

## Protective Efficacy against Respiratory Syncytial Virus following Murine Neonatal Immunization with BBG2Na Vaccine: Influence of Adjuvants and Maternal Antibodies

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Alum-adsorbed BBG2Na, a recombinant vaccine derived in part from the respiratory syncytial virus (RSV) subgroup A G protein, induced moderate antibody titers after 1 immunization in 1-week-old mice but conferred complete lung protection upon RSV challenge. The anti-BBG2Na IgG1-IgG2a neonatal isotype profile was suggestive of dominant Th2 responses compared with those in adults. Formulation of BBG2Na with a Th1-driving adjuvant efficiently shifted neonatal responses toward a more balanced and adultlike IgG1-IgG2a profile without compromising its protective efficacy. BBG2Na-induced protective immunity was maintained even after early life immunization in the presence of high titers of maternal antibodies. Under these conditions, the protective efficacy (86%–100%) reflected the high capacity of the nonglycosylated G2Na immunogen to escape inhibition by RSV-A–induced maternal antibodies. Thus, immunization with BBG2Na protected against viral challenge despite neonatal immunologic immaturity and the presence of maternal antibodies, two major obstacles to neonatal RSV vaccine development.

Respiratory syncytial virus (RSV), a pneumovirus of the Paramyxoviridae family, is a major respiratory tract pathogen. It affects humans through repeated infectious episodes. Its clinical severity is maximal in infancy, in immunosuppressed persons, and in the elderly [1]. Worldwide, RSV affects most infants during their first winter season, leading to hospitalization of a significant (0.5%–2%) fraction [2–5]. Infant protection from RSV thus represents a major public health issue but is a challenge that has not been met. Protection in early life requires the use of vaccines that can rapidly induce protective immune responses despite the relative immaturity of the immune system and the persistence of antibodies of maternal origin that can interfere with infant vaccine responses. Both issues are significant obstacles to the development of an efficient RSV vaccine [6, 7]. In addition, there is fear that vaccine-induced disease enhancement at the time of RSV exposure (previously observed after immunization of seronegative infants with a formaldehyde-inactivated viral vaccine [8, 9]) would occur.

Among the vaccine strategies currently being explored, the recombinant fusion protein BBG2Na appears to be a promising RSV vaccine candidate. First, this recombinant protein contains an RSV (Long) G protein fragment (G2Na, aa 130–230), including a conserved subgroup A–specific protective epitope [10, 11] and a stretch of amino acid residues (aa 164–176) that are completely conserved in all known human A and B RSV isolates [12, 13]. Second, this G protein fragment is fused to the albumin-binding region (BB) of streptococcal protein G, which is capable of significantly enhancing the in vivo half-life of fusion partners and thus their exposure to the immune system [14–17]. This fusion partner also has carrier-related properties [15] and potentiates the immunogenicity of G2Na [16]. Third, BBG2Na is produced by prokaryotic expression in *Escherichia coli* as a nonglycosylated protein, thereby circumventing the limitations of the native G protein immunogenicity because of its heavy glycosylation pattern [17]. The strong immunogenicity and protective efficacy of alum (AlOH)–adsorbed BBG2Na have been demonstrated against intranasal RSV challenge in both adult BALB/c mice and cotton rats against both homologous and heterologous RSV challenge [18]. This new RSV vaccine candidate is now being evaluated in phase I clinical studies.

To evaluate whether BBG2Na could induce protection against RSV lower respiratory tract infections in early life, we previously used a murine model of neonatal immunization to evaluate the neonatal immunogenicity of AlOH-adsorbed BBG2Na. Intraperitoneal (ip) BBG2Na immunization of  $\leq 1$ -week-old BALB/c mice induced significant antibody responses

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Animals were maintained in an accredited facility, and experiments were done in accordance with the guidelines of local authorities.

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[19], but these antibody responses significantly differed from those in adult mice. First, IgG-specific BBG2Na responses to a single immunization at age 1 week reach antibody titers ( $\sim 3.5 \log_{10}$ ) that remain 100-fold lower ( $5.5 \log_{10}$ ) than those observed in response to adult immunization. Second, infant mouse antibody responses to AIOH-BBG2Na also differ qualitatively from adult responses, being almost exclusively of the IgG1 isotype, whereas similar levels of IgG1 and IgG2a antibodies are generated in adult mice [19]. In view of the quantitative and qualitative differences in immune responses induced by neonatal versus adult immunization, in the present study we evaluated whether neonatal BBG2Na immunization via various immunization routes could protect against an RSV challenge and whether adultlike, more balanced Th1-Th2 responses to BBG2Na could be achieved in neonates by changing the adjuvant formulation. We also evaluated the protective efficacy of neonatal BBG2Na immunization when given in the presence of maternal antibodies to RSV-A, whether induced by maternal exposure to RSV-A or BBG2Na immunization, another critical issue for vaccine-mediated protection in early life.

## Materials and Methods

**Mice.** Specific pathogen-free adult BALB/c inbred mice were purchased from IFFA-CREDO (L'Arbresle, France), kept under specific pathogen-free conditions at the WHO Center for Neonatal Vaccinology, and manipulated according to national and European guidelines. Breeding cages were checked daily for new births, and the day of birth was recorded as the day the litter was found. Pups were kept with mothers until weaning at age 4 weeks.

**Vaccine antigen, viruses, and cells.** The recombinant fusion protein BBG2Na was expressed and purified in *Escherichia coli* as described [18, 20]. Twenty-microgram doses were used per immunization after resuspension either in an isotonic solution containing AdjuPhos (400  $\mu\text{g}$  of aluminum dose; Superfos BioSector, Vedbaek, Denmark) or in a formulation containing the CRL8941 adjuvant in squalene (TiterMax; gift of CytRx, Norcross, GA) according to the manufacturer's instructions. RSV subgroup A (RSV-A) Long strain (ATCC VR-26; American Type Culture Collection, Rockville, MD) propagation in HEP-2 cells (ECACC 86030501; European Collection of Animal Cell Cultures, Salisbury, UK), along with the production of viral and control cell ELISA antigens were undertaken as described [20].

**Immunization and challenge procedures.** Litters of 6–8 mice were immunized by the routes and at the ages indicated in the figure legends. Mice were bled at regular intervals to determine BBG2Na- and RSV-A-specific serum antibody titers. When indicated, a booster immunization was given 3 weeks later. For maternal immunization, 2 doses of BBG2Na (20  $\mu\text{g}$ ) or 3 doses of AIOH-adsorbed RSV-A (Long strain) were given ip at 3-week intervals prior to mating. Offspring were challenged with TCID<sub>50</sub> RSV-A by intranasal instillation after being anesthetized with 2.5 mL/kg of a 4/1 mixture (vol/vol) of ketamine (Imalgene 500; Rhône Mérieux, Lyon, France) and xylazine (Rompun 2%; Bayer, Cu-teaux, France). Mice were sacrificed 5 days after challenge, coincident with previously characterized peak RSV lung titers [21].

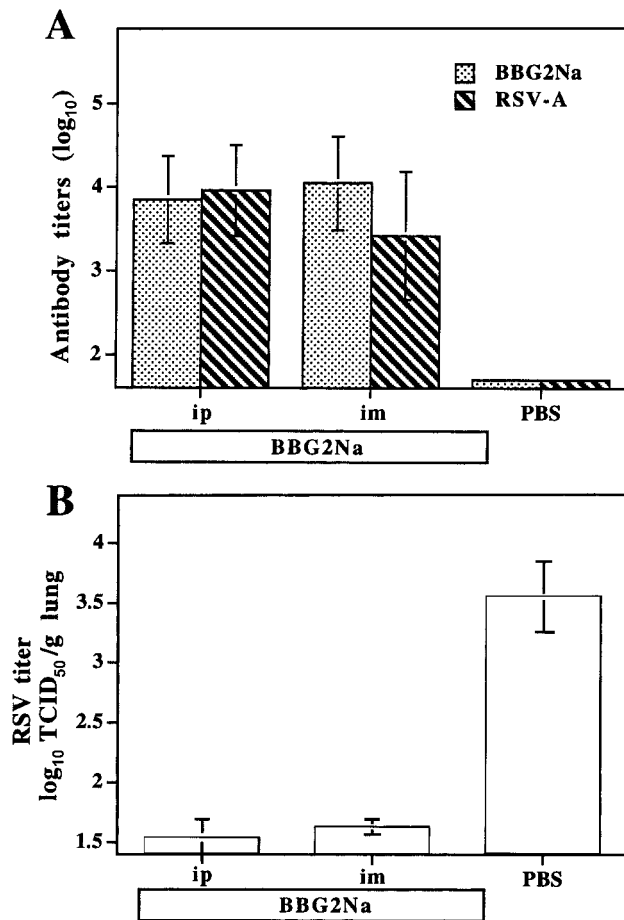
**Quantification of vaccine- and RSV-specific antibodies.** BBG2Na- and RSV-A-specific IgG and subclass antibodies were determined by ELISA. In brief, flat-bottomed wells of microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50  $\mu\text{L}$  of a 4  $\mu\text{g}/\text{mL}$  solution of BBG2Na in PBS or at 37°C and low humidity with 50  $\mu\text{L}$  of RSV-A or HEP-2 cell protein preparations previously calibrated according to their reactivity with relevant reference sera. After a wash with PBS-0.05% Tween 20 and blocking with 4% milk solids, serial dilution of sera were added and incubated for 2 h at room temperature. The relevant isotype-specific peroxidase-conjugated goat or rabbit anti-mouse (Zymed Laboratories, South San Francisco) was added for 2 h at 37°C prior to incubation with substrate. Optical density results were expressed as antibody titers by reference to a titered reference serum representing a pool of sera from immunized adult mice. As the virus stocks contained cellular antigen, anti-RSV-A-specific antibody titers of mice infected with RSV-A were calculated by subtracting anti-HEP-2 optical density values from those of anti-RSV-A.

**Animal sample preparation and virus titration.** Animals were anesthetized as described above and exsanguinated by cardiac puncture. Lung removal, lung homogenate preparation, and virus titration in the homogenates were done as previously described [18]. The limit of detection for lung tissues was  $\log_{10}$  1.45 TCID<sub>50</sub>/g lung, except where insufficient lung homogenate was available. When no virus was detected, actual detection limits were used for statistical analyses. Thus, standard deviations  $>0$  were occasionally recorded for lung titers of some virus-free animal groups. Lungs were considered protected when virus titers were reduced by  $\geq 2 \log_{10}$  relative to control mice.

**Statistical analysis.** Unless otherwise indicated, significance analyses of antibody levels from various groups of mice were done by Wilcoxon rank test. For statistical analyses of viral titers, we used *t* or Kolmogorov-Smirnov (for low sample numbers) tests and  $\chi^2$  tests (Statigraphic software program; Manugistics, Rockville, MD). *P*  $> .05$  was considered insignificant.

## Results

**Protective efficacy of neonatal immunization with BBG2Na.** To assess whether BBG2Na immunization could induce protective immune responses in the first week of life, BALB/c pups were immunized at age 1 week with a single dose of BBG2Na (20  $\mu\text{g}$ ) adsorbed to aluminum phosphate (AlPO<sub>4</sub>), the adjuvant formulation selected for clinical trials. Immunization was done either ip or intramuscularly (im), routes likely to be adopted in humans. Age-matched control pups were given PBS ip. Mice were bled 4 weeks after immunization to determine BBG2Na- and RSV-A-specific antibody titers. Significant IgG antibody responses were elicited by a single neonatal immunization in 22 mice (figure 1A); no vaccine-specific antibodies were detected in 1 mouse. This mouse was considered inefficiently immunized and excluded from further analyses. Under the conditions described, vaccine-specific and RSV-A-specific IgG antibody responses were similar regardless of immunization route. All PBS-injected controls remained seronegative (figure 1A). However,



**Figure 1.** Immunogenicity and protective efficacy of neonatal BBG2Na immunization. Mice were immunized intraperitoneally (ip) or intramuscularly (im) at age 1 week with 20  $\mu$ g of BBG2Na resuspended in AlPO<sub>4</sub>. Age-matched control pups received PBS ip. *A*, BBG2Na- and RSV-A-specific antibodies were measured by ELISA 4 weeks after immunization and expressed as antibody titers by reference to titered reference serum. *B*, Mice were sacrificed 5 days after intranasal RSV-A challenge for virus titration in lung homogenates as described in Materials and Methods. Results are expressed as log<sub>10</sub> TCID<sub>50</sub>/g lung tissue.

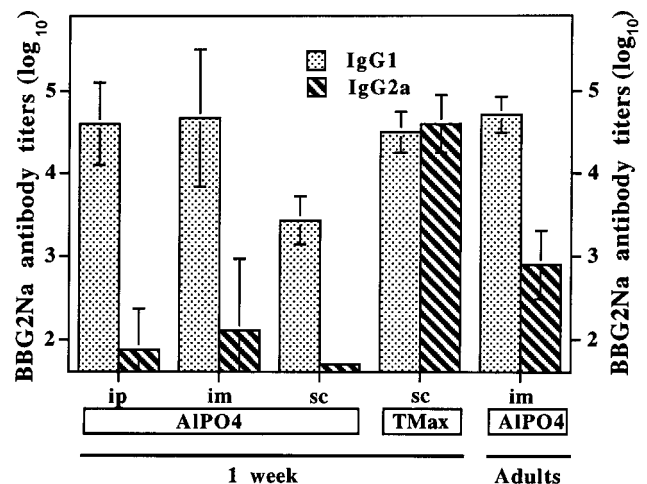
as previously observed in mice immunized ip with AlOH-BBG2Na, BBG2Na antibody responses remained significantly lower than those elicited in 28 adult mice whose antibody titers were already 10-fold higher ( $4.98 \pm 0.33$  log<sub>10</sub>) 2 weeks after ip immunization.

Immune and control litters were simultaneously challenged intranasally 4 weeks after immunization (i.e., at age 5 weeks). All animals remained asymptomatic and were sacrificed 5 days later to measure RSV-A lung titers. High lung virus titers were measured in all PBS control pups (figure 1B). In contrast, lung protection was observed in all 20 pups immunized ip or im with BBG2Na; 19 of 20 pups were virus-free (the remaining pup was positive for virus at the limit of detection of the assay).

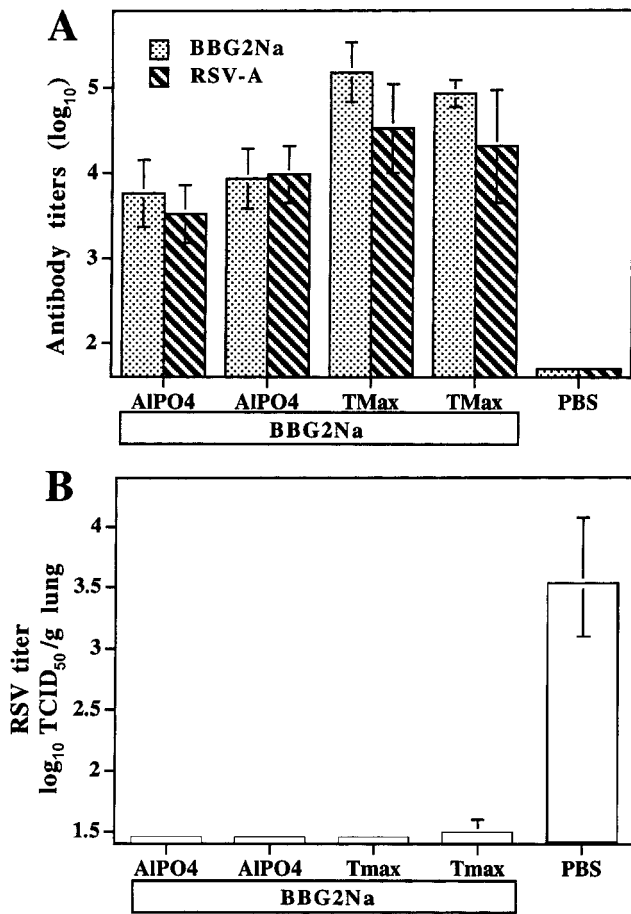
No difference in protective efficacy was observed with ip or im immunization.

*Influence of adjuvant formulation on immunogenicity and protective efficacy of neonatal BBG2Na immunization.* We did comparative analyses of the IgG1 and IgG2a subclass distribution of vaccine-specific antibodies (selected as indirect markers of Th1-Th2 response type) 4 weeks after immunization. After neonatal AlPO<sub>4</sub>-BBG2Na immunization, vaccine antibodies were almost exclusively among the IgG1 and only rarely (4/23 weakly positive mice) among the IgG2a isotypes (figure 2). This was not significantly influenced by the immunization route and contrasted with the presence of IgG2a antibodies in all 28 mice immunized as adults (figure 2).

We thus asked whether a change of adjuvant formulation (selecting a product known to induce strong Th1 responses in either adult or young mice [22] in contrast to aluminum salts, which preferentially support Th2 responses both in mice and humans [23]) would affect immunogenicity and protective efficacy of neonatal BBG2Na immunization. We immunized 1-week-old pups with BBG2Na resuspended in TiterMax, an adjuvant formulation composed of block copolymers in a water-in-oil emulsion, prior to subcutaneous (sc) immunization. Control pups were given AlPO<sub>4</sub>-BBG2Na sc. Following sc AlPO<sub>4</sub>-BBG2Na immunization, vaccine antibodies were again exclusively of the IgG1 isotype (figure 2). In contrast, in pups immunized in the presence of TiterMax, BBG2Na-specific antibodies were similarly distributed among the IgG1 and IgG2a isotypes, suggesting successful induction of balanced Th1-Th2 responses (figure 2).



**Figure 2.** Isotype distribution of antibodies after neonatal BBG2Na immunization. Mice were immunized at age 1 week or as adults with 20  $\mu$ g of BBG2Na resuspended in AlPO<sub>4</sub> and given intraperitoneally (ip), intramuscularly (im), or subcutaneously (sc) or with the same dose of BBG2Na resuspended in TiterMax (TMax). IgG1 and IgG2a BBG2Na-specific antibodies were measured by ELISA 4 weeks after immunization and expressed as antibody titers by reference to titered reference serum.



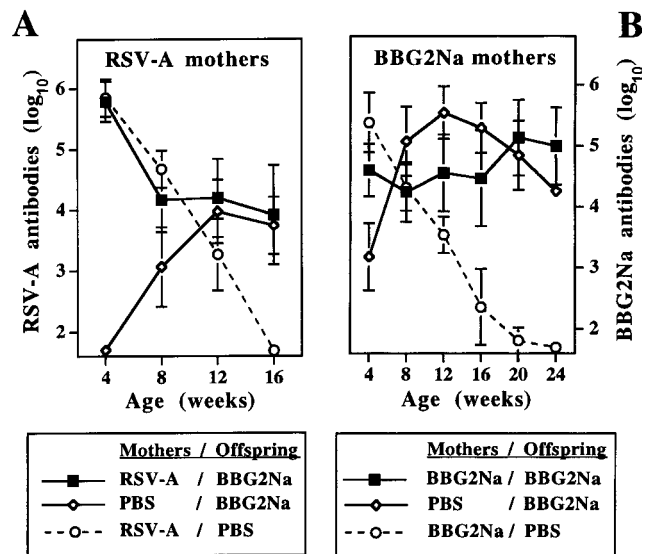
**Figure 3.** Influence of adjuvant on immunogenicity and protective efficacy of neonatal BGG2Na immunization. Mice were immunized subcutaneously (sc) at age 1 week with 20  $\mu$ g of BGG2Na resuspended in AIPO<sub>4</sub> or in TiterMax (Tmax) as indicated. *A*, Age-matched control pups received PBS sc; BGG2Na- and RSV-A-specific antibodies were measured by ELISA 4 weeks after immunization and expressed as antibody titers by reference to titered reference serum. *B*, Mice were sacrificed 5 days after intranasal RSV-A challenge, and virus titers in lung homogenates were determined as described in Materials and Methods. Results are expressed as log<sub>10</sub> TCID<sub>50</sub>/g lung tissue.

To assess whether this change of immunogenicity would affect responses to intranasal RSV challenge, litters immunized sc with BGG2Na-AIOH (2 litters) or BGG2Na-TiterMax (2 litters) and PBS-immunized controls were challenged simultaneously 4 weeks after immunization. All mice remained asymptomatic. In correlation with induction of vaccine-specific and RSV-A-specific antibodies (figure 3A), high lung virus titers were measured in all seronegative nonimmune control pups (figure 3B). In contrast, lung protection was again observed in pups immunized with BGG2Na, regardless of the adjuvant formulation used for neonatal immunization: all 28 challenged pups were protected, and 27 of 28 remained virus-free.

*Influence of maternal antibodies on protective efficacy of neo-*

*natal BGG2Na immunization.* To assess whether high levels of RSV-A antibodies of maternal origin would influence BGG2Na vaccine-induced protection, BALB/c female mice were immunized either 3 times with RSV-A or twice with BGG2Na prior to mating to induce high levels (5–6 log<sub>10</sub>) of RSV-A antibodies. Pups born to immune or control nonimmune mothers were immunized im with AIPO<sub>4</sub>-BGG2Na at age 1 week and boosted 3 weeks later, following a protocol previously shown to induce long-lasting vaccine responses in pups of nonimmune mothers (not shown). Control pups born to immune mothers received only PBS to allow evaluation of the kinetics of maternal antibody decline. All mice were bled at regular intervals for determination of RSV-A-BGG2Na antibodies.

In pups of RSV-A-immunized mothers (figure 4A), RSV-A antibodies of maternal origin were initially very high (5.5–6.0 log<sub>10</sub>). As expected, they steadily declined over 16 weeks in nonimmunized control pups. In contrast, in pups of RSV-A-immunized mothers that were immunized with BGG2Na in early life, RSV-A antibodies initially declined over 8 weeks but remained elevated when they reached the antibody titer (~4 log<sub>10</sub>) that corresponded to normal antibody responses induced in pups of nonimmune mothers (figure 4A). Similar patterns were observed in experiments with pups of BGG2Na-immunized mothers (figure 4B). Vaccine antibodies of maternal origin



**Figure 4.** Influence of maternal antibodies on immune responses to BGG2Na. Pups born to RSV-A- or BGG2Na-immunized mothers (*A* and *B*, respectively) were immunized intramuscularly at age 1 week with AIPO<sub>4</sub>-BGG2Na and boosted 4 weeks later; controls received PBS. Age-matched pups of control seronegative mothers were immunized as controls. Mice were bled monthly for determination of RSV-A (*A*) and BGG2Na (*B*) antibodies by ELISA. Results (representative of 2–4 litters) are expressed as antibody titers by reference to titered reference serum.

slowly declined from 5.5 log<sub>10</sub> to undetectable levels in non-immunized control pups of immune mothers; they remained at high levels in BBG2Na-immunized pups of immune mothers. Although BBG2Na antibody responses occasionally and transiently reached higher titers in control pups of nonimmune mothers, they stabilized at the same antibody level as that induced in pups of immune mothers.

To allow evaluation of the protection induced by neonatal immunization without any interference from residual antibodies of maternal origin, which can confer passive protection [19], intranasal RSV-A challenge was scheduled only after disappearance of maternal antibodies in nonimmunized pups of RSV-A-BBG2Na immune mothers, that is, at >16–20 weeks. Challenge was done in 4 successive experiments: each included immunized and control pups of immune mothers and immunized pups of control mothers. For offspring of RSV-A-immunized mothers (table 1, part A), high RSV-A virus titers were present after challenge in lungs of nonimmunized pups of immune mothers, confirming that antibodies of maternal origin had fallen below protective levels. In contrast, all 22 BBG2Na-immunized pups were protected, whether immunization was done in the absence or presence of high RSV-A maternal an-

tibodies. Twenty-one (96%) of these 22 immunized pups were virus-free when tested 5 days after challenge.

In the second series of experiments, we evaluated protective efficacy in pups of BBG2Na-immune or control mothers (table 1, part B). High RSV-A virus titers were present in the lungs of 12 of 13 nonimmunized pups of BBG2Na-immune mothers. Among pups of control seronegative mothers, 16 of 16 challenged pups were protected from RSV-A by BBG2Na immunization, while 13 (81%) of 16 were virus-free. For pups immunized in the presence of high levels of BBG2Na maternal antibodies, 25 (86%) of 29 were protected from intranasal RSV-A challenge, while 20 (69%) of 29 were virus-free. Although the protective efficacy of BBG2Na immunization in pups of BBG2Na mothers (table 1, part B) was not significantly different ( $P = .1$ ) from that in pups of RSV-A-exposed mothers (table 1, part A), complete protection (i.e., no detectable virus 5 days after challenge) significantly differed between groups (96% vs. 69%,  $P = .02$ ). RSV-A antibodies present immediately before viral challenge in the 4 unprotected pups born to BBG2Na-immune mothers were low (2.1 and 2.4 log<sub>10</sub>) in 2 unprotected pups but reached significant levels (3.2 and 4.8 log<sub>10</sub>) in the remaining 2 mice.

**Table 1.** Influence of maternal antibodies on protective efficacy of neonatal BBG2Na immunization.

Immunogen		RSV-A titer (mean ± SD)		No. virus free/no. challenged
To mothers	To offspring	Antibody <sup>a</sup>	Lung <sup>b</sup>	
<b>Part A</b>				
Experiment 1				
a. RSV-A	PBS	<1.7 ± 0.0	4.45 ± 0.29	0/4
b. RSV-A	BBG2Na	3.8 ± 0.7	1.45 ± 0.0 <sup>c</sup>	4/4
c. None	BBG2Na	3.1 ± 1.0	1.56 ± 0.3 <sup>c</sup>	5/6
Experiment 2				
a. RSV-A	PBS	<1.7 ± 0.0	4.40 ± 0.33	0/5
b. RSV-A	BBG2Na	4.0 ± 0.9	1.45 ± 0.0 <sup>d</sup>	7/7
c. None	BBG2Na	3.9 ± 0.5	1.47 ± 0.0 <sup>d</sup>	5/5
<b>Part B</b>				
Experiment 3				
a. BBG2Na	PBS	<1.7 ± 0.0	3.77 ± 1.18	0/7
b. BBG2Na	BBG2Na	3.2 ± 0.7	1.53 ± 0.2 <sup>d</sup>	5/6
c. BBG2Na	BBG2Na	3.6 ± 0.3	1.45 ± 0.0 <sup>d</sup>	7/7
d. None	BBG2Na	3.5 ± 0.3	1.50 ± 0.1 <sup>d</sup>	4/5
e. None	BBG2Na	4.0 ± 0.5	1.45 ± 0.0 <sup>c</sup>	4/4
Experiment 4				
a. BBG2Na	PBS	<1.7 ± 0.0	4.45 ± 0.27	0/6
b. BBG2Na	BBG2Na	3.2 ± 0.9	2.01 ± 0.95 <sup>d</sup>	3/8
c. BBG2Na	BBG2Na	3.9 ± 0.6	1.92 ± 1.0 <sup>d</sup>	5/8
d. None	BBG2Na	4.5 ± 0.5	1.45 ± 0.0 <sup>d</sup>	5/7

NOTE. Offspring of immune and control mothers, previously injected at age 1 week with BBG2Na or PBS and boosted 3 weeks later, were bled for determination of RSV-A antibody titers. Litters were challenged through 4 independent experiments after disappearance of maternal antibodies in unimmunized control pups (>16 weeks), which were challenged simultaneously. Significance was determined for each litter (a–e) within each experimental group (experiments 1–4) relative to nonimmunized controls from immune mothers (litters a).

<sup>a</sup> ELISA titer, log<sub>10</sub>.

<sup>b</sup> RSV-A titer, log<sub>10</sub> TCID<sub>50</sub>/g lung.

<sup>c</sup>  $P < .05$  (nonparametric test; Kolmogorov-Smirnov test because of low sample numbers).

<sup>d</sup>  $P < .002$  ( $t$  test using null hypothesis).

## Discussion

The immaturity of the neonatal immune system and the presence of high titers of antibodies of maternal origin are two major obstacles to the development of vaccine-mediated protection against RSV lower respiratory tract infections [6, 7]. The immaturity of the immune system affects both innate and specific immune responses required for viral neutralization and clearance through mechanisms as yet only partly understood (reviewed in [24]). Among them, limitation of antigen-specific antibody responses is an important parameter. It affects infant responses to RSV antigens and is responsible for limited serologic responses of infants <6–9 months after RSV infection [7, 25] or immunization with RSV vaccine candidates [6]. Limitation of early life antibody responses to vaccines is also observed in murine models of neonatal immunization [26], which thus appear to be of interest for preclinical evaluation of vaccine antigens or formulations early in life. Accordingly, BBG2Na vaccine responses triggered in the first weeks of life were slower and 100-fold weaker than those induced in adult controls [19]. We show here that a single immunization with BBG2Na (whether ip, im, or sc) conferred complete protection against intranasal RSV challenge in mice, even when immunization was done at a stage of immune immaturity (i.e., age 1 week) considered to best approximate the immune capacity of human newborns (unpublished observations). Should the characteristics of BBG2Na allow the induction of equally strong protective immune responses in humans, which is currently being evaluated by clinical testing, its capacity to induce protective immune

responses early in life would meet an important requirement for prevention of severe RSV disease in infants.

Another parameter of the immaturity of early life immune responses is recognized in murine models as the preferential polarization of neonatal vaccine responses toward the Th2 phenotype, even in response to vaccine antigen formulations or delivery systems that induce balanced Th1-Th2 responses in adults [26–28]. This is currently thought to reflect the higher activation requirements of neonatal versus adult antigen presenting cells (APCs), which result in suboptimal APC–T cell interactions, rather than reflecting intrinsic limitations of neonatal T cells (reviewed in [24]). The bias of neonatal vaccine responses to ALOH-BBG2Na ip immunization [19] was also observed here by use of AlPO<sub>4</sub>-adsorbed BBG2Na, whether administered ip, im, or sc. Of importance, however, this Th2 polarization of neonatal responses did not affect BBG2Na protective efficacy, which is as strong after a single vaccine dose in early life as in adult mice [18].

Priming for preferential Th2 vaccine responses upon neonatal immunization also raises the concern of immunopotentialization of RSV disease at the time of subsequent natural viral infection. Components of the phenomenon observed in seronegative infants during the clinical trials of FI-RSV [8, 9] were similarly inducible in BALB/c mice and correlated with an intensified production of Th2-derived cytokines [29]. Furthermore, experiments that used vaccinia virus recombinant vectors [30–32] or purified protein preparations revealed that native G protein strongly induced Th2-type cytokine synthesis and lung immunopathology, even when used with the Th1-driving QS-21 adjuvant [32]. In addition, aluminum salts (the single adjuvants currently authorized for infant immunization) further contribute to a preferential Th2 polarization of vaccine responses [23]. Thus, it was important to ask whether a change in the adjuvant formulation could induce adult-like balanced Th1-Th2 responses to BBG2Na in early life. A change in the adjuvant formulation of BBG2Na was sufficient to restore the balanced IgG1-IgG2a antibody responses observed in adult mice, and this change of immunogenicity did not affect the protective capacity of neonatal immunization. Thus, a Th2 polarization of responses cannot be considered as an intrinsic limitation of all G protein-derived vaccines [30–32], and BBG2Na could be used with Th1-driving adjuvants if required for safety reasons. However, parallel studies recently showed that even AIOH-adsorbed BBG2Na did not prime for deleterious Th2-type anti-RSV immunopathologic responses at the time of RSV exposure in an adult BALB/c mouse model [33, 34]. If this immunopotentialization model can be adapted to the neonatal period as currently evaluated, it would enable further preclinical evaluation of the safety of neonatal BBG2Na immunization.

Because of the ubiquitous nature of RSV and the frequency of reinfections in adults, RSV antibodies of maternal origin are present at birth at low, moderate, or sometimes even high titers [25]. Passively transferred antibodies can confer protection from

RSV disease both in humans [35] and in murine models of maternal immunization with BBG2Na [19]. However, the protection conferred by maternal antibodies is transient, and prolonged protection requires associated infant immunization. Unfortunately, maternal antibodies may interfere with neonatal vaccine responses. This inhibition of antibody responses affects infant responses to RSV antigens [6, 7], as shown in rodent models that used either live RSV-A [36, 37], F- and G-expressing recombinant vaccinia viruses [38], or purified F and G glycoproteins [39]. In striking contrast, even high titers of RSV-A maternal antibodies (5 log<sub>10</sub>) did not inhibit induction of murine antibody responses to BBG2Na [19]. Accordingly, we show here that the protective capacity of neonatal BBG2Na immunization reached 100% in the presence of maternal antibodies induced by repeated exposure to RSV-A: 96% of pups were free of virus 5 days after challenge. We previously showed the capacity of BBG2Na immunization to induce strong antibody responses even in the presence of passively transferred maternal antibodies induced by intranasal RSV infection [19]. Intraperitoneal use of live RSV in alum induced antibody titers that were higher (5.5–6.0 log<sub>10</sub>) than those induced after repeat intranasal infection and readily transferred to the offspring, thereby resulting in higher levels of maternal anti-RSV-A antibodies in the neonates. To our knowledge, this is the first description of vaccine-induced protection from RSV challenge observed after early life immunization in the presence of high levels of RSV-A maternal antibodies.

Pups of BBG2Na-immunized mothers raised similarly high antibody responses to BBG2Na, and protective efficacy of neonatal BBG2Na immunization was high (86%). However, complete protection (no detectable virus 5 days after challenge) was observed significantly less often than in pups of RSV-A-exposed mothers (69% vs. 100%). This difference in BBG2Na neonatal protective efficacy following RSV-A or BBG2Na maternal immunization is unlikely to reflect differences in induction of T cell vaccine responses, since maternal antibodies were shown in parallel immunization models not to alter neonatal T cell responses to vaccines [40]. Differences in maternal antibody titers present in pups at the time of immunization cannot account for this difference in protective efficacy. A more attractive hypothesis is that the capacity of BBG2Na to largely escape inhibition by RSV-A maternal antibodies reflects an immunogenicity of the nonglycosylated G2Na fragment that is partly different from that of the native RSV G protein. Since inhibition of infant antibody responses by maternal antibodies is epitope-specific, their influence is indeed greater when the same vaccine antigen is used in both mothers and infants [41]. We therefore suggest that binding of BBG2Na-induced maternal antibodies to certain specific protective epitopes of BBG2Na could inhibit the induction of infant responses to such epitopes, occasionally reducing protective efficacy. Given the interest in maternal immunization to prevent early RSV infection, analysis of the hierarchy of epitope recognition following neonatal

BBG2Na immunization of pups of control, RSV-A- or BBG2Na-immunized mothers by use of peptide-based ELISA and pepscan analyses is ongoing. Should a modification of epitope recognition be identified following immunization in the presence of high levels of maternal antibodies to BBG2Na, it could contribute to the identification of protective B cell epitopes in BBG2Na.

In conclusion, this study provides a promising preclinical evaluation of the capacity of BBG2Na immunization to induce protection from RSV lower respiratory disease in early life. Its unique features allow BBG2Na to induce protective immune responses even after a single immunization given at an early stage of immune maturation, whether adsorbed to aluminum salts or given with Th1-driving adjuvant formulations. It also suggests that the distinct immunogenicity of BBG2Na could allow induction of protective vaccine responses early, even in presence of high titers of RSV-A maternal antibodies. These distinct properties make BBG2Na an interesting RSV vaccine candidate.

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