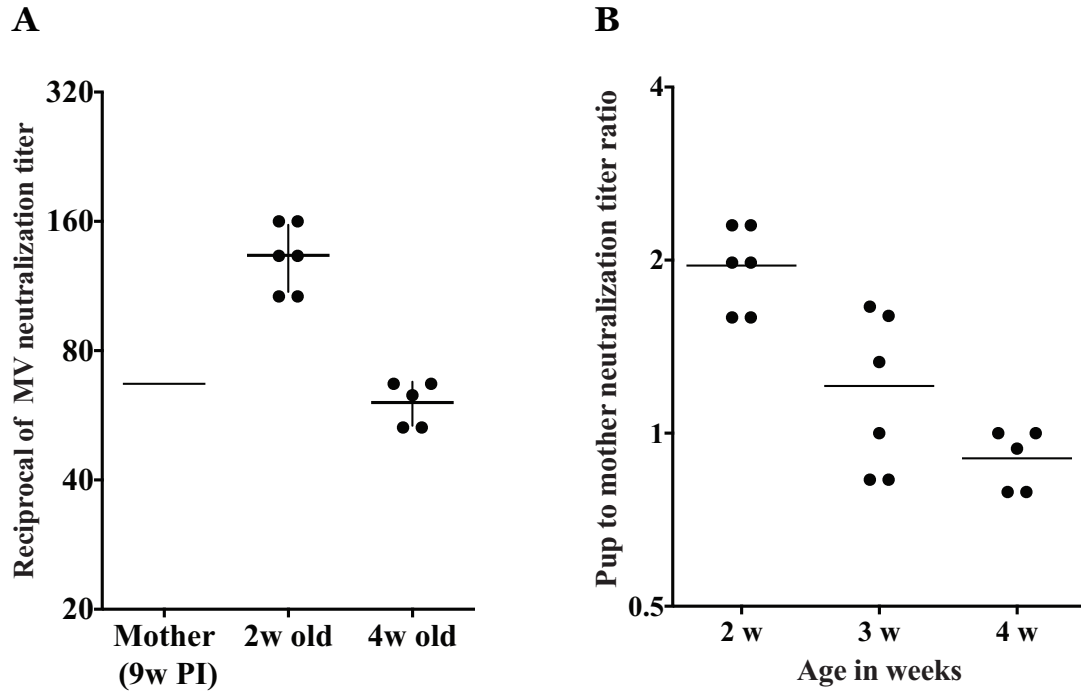
Figure 3.3 Transfer of maternal anti-MV humoral immunity from dam to pup in HuCD46IFNar^{KO} mice. (A) A single HuCD46Ge-IFNar^{KO} female was vaccinated with an MVvac2 vector and mated to a male of the same strain. Sera were obtained from sequential litters from this dam at two or four weeks of age to assay anti-MV immunity, reported as the reciprocal of neutralization titer. Serum obtained from the dam was also assayed for comparison. (B) Pup anti-MV neutralization titers from (A) are reported as the ratio of pup to mother neutralization titer. To obtain an additional time point, three additional HuCD46Ge-IFNar^{KO} females were vaccinated with MVvac2 and mated to a male of the same strain. Sera obtained from pups when they reached three weeks of age were assayed for anti-MV neutralization, expressed at the ratio of pup to mother neutralization titer. w, weeks; PI, post-immunization.

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CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

All in all, this work explores the competence of measles virus for enhanced expression of its hemagglutinin and the consequences of this modification. In Chapter 2, I describe the generation of two novel modified MVs with increased expression of incorporated, full-length or soluble, truncated H glycoprotein. The first recombinant MV is able to incorporate additional H protein to levels up to three-fold higher than observed in its parental strain, and this additional H incorporation likely accounts for the relative resistance of this modified virus to neutralization *in vitro* by immune serum without impacting its thermostability. The second recombinant MV is capable of secreting H glycoproteins in a measurable manner. I describe also the increased immunogenicity of these vectors with higher H expression in non-susceptible and, for the virus with increased H incorporation, in MV-susceptible mice. In Chapter 3, I document that the vector with higher incorporation of full-length H is more immunogenic than its parental strain in MV-susceptible mice at young ages and in the presence of artificially introduced passive immunity. These two features recapitulate barriers that prevent successful immunization of young infants using current MV vaccines, and constitute a major hurdle towards measles eradication. I additionally present measurements of the transfer of neutralizing maternal immunity in the gold standard MV-susceptible small animal model, and suggest the value of this genetically-modified mouse for ongoing and future evaluation of alternative pediatric MV vaccines that can circumvent maternal immunity either by enhanced immunogenicity or by surpassing the waning maternally-acquired

immunity that contraindicates the administration of current measles vaccine to very young infants.

This effort intrinsically depended on the robustness of the measles virus reverse genetics system, which has provided a platform for production of recombinant and modified MVs (1), as well as a full-length, infectious cDNA encoding a Moraten-equivalent genome with engineered unique restriction sites for insertion of additional coding sequence (2). Immunogenicity studies presented in Chapters 2 and 3 also depended upon the availability of a reliable and relevant small animal model for preclinical evaluation of MV vaccines, the HuCD46Ge-IFNar[KO] mouse (3).

This dissertation contributes to the field of vaccinology in three clearly discernible ways. First, while enhancement of glycoprotein expression has been reported for other non-segmented negative sense RNA viruses, this work represents the first such report for MV. Measles thus joins rabies, vesicular stomatitis, and respiratory syncytial viruses among vectors of the *Mononegavirales* that are amenable to such manipulation. For RV and VSV as for MV, increasing full-length glycoprotein expression can also improve immunogenicity (4-6), suggesting that this strategy might be more broadly applied among attenuated *Mononegavirales* vaccines to improve immune responses. This group of viruses has members that represent very important human pathogens. Nipah and Hendra viruses are the cause of lower respiratory-tract infections with high mortality and epidemic potential (7). Parainfluenza and Metapneumoviruses are the most frequent causes of viral pneumonia for children and adults (8, 9). Intriguingly, among the vectors whose glycoprotein expression has been increased by similar means as reported in this dissertation, increasing expression of the glycoprotein (G) of RSV failed to increase

either higher G-specific IgG or neutralizing responses, and increasing its fusion (F)

protein expression similarly failed to increase neutralizing responses (10). As RSV

natively produces an soluble version of its G protein through alternative initiation of

translation (11), and we similarly observed relatively inefficient induction of immunity

by our modified MV vectoring soluble H protein, we may speculate that high level

production of soluble glycoprotein by Paramyxoviruses is not an effective strategy to

enhance vaccine immunogenicity. To our knowledge this dissertation reports the first

application of a virus with modified levels of envelope protein expression to avoid

neutralizing immunity. While the vectors above have been evaluated for their

immunogenicity in animal models, none have been tested for performance in the presence

of passive immunity; we believe that such experimentation would be particularly

interesting for these other, currently available vectors, and particularly for RSV with

increased glycoprotein expression. For the MV with another copy of full-length H, we

documented an up to three-fold increase of H incorporation into virions, but cellular

expression of H was increased even to higher levels, which may suggest that this is the

maximal increase of H expression that the MV envelope can incorporate. It remains of

interest of us to see how modulation of H incorporation changes the neutralization

susceptibility and immunogenicity of this virus. It would also be interesting to see the

effect of modulating the level of MV F protein expression, given that this other envelope

glycoprotein represents the other, albeit more minor, target of neutralizing immunity

against measles.

In a second broad contribution to the field, this dissertation suggests that new

vaccines based on current attenuated MV strains represent a viable option for immunizing

infants against measles at a younger age. To our knowledge, this is the first report of a current MV vaccine-based strategy targeted as an alternative pediatric vaccine since the clinical trials that documented the improved immunogenicity of high titer attenuated measles vaccines (reviewed in Chapter 1). Development of alternative pediatric MV vaccines since that time has centered on DNA vaccines, non-MV vectors, and immune-stimulating complexes, each delivering MV antigens (reviewed in Chapter 2). Apart from the potential of our MV vaccine with an additional copy of full-length H, we suggest that this work lays the foundation for other experimentation with the glycoprotein-coding capacity of current MV vaccines to attempt to improve their immunogenicity. For RV, for example, increased glycoprotein expression was initially achieved by increasing the coding gene's copy number, but has also been increased by codon optimization of the single copy of the native gene (12), and this strategy could be applied to MV. It is also possible that changing, or increasing the diversity of, the genetic background of the MV H-coding gene in multiple copies could improve immunogenicity. For example, we considered inserting the H-coding sequence from the Edmonston-Zagreb vaccine, which induces a greater proportion of seroresponders in six-month-old infants than the Schwarz vaccine induces (13), into our Moraten vaccine strain-equivalent genetic background, which, notably, is also identical in terms of protein-coding capacity to the Schwarz vaccine. While four amino acids differ between the Edmonston-Zagreb and Moraten/Schwarz H proteins (differences L117F, V280A, T484N, S546G, using MVvac2 H as reference for comparison to Edmonston H from GenBank AAF85697.1), they have not been predicted to contribute majorly to neutralizing immunity, and so we have not yet pursued this strategy, but it nonetheless provokes interesting possibilities. Substitution of

attenuated H for wild-type H in a wild-type MV background MV had an attenuating effect on the virus (14), but to our knowledge the inverse exchange (a wild-type H in a vaccine strain MV background) has not been documented. We may speculate that introduction of another copy of wild-type H in our Moraten-equivalent background might elicit stronger neutralizing immunity. Insertion particularly of another copy of H-coding sequence from a contemporaneous MV into the Moraten background might provide better protection. Finally, it has long been suggested that a non-MV Morbillivirus, like rinderpest, could avoid inhibition by maternal antibodies and elicit broadly protective anti-Morbillivirus immunity (Norrby, E., original communication). In similar manner, MV itself has long been used to vaccinate puppies against canine distemper virus in the presence of maternal immunity and has been shown to provide a degree of protection against challenge (15), and there is some evidence that immunity raised against other Morbilliviruses can cross-neutralize MV. For example, sera from cattle exposed to virulent rinderpest weakly neutralized MV *in vitro* (16). The eradication of rinderpest makes use of this virus itself an unfeasible strategy, but H-coding sequence from rinderpest or other Morbilliviruses might be inserted in multiple copies to the Moraten-equivalent background to elicit a broad protective immune response. Overall on this point, we argue that modification of current MV vaccines represents a viable and, as yet, relatively unexplored strategy to achieve a successful vaccine for young infants.

Third, and significantly, this dissertation identifies a promising candidate for an alternative pediatric measles vaccine. MVvac2-H2, so named for its increased expression of full-length H, induces significantly stronger immune responses than its parental current vaccine-equivalent in mature and young mice and, most remarkably, in the presence of

passive immunity. Currently, we are testing the performance of MVvac2-H2 compared to

its parental strain, MVvac2, in the MV-susceptible mouse model of natural passive

transfer of maternal immunity introduced in Chapter 3, where we anticipate that

MVvac2-H2 will be able to induce protective levels of active neutralizing immunity in

the presence of maternal antibodies that entirely inhibit pup responses to MVvac2.

Moving forward, we suggest three main experimental thrusts that will together provide

enough evidence for advancement of this vaccine to clinical trials. First, given the

established importance of vaccine dose to infant responses, reviewed in Chapter 1, the

dose relationship between MVvac2 and MVvac2-H2 in MV-susceptible mice should be

established. Intriguingly, in this work we have shown MVvac2-H2 to induce significantly

stronger neutralization responses than MVvac2 induces at a standard titer dose, but

slightly lower responses than MVvac2 at a high titer dose. It remains of interest to us to

measure the immunogenicity of MVvac2-H2 at an intermediate dose of 10^4 $TCID_{50}$, the

maximum allowable MV vaccine dose mandated by the World Health Organization (17).

We aim also to evaluate whether there is a dosage of MVvac2-H2 that elicits a

comparable neutralizing response to $10^{5.3}$ PFU of MVvac2, which corresponds to the

dose of the Schwarz vaccine that significantly improved 6 months old infant responses.

Second, MVvac2-H2 should be evaluated for performance at a human-range dose of 10^3

to 10^4 $TCID_{50}$ in adult non-human primates. Macaques are susceptible to MV infection,

allowing challenge after vaccination, and replicate many of the clinical and

immunological features of MV infection in humans (18). Macaques have also been

shown to develop an atypical measles-like syndrome upon challenge after administration

of formalin-inactivated MV vaccine, and thus reproduce an important measles

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complication of humans (19). Testing in adult macaques will thus allow evaluation of the reactogenicity in addition to the immunogenicity of MVvac2-H2, and will also allow assessment of protection from wild-type challenge. The third and most important arm of preclinical evaluation of MVvac2-H2 will consist of testing the efficacy of the vaccine in neonatal and infant macaques of an MV-vaccinated colony. This final experiment directly tests our central hypothesis, that MV with increased H expression will prove a better inducer of protective immunity than current vaccines in the very young, in the most relevant non-human model for measles. MVvac2-H2 should be administered in a standard dose of 10^3 to 10^4 TCID$_{50}$ to young macaques divided into monthly cohorts from newborns to 12-month-old infants, with the presence of maternal immunity assessed in recipients just prior to vaccination. Immune responses of the young animals to vaccination should be measured, as well as protection from challenge one year after vaccination. We expect that MVvac2-H2 will evoke protection from infection at earlier ages than MVvac2 will. These experiments may provide a foundation for advancement to testing in humans.

In sum, this dissertation explores modifications to the H-coding capacity of MV and most importantly presents a promising candidate, MVvac2-H2, for a next generation measles vaccine targeted to very young infants. I sincerely hope that this work will prove generative to the intelligent design of vaccines against measles and other viral pathogens.

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