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Neonatal Immunization with a Sindbis Virus-DNA Measles Vaccine Induces Adult-Like Neutralizing Antibodies and Cell-Mediated Immunity in the Presence of Maternal Antibodies

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Infants younger than age 9 mo do not respond reliably to the live attenuated measles vaccine due the immaturity of their immune system and the presence of maternal Abs that interfere with successful immunization. We evaluated the immune responses elicited by Sindbis virus replicon-based DNA vaccines encoding measles virus (MV) hemagglutinin (H, pMSIN-H) or both hemagglutinin and fusion (F, pMSINH-FdU) glycoproteins in neonatal mice born to naive and measles-immune mothers. Despite the presence of high levels of maternal Abs, neonatal immunization with pMSIN-H induced long-lasting, high-avidity MV plaque reduction neutralization (PRN) Abs, mainly IgG2a, that also inhibited syncytium formation in CD150+ B95-8 cells. IgG secreting plasma cells were detected in spleen and bone marrow. Newborns vaccinated with pMSINH-FdU elicited PRN titers that surpassed the protective level (200 mIU/ml) but were short-lived, had low syncytium inhibition capacity, and lacked avidity maturation. This vaccine failed to induce significant PRN titers in the presence of placentally transferred Abs. Both pMSIN-H and pMSINH-FdU elicited strong Th1 type cell-mediated immunity, measured by T cell proliferation and IFN-γ production, that was unaffected by maternal Abs. Newborns responded to measles DNA vaccines with similar or even higher PRN titers and cell-mediated immunity than adult mice. This study is the first demonstration that a Sindbis virus-based measles DNA vaccine can elicit robust MV immunity in neonates bypassing maternal Abs. Such a vaccine could be followed by the current live attenuated MV vaccine in a heterologous prime-boost to protect against measles early in life.

DNA vaccines encoding measles virus (MV)4 Ags have been pursued as promising measles vaccine candidates. Several studies have shown a degree of protective efficacy using a variety of DNA plasmids encoding MV hemagglutinin and fusion glycoproteins in cotton rats (3, 4) and rhesus macaques (5–7).

DNA vaccination is particularly appealing for early life immunization for several reasons: 1) in neonatal animal models DNA vaccines have been shown to generate long-lived, adult-like protective immunity against viral diseases (8); 2) vaccine components such as CpG motifs present in the backbone plasmid are known to activate and enhance maturation of neonatal dendritic cells (DC) and B cells, favoring the development of Th1 type immunity and increasing Ab titers and avidity maturation (9); and 3) the vaccine-encoded Ag is synthesized within the hosts cells, shielded from blocking maternal Abs, and it can either be directly presented or cross-presented (through release of apoptotic bodies) by DC to stimulate naive T cells (10–13).

A measles DNA vaccine could be most useful to protect newborns and young infants from severe measles during the period of susceptibility; this would still allow for a boost immunization with the currently used live attenuated MV vaccine, as recommended, after 9 mo of age (14). MV DNA vaccination of newborn monkeys using conventional plasmid expression vectors in the presence of measles Abs elicited cell-mediated immunity (CMI) that reduced postchallenge viremia (12) and conferred 60–90% protection when an IL-2 molecular adjuvant was given 48 h later (12) or at the time of vaccination (15). These animals, however, did not develop neutralizing Abs (15), the accepted correlate of protection against measles (16).

4 Abbreviations used in this paper: MV, measles virus; DC, dendritic cell; ASC, Ab-secreting cell; BM, bone marrow; CMI, cell-mediated immunity; PRN, plaque reduction neutralization; SIA, syncytium inhibition assay; SFC, spot-forming cell.

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Pursuing a more efficient DNA vaccine approach to tackle the neonatal immune system, we developed Sindbis virus-based DNA plasmids encoding the MV hemagglutinin (3) and both the MV hemagglutinin and fusion glycoproteins (3, 17). A modified plasmid replicon pSINCP that incorporates nonstructural protein gene sequences from a human DC tropic Sindbis-virus was engineered to contain MV hemagglutinin, or both the hemagglutinin and fusion genes, resulting in plasmids pMSIN-H (3) and pMSINH-FdU (17). These constructs were selected for two reasons. First, MV neutralizing Abs, the main correlate of protection, are primarily directed to the hemagglutinin glycoprotein (18). Second, albeit the fusion Ag alone might be insufficient for protection, CMI can be enhanced if fusion is given along with the hemagglutinin Ag (17), and such responses may synergize the protective effect of neutralizing Abs. A single DNA construct encoding both hemagglutinin and fusion Ags has practical advantages over coadministration of two different plasmids (17).

Sindbis virus replicons represent a new generation of improved DNA vaccines in which cDNAs driven by eukaryotic promoters are transcribed into replicon RNA vectors that self-amplify in the cytoplasm through dsRNA intermediates and produce high-level Ag expression (reviewed in Refs. 19, 20). Cells transfected with Sindbis virus-based plasmids undergo apoptotic death releasing antigenic material for cross-presentation and dsRNA, which provides additional proinflammatory immune stimulation (20, 21).

Furthermore, Sindbis virus-derived dsRNA can activate and enhance maturation of DC (22), a major requirement to generate Th1 type immunity early in life (23).

We report the capacity of pMSIN-H and pMSINH-FdU to elicit MV-specific neutralizing Abs and T cell responses in neonatal mice in the presence or absence of maternal Abs. We characterized these responses in terms of virus-neutralizing capacity, inhibition of syncytium formation, avidity maturation, induction of CMI, Th1/Th2 profile, and immune memory.

Materials and Methods

Plasmid constructs

The Sindbis virus-derived replicon pSINCP (Chiron) was used as the backbone for the construction of DNA plasmids encoding full-length MV hemagglutinin (pMSIN-H), MV fusion without the 5′ untranslated region (pMSIN-FdU), both under the control of a subgenomic promoter (3, 17), and MV hemagglutinin and fusion in a bicistronic construct (pMSINH-FdU) containing two separate subgenomic promoters (17). Plasmids were purified using endo-free Qiagen columns as indicated by the manufacturer and dissolved in sterile PBS to a final concentration of 0.6 and 1 mg/ml for vaccination of neonatal and adult mice, respectively.

Western immunoblot and densitometric analysis

BHK-21 cells (American Type Culture Collection (ATCC) no. CCL-10) grown in DMEM supplemented with 10% FCS (Invitrogen Life Technologies) were transfected with Sindbis-based constructs using LipofectAMINE (Invitrogen Life Technologies) as previously described (17). Briefly, 2 μg of plasmid DNA were added to 5 × 10^3 cells/well in a 6-well tissue culture plate. Cells were recovered after 48 h of incubation and resuspended in lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 10 mM Tris (pH 7.4)). Cell lysates diluted in sample buffer (Bio-Rad) were separated in 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were blocked with 10% nonfat dry milk (Nestle) in PBS and incubated for 1 h at 37°C with anti-MV hemagglutinin or anti-MV fusion mAbs (provided by Dr. D. Griffin, Johns Hopkins University, Baltimore, MD) diluted in 5% nonfat dry milk in PBST (PBS containing 0.05% Tween). Ag-Ab complexes were detected with HRP-labeled goat anti-mouse serum followed by ECL Plus detection system (GE Healthcare Technologies). Densitometric analysis of bands was performed from digital images using Sigma-Gel software (Jandel Scientifics). The integrated area of each band and intensity was compared with the band of -actin in the same sample. The amount of protein was determined using a standard curve of known concentrations of actin (Sigma-Aldrich) vs band intensity.

Mice and immunizations

Newborns. BALB/c mice (8–10 wk old) purchased from Charles River Breeding Laboratories were bred to produce pups. Breeding cages were checked daily and new births were recorded. Experimental groups contained 1–2 litters (average 6 pups/litter). Newborn mice were immunized i.m. on day 7 after birth with 30 μg of naked DNA plasmids pMSIN-H, pMSIN-FdU, and pMSINH-FdU injected in the tibial muscle (25 μl in each leg). For immunological comparison, 7-day-old mice are believed to approximate the stage of immune maturation of a newborn human (24). Pre-immunization sera were obtained from age-matched naive pups. A second dose of plasmids was given in identical manner on day 22 after birth. Additional bleedings were performed from the retro-orbital sinus. To assess vaccine immunogenicity in the presence of maternal Abs, neonatal mice born to immune mothers who had been vaccinated with pMSIN-H or pMSINH-FdU were randomly assorted into three groups (average 6 pups/group) and immunized i.m. with pMSIN-H, pMSINH-FdU, or pSINCP (control) on days 7 and 22 after birth. Soon after birth, pups were transferred to a naive foster mother for nursing to avoid additional transfer of maternal Abs through milk, which occurs in rodents but not in humans (25).

Adults. Female BALB/c mice (8–10 wk old) were immunized i.m. with 100 μg of pMSIN-H, pMSINH-FdU, or pSINCP suspended in PBS (50 μl per hind leg) as previously described (3). All mice received a booster dose 14 or 28 days after the initial immunization. Blood samples were collected from the retro-orbital sinus before each vaccination and every 2 wk thereafter until day 70 after primary immunization. All animal studies were approved by the University of Maryland Institutional Animal Care and Use Committee.

MV-specific IgG and IgG subclasses

Serum IgG, IgG2a, and IgG1 against MV were measured by ELISA as previously described (3). Immunol II plates were coated with MV Edmonston strain lysate (Advanced Biotechnologies) at 5 μg/ml in carbonate buffer (pH 9), for 3 h at 37°C, and blocked overnight with 10% dry milk (Nestle) in PBS. After each incubation, plates were washed six times with PBST. Serum samples diluted 2-fold in 10% milk-PBST were incubated for 1 h at 37°C. After washing, plates were incubated with 100 μl of HRP-goat anti-mouse IgG, IgG1, or IgG2a (Roche) diluted 1/1000 in 10% milk-PBST for 1 h at 37°C. The substrate used was TMB Microwell Peroxidase (Kirkegaard & Perry Laboratories). After 5 min of incubation, the reaction was stopped by the addition of 100 μl of 1 M H2PO4 and OADase were measured. Sera were run in duplicate; negative and positive control sera were included in each assay. Optical densities vs serum dilutions were analyzed by linear regression, and titers were calculated from regression parameters as the inverse of the dilution that produces an optical density of 0.2 above the blank (ELISA in units per milliliter).

Avidity of MV-specific Abs

Avidity of measles serum IgG was determined using an ELISA with an urea elution step as previously described (17, 26, 27). Briefly, serum samples were tested for measles IgG as described in duplicate plates. In one of the plates, a 10-min overlay with 6 M urea (Sigma-Aldrich) was added after incubation with serum samples. Results are expressed in avidity index, calculated as the percentage of residual activity (end point titer) after treatment with urea. The assay was validated using a large number of positive and negative sera that were tested simultaneously for plaque reduction neutralization (PRN) and avidity. PRN titers above 200 mIU/ml showed avidity indices above 30, similar to what has been described in human samples (26, 27).

PRN assay

PRN titers were measured as previously described (3). Briefly, serum samples in serial dilutions were incubated with 100 PFU of wild-type MV Edmonston strain for 1 h at 37°C, 5% CO2, and plated in duplicate onto confluent Vero cells (ATCC no. CCL-81) in 12-well plates. After 1 h incubation, cells were overlaid with 2 ml of aqua/well and incubated for 5 days. Wells were stained with neutral red (Invitrogen Life Technologies), incubated overnight, and plaques were counted. The World Health Organization (WHO) measles serum standard 66/202 and internal controls calibrated against the WHO standard were tested in parallel with the samples, thereby allowing PRN titers to be expressed in mIU/ml. Titers >200 mIU/ml were considered protective (28).

Syncytium inhibition assay (SIA)

The SIA was performed as described by Ward et al. (29) with minor modifications. Briefly, serum samples were serially diluted with complete RPMI medium (RPMI 1640 containing 10% FCS, 200 mIU/ml glutamine, and penicillin-streptomycin, all from Invitrogen Life Technologies) in 96-well
after birth. Arrows indicate each immunization. Data represent individual vaccines pMSIN-H, pMSINH-FdU, or pSINCP (control) on days 7 and 22 after birth. Kinetics of serum Abs to MV Ags measured by ELISA and PRN. Significant differences between groups are indicated. Daily wells were already subtracted. A positive response was defined as 4 spots per 106 cells. IFN-γ-secreting cells

Spleens were harvested on days 15 and 70 after birth, and single cell suspensions were prepared as previously described (30). To harvest BM cells, skin-peeled hind legs were removed and the bone cavity of femurs and tibiae were flushed with 3–5 ml of complete RPMI medium; cells centrifuged and resuspended in complete medium. The frequency of IgG ASC were measured as previously described (30). Briefly, microtiter plates were coated with 5 µg/ml MV lysate, washed with PBS, and blocked with complete medium. Cells in 2-fold dilutions (5 × 10^3 to 6.25 × 10^5) were incubated in measles Ag coated wells overnight at 37°C, 5% CO₂. Specific Abs were revealed with HRP-labeled goat anti-mouse IgG (Roche) diluted 1/500 in PBS containing 1% BSA. Spots were developed using True Blue substrate (Kirkegaard & Perry Laboratories) in agarose overlay and enumerated using a stereomicroscope. Results are expressed as mean IgG ASC counts per 10^6 cells from replicate wells. Spots from control wells were subtracted from experimental wells. A positive response was defined as ≥4 spots per 10^6 cells.

**MV-specific T cell proliferation**

Single cell suspensions from spleens of vaccinated and control mice were cultured in triplicate wells (2 × 10^6 cells/well) with 10 µg/ml MV lysate or BSA (control) for 6 days at 37°C, 5% CO₂. Cell proliferation was measured by incorporation of [3H]thymidine as previously described (30). Results are expressed as stimulation index, calculated as counts per minute in cells incubated with Ag per counts per minute of cells incubated with medium alone ± SEM of replicate cultures. A stimulation index ≥3 was considered a significant response according to the measles literature (31, 32).

**Statistical analysis**

Ab titers, frequencies of IgG ASC, IFN-γ SFC, and stimulation index, measured at different time points in vaccinated and control mice, were compared using t test or Mann-Whitney U test (if normality failed). Differences with p < 0.05 were considered significant. Statistical analysis was performed using SigmaStat 3.1 (Systat software).

FIGURE 1. Kinetics of serum Abs to MV Ags measured by ELISA and PRN. A, kinetics of MV-specific Ab titers measured by ELISA and PRN in mice immunized as neonates with measles DNA vaccines. Newborn mice were immunized i.m. with 30 µg of Sindbis virus-repli-con DNA vaccines pMSIN-H, pMSINH-FdU, or pSINCP (control) on days 7 and 22 after birth. Arrows indicate each immunization. Data represent individual
Results

Ab responses in newborn and adult mice immunized with MV DNA vaccines

BALB/c mice born to measles-naive mothers were immunized at 7 and 22 days of age with 30 μg of pMSIN-H and pMSINH-FdU i.m. Empty pSINCP was used as a control. Kinetics of serum Abs to MV Ags measured by ELISA and PRN are shown in Fig. 1A. Both pMSIN-H and pMSINH-FdU elicited robust serum Ab responses, whereas no responses were observed in the control group. Mice immunized with pMSIN-H exhibited mean neutralizing Ab levels (PRN) that surpassed the human protective threshold (200 mIU/ml) after the first dose. These titers increased after the boost (p < 0.05). Peak responses, with 40- to 300-fold increases in individual animals post vaccination, were observed on day 49 after birth; these high titers persisted for at least 6 mo. Mice that received the bicistronic construct pMSINH-FdU also induced MV neutralizing Abs following each dose, although two doses of this constructs were required to achieve protective levels (>200 mIU/ml). Peak titers were observed on day 49, with 20- to 270-fold increases postvaccination. Ab titers, however, appeared to be lower than those achieved by pMSIN-H, although this difference was not statistically significant until day 63 after birth. In contrast to the PRN titers elicited by pMSIN-H, titers induced by pMSINH-FdU decreased over time (p < 0.05). There was also a large variation among Ab titers within the same group.

We next compared the responses elicited by newborn mice vaccinated with pMSIN-H and pMSINH-FdU with those generated in adult mice. DNA vaccines are typically given to adult mice in two doses, ~1 mo apart. Because our neonatal vaccination schedule consists of two doses of DNA vaccines given 2 wk apart, we immunized adult mice reproducing this schedule (i.e., 14 days between doses) and with the traditional schedule (i.e., 28 days between doses). ELISA and PRN titers were measured 70 days after primary immunization. Results are shown in Fig. 1B.

Adult mice receiving either plasmid achieved high levels of measles Abs measured by ELISA and PRN when delivered 28 days apart. Lower responses were observed when the DNA vaccines were given 14 days apart. This difference was not significant for titers measured by ELISA but was clearly evident for those measured by PRN, which indicates that shortening the interval between doses reduced the neutralization capacity of the Abs. This effect was more pronounced in mice immunized with pMSINH-FdU; PRN titers in mice immunized with this construct 14 days apart never reached the protective threshold. Notably, when neonates were given the same DNA vaccines 14 days apart, they developed ELISA and PRN titers of similar magnitude to those of adult mice vaccinated 28 days apart following the traditional immunization schedule. More importantly, using the 14-day vaccination schedule, neonates induced significantly higher PRN titers (p < 0.05) compared with adults.

The fact that Ab responses to pMSIN-H were consistently superior to those elicited by pMSINH-FdU raised the question of whether neonates would also respond efficiently to a Sindbis replicon DNA encoding only MV fusion. High Ab responses to fusion glycoprotein, as those raised by Sindbis encoding hemagglutinin glycoprotein alone, would support the hypothesis that the decline of responses is somehow associated with coexpression of these Ags. To address this question, newborn mice were immunized on days 7 and 21 after birth with 30 μg of pMSIN-FdU. Measles Abs were measured by PRN and ELISA and results are shown in Fig. 2. Neonates developed low levels of ELISA MV-specific Abs that did not show neutralizing capacity. The next question was whether the bicistronic construct was producing the same amount of MV hemagglutinin protein than pMSIN-H, reasoning that its inferior immunogenicity could be due to inadequate expression of MV hemagglutinin protein. The amounts of MV hemagglutinin and fusion proteins expressed by transfected BHK-21 cells were measured by ELISA and PRN, which indicates that shortening the interval interpolating in a curve of known concentrations of β-actin. Data shown are mean protein concentration ± SD from two different experiments.

FIGURE 2. MV-specific Ab titers measured by ELISA and PRN in mice immunized as neonates with a measles DNA vaccine encoding MV fusion glycoprotein. Newborn mice were immunized with pMSIN-FdU as indicated in Fig. 1. Data represent individual titers from nine pups. Lines are plotted upon mean Ab titers.

FIGURE 3. Quantification of MV hemagglutinin and fusion proteins expressed by transfected BHK-21 cells. Cell lysates were obtained 48 h after transfection with different Sindbis-replicon DNA constructs. MV hemagglutinin and fusion proteins were separated by SDS-PAGE, transferred to membranes, and revealed with mAbs. A lysate of MV-infected Vero cells (3.5 μg) was used as control. Protein quantification was performed by densitometric analysis of each band (integrated area and intensity) interpolating in a curve of known concentrations of β-actin. Data shown are mean protein concentration ± SD from two different experiments.
Albeit the expression of both hemagglutinin and fusion proteins in the single plasmids was slightly higher than in the bicistronic construct, the differences were not statistically significant. Because of the failure of pMSIN-FdU to elicit neutralizing Abs, we did not perform further analysis with this plasmid.

Preferential synthesis of IgG2a in newborn and adult mice after MV DNA vaccination

To further characterize the neonatal responses to pMSIN-H and pMSINH-FdU, we examined the MV-specific IgG subclasses as a marker for Th1/Th2-type responses. Fig. 4A shows the kinetics of IgG subclass distribution at different time points. Both plasmids induced mainly IgG2a after each vaccine dose. Neonates immunized with pMSIN-H had a significantly higher IgG2a after the first vaccination (on day 21 after birth) compared with those that received pMSINH-FdU; no further differences were observed thereafter. We also compared the IgG subclass distribution between neonatal and adult mice immunized with pMSIN-H and pMSINH-FdU (Fig. 4B). Adult mice also responded with significantly higher levels of IgG2a ($p < 0.05$) to both vaccines, regardless of the immunization schedule (interval between doses) used.

Avidity profile and syncytium inhibition capacity of MV Abs in neonatally vaccinated mice

We investigated the quality of Abs elicited by pMSIN-H and pMSINH-FdU in terms of avidity and capacity to neutralize MV-induced syncytium formation using B95-8 cells that express the mammalian wild-type MV receptor CD150 or SLAM. These attributes of MV Abs appear to be critical for protection and have been singled out as important parameters to investigate for any new measles vaccine candidate (33).

Avidity and syncytium inhibition capacity of MV Abs were measured in serum samples collected on day 63 after birth. Results are shown in Fig. 5. Neonatal immunization with pMSIN-H or pMSINH-FdU elicited Abs clearly different in their avidity profiles. Abs produced by pMSIN-H had higher avidity than those elicited by pMSINH-FdU; this observation was consistent in several experiments. Although the difference in avidity index was marginal in the subset of samples presented, it was significant when we compared larger number of samples from different experiments. Increase of avidity over time for pMSIN-H-induced Abs was also evident when we studied the kinetics of avidity maturation in the presence of maternal Abs (Fig. 6B). Abs raised by pMSIN-H vaccination were comparable in their high avidity to the Abs produced by human infants 1 mo after receiving the licensed live-attenuated measles vaccine (data not shown). Avidity indices were also $>30$, which together with a protective PRN level, have been singled out as parameters indicative of a memory (secondary) response in human adults following vaccination (26, 27).
The difference in avidity of Abs induced by pMSIN-H and pMSINH-FdU was reflected in their capacity to inhibit MV-induced syncytia formation in B95-8 cells. Abs produced by pMSIN-H more efficiently prevented virus-infection and formation of syncytia in CD150<sup>+</sup>/H11001 cells, compared with Abs raised by pMSINH-FdU. Both vaccines, however, showed mean SIA titers above 16, which has been proposed as the SIA cutoff value to identify fully susceptible individuals with PRN titers below the protective levels 120–200 mIU/ml (29). The mean PRN titers for these samples were 1335 and 633 mIU/ml for pMSIN-H and pMSINH-FdU, respectively.

**FIGURE 6.** Kinetics of MV PRN Ab production (A), avidity maturation (B), and subclass distribution (C) in neonatal mice born to naive or measles-immune mothers after measles DNA vaccination. Pups from naive (left column), pMSIN-H-immunized (center column), or pMSINH-FdU-immunized (right column) mothers received two doses of pMSIN-H, pMSINH-FdU, or pSinCP (control) as described in Fig. 1. PRN Ab titers, avidity index, and subclass profile (expressed as log<sub>10</sub> IgG2a to IgG1 ratio) were measured in mothers (y-axis by gray area) at the time of delivery and in the pups at different times points. Arrows indicate each vaccination. Data represent geometric mean titers for each group ± SD. PRN protective titer (200 mIU/ml) is indicated (horizontal dashed line) in A. Significant differences (*, p < 0.05) for PRN titers and avidity measurements between mice vaccinated with pMSIN-H or pMSINH-FdU are indicated.

It is known that even low concentrations of placentally transferred measles Abs interfere with immunization using the live-attenuated MV vaccine (34), creating a major obstacle for early life immunization. These maternal Abs are believed to reduce the amount of Ag available for priming and to interfere with its processing and presentation (13, 34). We investigated the responses to pMSIN-H and pMSINH-FdU in newborn mice in the presence of maternal Abs. Pups were bred from females that had been immunized when they...
were neonates with pMSIN-H or pMSINH-FdU and who placentally transferred their Abs to their offspring. Mothers immunized with pMSIN-H maintained high levels of measles neutralizing Abs, whereas those who received pMSINH-FdU had lower levels at the time of delivery. Soon after birth pups were transferred to a naive surrogate mother for nursing, to avoid additional transfer of Abs by milk, more closely recreating the transfer of maternal immunity in humans. Litters from measles immune and naive mothers were immunized on days 7 and 22 with pMSIN-H, pMSINH-FdU, or pSINCP (control). The kinetics of PRN MV Ab responses for pups with different maternal background are shown in Fig. 6A.

As expected, in the absence of maternal Abs, newborn mice responded vigorously to both DNA vaccines (Fig. 6A, left). All mice seroconverted, reaching high levels of PRN Abs, particularly the group vaccinated with pMSIN-H. No responses were observed in the control group that received pSINCP. Newborn mice from immune mothers who received pSINCP displayed the natural progressive decay of maternal Abs, which had completely disappeared 49 days after birth. Neonaotes born to pMSIN-H-immunized mothers responded with high PRN titers after vaccination with pMSIN-H. Although an initial decline in PRN titers was observed after pMSIN-H delivery to measles immune pups during the first 2 wk after birth, those levels mainly reflect the decrease of maternal Abs as well as the early vaccine-induced Abs. Importantly, the mean PRN titer measured 14 days after the first dose of pMSIN-H was significantly higher (p < 0.05) than the titer of the control group who received pSINCP and almost identical to the mean PRN titers raised by pMSIN-H in neonates born to naive mothers at the same time point. After the second pMSIN-H dose, PRN levels further increased (displaying a sharp turn around in the kinetics curves), reaching levels well beyond the protective threshold (200 mIU/ml), and similar to those elicited in neonates born to naive mothers. Siblings from the same mother who received pMSINH-FdU, however, had significantly lower responses (p < 0.05 on days 35, 49, and 63 after birth) compared with those who received pMSIN-H. The PRN titers raised by pMSINH-FdU failed to boost after the second vaccination, falling below the protective level (<200 mIU/ml). Although these MV Abs did not totally disappear as in control pSINCP-immunized or unvaccinated mice (data not shown), they never increased and remained at a plateau beyond that point.

Similar results were observed in pups that placentally acquired maternal measles hemagglutinin and fusion Abs from pMSINH-FdU-immunized mothers (Fig. 6A, right). Only pups that received pMSIN-H were able to overcome the inhibitory effect of maternal Abs and produce a significant neutralizing Ab response that surpassed the PRN protective level.

To further characterize the quality of the humoral responses against MV following pMSIN-H and pMSINH-FdU vaccination, we measured the avidity of MV IgG Abs in the context of pre-existing immunity. Kinetics of avidity maturation of MV-specific IgG induced by both vaccines are shown in Fig. 6B. In the absence of maternal passive immunity, the avidity of Abs induced by pMSIN-H and pMSINH-FdU increased steadily over time. Consistent with previous observations, Abs of higher avidity were induced by pMSIN-H (Fig. 6B, left). A similar increase in MV-IgG avidity was observed, upon immunization with pMSIN-H, in neonates.

**FIGURE 7.** MV-specific IFN-γ production (A) and T cell proliferation (B) in spleen cells from neonatal mice born to naive or immune mothers after measles DNA vaccination. Newborn mice were immunized with pMSIN-H, pMSINH-FdU, or pSINCP (control) as described in Fig. 1. Spleens were harvested on day 15 after birth (8 days after the first dose), and on day 70 after birth (7 wk after the booster). A, The frequency of MV-specific IFN-γ-secreting cells was measured by ELISPOT upon ex vivo stimulation with MV lysate. Data represent mean IFN-γ SFC per 1 × 10^6 cells ± SD of replicate cultures. Frequencies of IFN-γ SFC elicited on day 15 in mice born to immune mothers in response to pMSIN-H (center) and pMSINH-FdU (right) were significantly higher (p < 0.05) than those elicited in mice born to naive mothers. B, T cell proliferation of spleen cells upon in vitro incubation with MV lysate were measured by [3H]thymidine incorporation and expressed as mean stimulation index ± SEM. Threshold (SI ≥3) for a positive response is shown at horizontal dashed line. T cell responses on day 15 in newborn from immune mothers in response to pMSINH-FdU vaccination were significantly higher (p < 0.05) than those of naive mother’s pups. Significant differences for both IFN-γ SFC and T cell proliferation in mice vaccinated as neonates with pMSIN-H or pMSINH-FdU are indicated.
born to immune mothers who had high levels of maternal hemagglutinin or hemagglutinin plus fusion Abs. Conversely, there was no enhancement of IgG avidity in mice immunized with pMSINH-FdU in the presence of maternal Abs of either specificity.

We also examined whether the presence of maternal Abs influenced the development of neonatal Th1 responses induced by these DNA vaccines. The kinetics of IgG2a/IgG1 production in pups born to naive and measles immune mothers is shown in Fig. 6C. In the absence of maternal Abs, both DNA vaccines induced a preferential synthesis of IgG2a over IgG1. Newborns from immune mothers showed a steady 100:1 IgG2a to IgG1 ratio after vaccination with pMSIN-H, regardless of the pre-existing hemagglutinin and fusion maternal Abs. A somewhat lower IgG2a to IgG1 ratio was observed in immune pups after vaccination with pMSINH-FdU in most of the time points; nonetheless, IgG2a levels that were at least 10 times higher than IgG1 were measured throughout the immunization schedule.

Together, these data indicate that regardless of the maternal immune status, neonatal mice vaccinated with DNA vaccine pMSIN-H can elicit de novo PRN responses that further augment to reach high levels after a second dose; these Abs were primarily IgG2a of high avidity and long-lived. Overall, these responses were strikingly similar to those raised in adult mice. The vaccine pMSINH-FdU also raised PRN responses, albeit less robust ones, in naive mice, but failed to overcome maternal Ab inhibition.

CMI in mice immunized as neonates with MV DNA vaccines despite the presence of maternal Abs

We evaluated the induction of MV-specific T cell responses induced by measles DNA vaccines in newborns from naive and measles immune mothers by measuring the frequency of IFN-γ-producing cells and the degree of T cell proliferation in the spleen on day 15 (true neonatal period) and day 70 after birth. Results are summarized in Fig. 7.

Neonates born to naive mothers developed significant MV-specific IFN-γ responses after two doses of both pMSIN-H and pMSINH-FdU, whereas no responses were observed in the control group that received pSINCP. The mean frequency of IFN-γ-producing cells (measured on day 70 after birth) in mice immunized with pMSINH-FdU was higher than those of mice immunized with pMSIN-H (p = 0.02) (Fig. 7A, left and center).

Neonates born to immune mothers also exhibited potent IFN-γ secretion in response to either vaccine, despite the presence of maternally derived MV hemagglutinin and fusion Abs. These responses were already observed after the first dose (day 15 after birth). Interestingly, higher IFN-γ responses were observed in pups born to immune mothers in comparison with those born to naive mothers after priming with one dose of DNA vaccine (day 15 after birth, p < 0.05). Mean IFN-γ SFC frequencies were 31 and 34 for pups immunized with pMSIN-H, and 43 and 33 for those immunized with pMSINH-FdU, when they were born from hemagglutinin and hemagglutinin plus fusion immune mothers, respectively; whereas newborns from naive mothers showed mean IFN-γ SFC values of 13 and 14 after vaccination with pMSIN-H and pMSINH-FdU. These responses further increased after the boost (p < 0.01). Higher frequency of IFN-γ SFC were measured on day 70 in mice immunized as neonates with pMSINH-FdU (mean IFN-γ SFC frequency range 112–133) vs those immunized with pMSIN-H (mean IFN-γ SFC range 67–78), regardless of the presence of maternal Abs at the time of vaccination.
Mice immunized as neonates with pMSIN-H or pMSINH-FdU also exhibited MV-specific T cell proliferative responses compared with the control group. Although at low levels, these responses were present after the first dose and increased after the boost, regardless of the maternal immune background. A single dose of pMSINH-FdU produced higher T cell proliferation in newborns from immune mothers ($p < 0.03$).

**Plasma IgG ASC in spleen and BM after neonatal vaccination with MV DNA vaccines in the presence or absence of maternal Abs**

Serum Abs are produced by plasma cells that have been differentiated into plasmablasts in secondary lymphoid tissues (35). These ASC migrate to the BM where they will differentiate into long-lived plasma cells that will be responsible for the maintenance of persistent Ab levels (36, 37). We investigated the frequency of B cells that can secrete MV-specific IgG Abs upon overnight Ag stimulation (plasma cells) in the spleen and BM of mice immunized as newborns with pMSIN-H, pMSINH-FdU, or pSINCP (control). Results are shown in Fig. 8. Significant levels of measles IgG ASC were detected in the spleens on day 15 after birth, in response to one dose of DNA vaccine (during the neonatal period); the frequency of these MV-specific plasma cells increased significantly after the boost. Most importantly, the induction of MV IgG ASC was not affected by the presence or specificity of maternal Abs. High frequency of MV-specific IgG ASC were found in the BM after neonatal vaccination with pMSIN-H and pMSINH-FdU, despite the presence of maternal Abs at the time of immunization; these plasma cells were detected on day 70 after birth. No responses were observed in the control group (pSINCP). Somewhat lower BM IgG ASC were raised in response to pMSINH-FdU by neonates born to naive and pMSINH-immunized mothers ($p < 0.05$). The plasma cells housed in the BM persist longer than those of the spleen and considered to be ASC memory cells (36, 38, 39).

**Discussion**

We demonstrate for the first time that Sindbis virus replicon-based DNA vaccines encoding MV hemagglutinin (pMSIN-H) or hemagglutinin plus fusion proteins (pMSINH-FdU) elicit neutralizing Ab levels above the protective threshold and cellular immune responses, despite the presence of maternal Abs in a neonatal mouse vaccination model. The PRN levels in mice vaccinated as neonates with pMSIN-H, pMSINH-FdU, or pSINCP (control). Results are shown in Fig. 8. Significant levels of measles IgG ASC were detected in the spleens on day 15 after birth, in response to one dose of DNA vaccine (during the neonatal period); the frequency of these MV-specific plasma cells increased significantly after the boost. Most importantly, the induction of MV IgG ASC was not affected by the presence or specificity of maternal Abs. High frequency of MV-specific IgG ASC were found in the BM after neonatal vaccination with pMSIN-H and pMSINH-FdU, despite the presence of maternal Abs at the time of immunization; these plasma cells were detected on day 70 after birth. No responses were observed in the control group (pSINCP). Somewhat lower BM IgG ASC were raised in response to pMSINH-FdU by neonates born to naive and pMSINH-immunized mothers ($p < 0.05$). The plasma cells housed in the BM persist longer than those of the spleen and considered to be ASC memory cells (36, 38, 39).

A major attribute of these vaccines is their capacity to induce a distinct ‘early life’ Th1 type immunity, characterized by high levels of MV-specific IgG2a and IFN-γ production. This profile of immune response was established during the first weeks of life, despite the presence of maternal Abs, and was maintained throughout adulthood. Identical Th1 type immunity is induced by pMSIN-H or pMSINH-FdU in adult mice. Notably, both DNA vaccines were able to stimulate a clear Th1 type profile during early phases of immune priming and T cells commitment; this result is important because once T cells are differentiated they can no longer switch to a different type of response later in life (40–43). In sharp contrast, newborn mice vaccinated with the licensed live-attenuated measles vaccine preferentially elicit a Th2 type response with high IgG1 and IL-5 (42).

Although PRN titers induced by both vaccines surpassed the threshold for protection in humans (200 mIU/mL), the Abs elicited by pMSIN-H persisted longer in circulation than those induced by pMSINH-FdU, which fell shortly after the boost. Abs elicited in response to pMSIN-H also showed sustained avidity maturation despite the presence of maternal Abs, and were able to block MV infection in B95-8 cells that express CD150, the human receptor for wild-type MV. In contrast, Abs produced by pMSINH-FdU had significantly lower avidity and virus neutralizing capacity, which failed to improve over time. The reasons for the lower immunogenicity of pMSINH-FdU are not totally understood. Our data show that this phenomenon is not due to impaired responses during the neonatal period, as pMSINH-FdU was also less immunogenic in adult mice (17). Neonates vaccinated with a plasmid encoding fusion alone failed to develop neutralizing Abs, indicating that Ab responses to pMSINH-FdU are directed primarily against MV hemagglutinin. BHK cells transfected in vitro with the bicistronic construct expressed somewhat lower levels of MV hemagglutinin compared with those transfected with pMSIN-H. It can be argued that less Ag expressed in vivo would lead to insufficient immune stimulation. However, this marginal difference in protein expression is unlikely to be the main reason for the impaired responses observed. In previous studies from our group conducted in adult mice, we observed that PRN titers remained unaltered even after reducing by 10³-fold (100 to 0.1 µg) the dose of pMSIN-H administered. It is likely that an interaction between hemagglutinin and fusion might be taking place when these glycoproteins are coexpressed within the same cell in vivo, somehow interfering with induction and maintenance of immune responses. Cells coexpressing fusion and hemagglutinin proteins but not cells expressing either of these proteins alone or a mixture of MV fusion and MV hemagglutinin expressing cells were found to inhibit cellular responses in vitro (44). MV hemagglutinin and fusion proteins present in the surface of MV-infected or transfected cells were found necessary and sufficient to induce immunosuppression in cotton rats (45), confirming that the MV fusion-hemagglutinin complex is also an immunosuppressive effector structure in vivo (44, 46). Several mechanisms have been proposed to explain such immunosuppression, including impaired terminal differentiation and Ag presentation by DC and direct negative signaling by MV glycoproteins to T cells (46, 47). Inadequate Ag presentation and/or T and B cell stimulation due to cell death or immunoregulatory signals or molecules could explain the diminished and low quality responses generated by pMSINH-FdU in our study. An impaired T cell helper function can account for the lack of avidity maturation and immune memory. The low immunogenicity of pMSINH-FdU could also be due to the exposure of immunosuppressive domains following proteolytic cleavage of fusion protein, shown by Weidman et al. (48) to diminish immune responses in the absence of syncytium formation.

The fact that MV-specific IgG ASC are detected in spleen and BM after pMSINH-FdU vaccination, despite low levels of systemic neutralizing Abs, leads us to conclude that expression of hemagglutinin and fusion is somehow interfering with the proper functioning of Th cells that fail to sustain B cell maturation. This would primarily affect the functional capacity, not just the level of Abs. The MV-specific ASC found in the BM might correspond to low avidity clones, already described in mice immunized as neonates with other vaccine Ags (49).

It is well known that early measles vaccination with live attenuated MV in the presence of even low levels of maternal Abs, results in low rates of seroconversion and a permanent state of hyporesponsiveness to subsequent booster doses (13, 34, 50). A major finding in our study is that pMSIN-H can induce robust PRN titers despite high levels of maternal Abs. Neonates also responded to a booster dose with increased levels of high quality Abs. Vaccination with pMSINH-FdU, in contrast, failed to overcome maternal Ab inhibition to achieve protective titers, even when the levels of placentally transferred Abs were low. Clearly, the intrinsic low immunogenicity of this vaccine, rather than the level of
pre-existing Abs, determined the outcome response. For other vaccines, the ratio between Ag to maternal Abs has been identified as the main determinant for overcoming maternal Ab inhibition (10, 51).

A deficient establishment of neonatal mature Ag-specific plasmablasts generated in the spleen as BM ASC pool was described by Pihlgren et al. (52, 53). Neonatal vaccination with pMSIN-H generated a large pool of plasma B cells regardless of the presence of maternal Abs, as indicated by the long-term persistence of high levels of PRN Abs and high frequencies of measles IgG ASC in spleen and BM. This advantage is important for a vaccine whose protective capacity lies mainly in the generation of long-lived plasmocytes that will be responsible for the continuous maintenance of adequate levels of neutralizing serum Abs (37, 54).

Mice immunized as neonates with two doses of pMSIN-H and pMSINH-FdU exhibited strong CMI regardless of the maternal background. Only neonates born to immune mothers exhibited MV-specific IFN-γ production and T cell proliferation after the first vaccination. The enhanced CMI in pups with placentally transferred immunity is likely due to facilitating maternal Abs that form immune complexes with MV hemagglutinin and fusion Ags and, in this form, are readily taken by APC for presentation to naive T cells. Sindbis virus-based replicons induce apoptosis, thereby providing additional hemagglutinin and fusion antigenic material that can bind maternal Abs and thus gain access to DC, leading to cross-presentation and enhanced T cell stimulation. Ag-Ab complexes that facilitate APC uptake and Ag presentation to T cells can also explain the strong CMI observed in 6-mo-old human infants born to immune mothers in response to live-attenuated measles vaccine (55). Specific enhancement of T cell responses in the presence of specific Abs has been described for other viral vaccines such as hepatitis B (50). It is intriguing that the lower capacity of pMSINH-FdU to produce humoral responses was not seen at the level of CMI. In fact, the IFN-γ and T cell responses following neonatal vaccination with pMSINH-FdU were similar or even higher in magnitude than those elicited by pMSIN-H.

Although part of the rationale for including the fusion Ag in a measles vaccine was to enhance CMI that may synergize the protective effect of neutralizing Abs, another reason was to enhance the safety profile of the new candidate measles vaccine. It has been argued that an imbalance in Abs against hemagglutinin and fusion Ags, mainly the absence of fusion Abs, was responsible for the atypical measles syndrome seen in the 1960s in children who received the formalin-inactivated measles vaccine and were later exposed to wild-type MV (56). However, studies performed by Polack et al. (33, 57) in nonhuman primates provide a different explanation for atypical measles, showing that this phenomenon is associated with the generation of high levels of complement-fixing Abs that exhibit low avidity for MV; such Abs form immune complexes and lead to immunopathology. These investigators also showed that juvenile rhesus macaques that received DNA vaccines encoding hemagglutinin and fusion Ags were protected from infection and did not develop atypical measles after challenge with wild-type MV. In these studies, MV DNA vaccines encoding fusion Ag primed animals to respond with a Th1-skewed profile upon challenge, whereas vaccination with hemagglutinin-encoding DNA vaccines primed for a Th2 response (58). Although both of our vaccines elicited a defined Th1-type immunity, stronger IFN-γ production was observed in pMSINH-FdU-vaccinated mice upon in vitro stimulation with MV Ags, in agreement with the nonhuman primate data.

Despite the weakened Ab responses, pMSINH-FdU was capable of inducing CMI that was even stronger than that elicited by pMSIN-H. Although vaccine-induced PRN Abs are deemed essential to block virus entrance to the host cell, T cell responses can control viral replication and confer protection, as demonstrated in vaccinated monkeys upon MV challenge (12, 15, 59, 60). The contribution of CMI to preventing measles infection in humans in the light of “failed” seroconversion has been increasingly recognized (31, 32, 55, 61, 62). Experiments to investigate the protective efficacy of our MV DNA vaccines in very young infant rhesus macaques are underway.

In conclusion, we demonstrate for the first time, in newborn mice, that a new generation Sindbis virus-based replicon DNA vaccine encoding measles hemagglutinin protein can stimulate the neonatal immune system circumventing the limitations to induce measles immunity in human neonates and young infants. Despite the presence of maternal Abs, pMSIN-H has been shown to induce remarkable levels of high avidity, neutralizing Abs and Th1-type immunity, similar to those elicited by adult mice. The outstanding immunogenicity of pMSIN-H was confirmed in very young infant rhesus macaques; 50 days old infant rhesus who received two doses of this vaccine developed protective PRN titers and were completely protected after wild-type measles challenge at 9 mo of age (M. F. Pasetti and M. M. Levine, unpublished observations). This vaccine appears to be a leading candidate for use in a prime-boost approach, where DNA priming during the first months of age can be followed by a boost with the live attenuated MV vaccine at the recommended 9–12 mo of age.

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Disclosures
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