

# Concomitant Administration of *Mycobacterium bovis* BCG with the Meningococcal C Conjugate Vaccine to Neonatal Mice Enhances Antibody Response and Protective Efficacy<sup>∇</sup>

Siggeir F. Brynjolfsson,<sup>1,2</sup> Stefania P. Bjarnarson,<sup>1,2</sup> Elena Mori,<sup>3</sup>  
Giuseppe Del Giudice,<sup>3</sup> and Ingileif Jonsdottir<sup>1,2,4\*</sup>

*Landspítali, Department of Immunology, Reykjavik, Iceland<sup>1</sup>; University of Iceland, Faculty of Medicine, Reykjavik, Iceland<sup>2</sup>;  
Novartis Vaccines and Diagnostics, Siena, Italy<sup>3</sup>; and deCODE genetics, Reykjavik, Iceland<sup>4</sup>*

Received 21 June 2011/Returned for modification 12 July 2011/Accepted 1 September 2011

***Mycobacterium bovis* BCG is administered to human neonates in many countries worldwide. The objective of the study was to assess if BCG could act as an adjuvant for polysaccharide-protein conjugate vaccines in newborns and thereby induce protective immunity against encapsulated bacteria in early infancy when susceptibility is high. We assessed whether BCG could enhance immune responses to a meningococcal C (MenC) conjugate vaccine, MenC-CRM<sub>197</sub>, in mice primed as neonates, broaden the antibody response from a dominant IgG1 toward a mixed IgG1 and IgG2a/IgG2b response, and increase protective efficacy, as measured by serum bactericidal activity (SBA). Two-week-old mice were primed subcutaneously (s.c.) with MenC-CRM<sub>197</sub>. BCG was administered concomitantly, a day or a week before MenC-CRM<sub>197</sub>. An adjuvant effect of BCG was observed only when it was given concomitantly with MenC-CRM<sub>197</sub>, with increased IgG response ( $P = 0.002$ ) and SBA (8-fold) after a second immunization with MenC-CRM<sub>197</sub> without BCG, indicating increased T-cell help. In neonatal mice (1 week old) primed s.c. with MenC-CRM<sub>197</sub> together with BCG, MenC-polysaccharide (PS)-specific IgG was enhanced compared to MenC-CRM<sub>197</sub> alone ( $P = 0.0015$ ). Sixteen days after the second immunization with MenC-CRM<sub>197</sub>, increased IgG ( $P < 0.05$ ), IgG1 ( $P < 0.05$ ), IgG2a ( $P = 0.06$ ), and IgG2b ( $P < 0.05$ ) were observed, and only mice primed with MenC-CRM<sub>197</sub> plus BCG showed affinity maturation and detectable SBA (SBA > 128). Thus, vaccination with a meningococcal conjugate vaccine (and possibly with other conjugates) may benefit from concomitant administration of BCG in the neonatal period to accelerate and enhance production of protective antibodies, compared to the current infant administration of conjugate which follows BCG vaccination at birth.**

*Neisseria meningitidis* remains a worldwide threat and annually causes ~1.2 million cases of meningococcal disease, claiming 135,000 lives (63). Although significant advances have been made in the development and coverage of vaccines, the disease burden remains high, highest in the meningitis belt in sub-Saharan Africa, where epidemics occur in waves lasting 3 to 4 years, the last in 2009 (64). Due to the immaturity of the immune system and decline of maternal antibodies (Abs), the incidence of meningococcal disease peaks in the first year of life, although it varies between countries due to differences in serogroup distributions (21, 30), and mortality is highest in infancy and for teenagers (50). Thus, it is of utmost importance to design early-life vaccination strategies that induce protective immunity in early infancy as well as long-lasting immunological memory against these major pathogens.

Meningococcal polysaccharide (PS) vaccines have been available since the 1960s (42). However, polysaccharides are T-cell-independent antigens, are poorly immunogenic in children under 2 years of age (54), and may induce hyporesponsiveness (11, 15, 32, 34).

In the 1980s conjugate vaccines were developed, in which the PS is conjugated to a protein carrier (46). Conjugate vac-

cines are T-cell dependent (TD) (53), and unlike PS vaccines they are immunogenic in young infants, induce immunological memory (45), reduce carriage (11, 60), and offer long-term protection (33, 34).

Similar to most protein vaccines (reviewed in reference 51), immune responses to conjugate vaccines are age dependent in both mice (4, 25) and humans (9). Meningococcal serogroup C (MenC) conjugate vaccines are immunogenic in infants and have successfully been introduced into many countries worldwide (55). Two quadrivalent meningococcal conjugate vaccines (serogroups A, C, W-135, and Y) are licensed. In clinical trials, their immunogenicity was considered modest (MenACWY-D) (44) or sufficient (52) in infants, the age group most vulnerable to meningococcal disease. However, their use is recommended in the United States for vaccination of all adolescents 11 to 18 years of age and for those aged 2 to 55 years who are at increased risk of meningococcal disease (41). In April 2011 Menectra was approved for use in infants over the age of 9 months but is not yet recommended by the CDC. Hence, well-tolerated effective adjuvants may enhance and accelerate immune responses to conjugate vaccines in neonates and infants (24, 25, 38), the age group most vulnerable to meningococcal disease. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is administered to human neonates in many countries worldwide. It is by far the most widely used vaccine worldwide, and since it was first introduced in 1921, more than 3 billion doses have been delivered (1). BCG is efficacious against tu-

\* Corresponding author. Mailing address: Department of Immunology, Landspítali, Hringbraut, 101 Reykjavik, Iceland. Phone: 354-543 5800. Fax: 354-543 4828. E-mail: ingileif@lsh.is.

<sup>∇</sup> Published ahead of print on 7 September 2011.

berculosis (TB) in infants and young children, but protection against adolescent and adult tuberculosis, the most prevalent form of the disease, is insufficient (6, 28). Newborns immunized with BCG show increased Th1-type responses, with similar gamma interferon (IFN- $\gamma$ ) production by CD4<sup>+</sup> T cells, as when BCG is given later in life (35, 61). BCG has also been shown to increase B- and T-cell responses to unrelated antigens in early life (39).

Maturation and responses of the immune system of 1- and 3-week-old mice correspond to those of human neonates and infants, respectively (31, 51). We have previously reported on age-dependent antibody production, B-cell, T-cell, and dendritic cell activation, and immunological memory elicited by pneumococcal conjugate vaccines in neonatal and infant mice (4, 20, 24, 25). If BCG has adjuvant effects on neonatal responses to meningococcal conjugate vaccines, it would be beneficial to administer these vaccines combined to neonates in areas where both *Mycobacterium tuberculosis* and *Neisseria meningitidis* are endemic and disease burden is high.

We investigated the ability of BCG to act as an adjuvant and enhance the antibody responses to the monovalent MenC-CRM<sub>197</sub> in mice primed as neonates, as well as its effect on antibody, affinity, and protective efficacy, measured as serum bactericidal activity (SBA). We also assessed the ability of BCG to direct the immune responses from a dominant Th2-associated IgG1 that is characteristic for neonatal responses toward a mixed response of IgG1 versus IgG2a and IgG2b (Th2 versus Th1 associated). In mice IgG2a and IgG2b are potent activators of complement and confer protection against encapsulated pathogens (58). Since meningococci do not infect mice, the effect of BCG on the protective efficacy of MenC-CRM<sub>197</sub> could not be studied *in vivo*. Therefore, SBA was measured and a titer of 128 used as a correlate of protection (5). Two-week-old mice were primed subcutaneously (s.c.) with MenC-CRM<sub>197</sub>. BCG was administered concomitantly, 1 day or 1 week before MenC-CRM<sub>197</sub> priming. The results show that BCG functions only as an adjuvant if given concomitantly with the MenC-CRM<sub>197</sub> conjugate. In the next set of experiments, mice were primed as neonates (1 week old) with MenC-CRM<sub>197</sub> with or without concomitant administration of BCG.

#### MATERIALS AND METHODS

**Animals.** Adult NMRI mice were purchased from M&B AS (Ry, Denmark) and kept in microisolator cages with free access to commercial food pellets and water. They were housed under standardized conditions at the Institute of Experimental Pathology at Keldur (Reykjavik, Iceland) with regulated daylight, humidity, and temperature. Breeding cages were checked daily for new births, and the pups kept with their mothers until weaning at the age of 4 weeks. The study was authorized by the Animal Experimental Committee of Iceland.

**Vaccines and adjuvants.** The monovalent meningococcal polysaccharide C conjugated to CRM<sub>197</sub> (MenC-CRM<sub>197</sub>), a nontoxic variant of diphtheria toxinoid produced by site-directed mutagenesis (10), was provided by Novartis Vaccines & Diagnostics (Siena, Italy). BCG was purchased from Statens Serum Institut (Copenhagen, Denmark).

**Immunizations.** For the initial set of experiments, 2-week-old mice, 8 per group, were immunized s.c. with 2.5  $\mu$ g MenC-CRM<sub>197</sub>. BCG (1 to 2  $\times$  10<sup>7</sup> CFU) was administered concomitantly, 1 day before or 1 week before MenC-CRM<sub>197</sub> immunization. Sixteen days later a second dose of MenC-CRM<sub>197</sub> without BCG was administered s.c. For a second set of experiments, neonatal (1-week-old) mice, 8 per group, were immunized with 2.5  $\mu$ g MenC-CRM<sub>197</sub> s.c., and BCG (2 to 8  $\times$  10<sup>5</sup> CFU) was given concomitantly. A 50- $\mu$ l vaccine solution was injected in the scapular girdle. Sixteen days later the mice received a second s.c. dose of MenC-CRM<sub>197</sub> (2.5  $\mu$ l). Age-matched mice that received saline were used as

controls. In a third set of experiments, mice primed as neonates with MenC-CRM<sub>197</sub> with or without BCG received a second and third MenC-CRM<sub>197</sub> immunization 16 and 28 days after priming. Mice were bled from the tail vein at 3 weeks of age (not possible to obtain samples earlier) and weekly until 6 weeks of age. Serum was isolated and stored at -20°C until analyses were performed. Each set of experiment was performed at least twice, with comparable results.

**Antibody measurement.** MenC-PS-specific antibodies (IgG, IgG1, IgG2a, and IgG2b) were measured essentially as previously described (13). Microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 5.0  $\mu$ g of purified meningococcal type C capsular PS (Novartis Vaccines and Diagnostics) in phosphate-buffered saline (PBS) with methylated human serum albumin (5  $\mu$ g/ml) (NIBSC, South Mimms, United Kingdom) and incubated overnight at 4°C. The plates were then blocked with 1% (wt/vol) gelatin (BDH Chemicals Ltd., Poole, United Kingdom) in PBS (pH 7.2) and incubated for 3 h at 37°C. Following fixation with 10% (wt/vol) saccharose (Merck, Darmstadt, Germany) and 4% (wt/vol) polyvinylpyrrolidone (Sigma, St. Louis, MO) for 2 h at room temperature, the plates were dried and stored at 4°C until use. Serum samples and standard were serially diluted in PBS-Tween (0.05%) containing 1% bovine serum albumin (BSA) (Sigma) in duplicate and incubated in MenC-PS-coated microtiter plates overnight at 4°C. After the plates were washed, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a, or IgG2b (Southern Biotechnology Associates, Inc., Birmingham, AL) was added and incubated for 3 h at 37°C. For development of the enzyme reaction, 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was incubated for 10 min according to the manufacturer's instructions, and the reaction stopped by 0.18 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450 nm in an enzyme-linked immunosorbent assay (ELISA) spectrophotometer (Titertek Multiscan Plus MK II; ICN Flow Laboratories, Irvine, United Kingdom).

The results were calculated from standard curves constructed by hyperimmunized reference serum. The titer of the reference corresponded to the inverse of the dilution, giving an optical density of 1.0. Results are expressed as mean log ELISA units (EU)/ml  $\pm$  the standard deviation (SD) for each group and time point.

**Avidity.** Avidity of MenC-specific IgG was measured by an ELISA as described above, including a potassium thiocyanate (KSCN) elution step (16). After the serum incubation step, serial dilutions of KSCN (7.5 to 0.117 M) or PBS-Tween (100% binding) were added to the wells and incubated for 15 min at room temperature. The remaining bound Ab was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc.). The reaction was developed with *p*-nitrophenylphosphate (*p*-NPP) (Sigma) in diethanolamine buffer (pH 9.8), and absorbance was read at 405 nm.

Results are expressed as follows: avidity index (AI) = M KSCN needed to displace 50% of the bound antibodies.

**SBA.** Bactericidal antibodies were determined in serum pools from each group (equal volumes from each mouse) as previously described (3, 36). Briefly, *N. meningitidis* strain C11 was grown overnight at 37°C on chocolate agar plates (starting from a frozen stock) with 5% CO<sub>2</sub>. Colonies were collected and used to inoculate 7 ml of Mueller-Hinton broth containing 0.25% glucose to reach an optical density at 600 nm (OD<sub>600</sub>) of 0.05 to 0.06. The culture was incubated for approximately 1.5 h at 37°C with 5% CO<sub>2</sub> with shaking until the OD<sub>600</sub> reached 0.23 to 0.24. Bacteria were diluted in Gey's balanced salt solution (Sigma) and 1% (wt/vol) BSA (Sigma) at the working dilution of 10<sup>5</sup> CFU/ml. The total volume of the final reaction mixture was 50  $\mu$ l, with 25  $\mu$ l of a serial 2-fold dilution of test serum, 12.5  $\mu$ l of bacteria at the working dilution, and 12.5  $\mu$ l of baby rabbit complement. Controls included bacteria incubated with complement and immune sera incubated with bacteria and complement inactivated by heating at 56°C for 30 min. Immediately after the addition of the baby rabbit complement, the controls were plated on Mueller-Hinton agar plates using the tilt method (time zero). The plates were incubated for 1 h at 37°C with 5% CO<sub>2</sub> with rotation. Each sample was transferred to Mueller-Hinton agar plates as spots, whereas the controls were transferred to Mueller-Hinton agar plates using the tilt method (time 1). Agar plates were incubated for 18 h at 37°C with 5% CO<sub>2</sub>, and the colonies corresponding to time zero and time 1 were counted. The data were used to calculate the reciprocal serum dilution at which 50% of the bacteria are killed. The SBA detection limit is 16 and is indicated with a dotted line in Fig. 2b. Samples with undetectable SBA were arbitrarily assigned an SBA titer of 8, corresponding to half the detection limit. An SBA titer of 64 is considered protective (5).

**Statistical analysis.** The nonparametric Mann-Whitney test was used to compare log antibody titers and the AI between groups and time points. A *P* value of <0.05 was considered statistically significant.

## RESULTS

The ability of BCG to act as an adjuvant and enhance the immune responses to MenC-CRM<sub>197</sub> in an early-life mouse model was investigated. In the first set of experiments, 2-week-old mice were immunized with MenC-CRM<sub>197</sub> and the effect of BCG administration a week before priming and a day before or at the same time as MenC-CRM<sub>197</sub> immunization on immune response was assessed by comparing these mice with mice that received MenC-CRM<sub>197</sub> without BCG. The second immunization with MenC-CRM<sub>197</sub> was administered 16 days later to allow the germinal center reaction to be completed (40) and antibody levels to rise. Age-matched control mice received saline at both time points.

**The adjuvant activity of BCG is optimal if given concomitantly with MenC-CRM<sub>197</sub>.** Two-week-old mice that received BCG ( $1$  to  $2 \times 10^5$  CFU) concomitantly with MenC-CRM<sub>197</sub> showed significantly higher MenC-PS-specific IgG levels after the first dose than mice pretreated with BCG 1 day ( $P < 0.001$ ) or a week ( $P = 0.015$ ) before priming with MenC-CRM<sub>197</sub> (Fig. 1a). Only mice primed with MenC-CRM<sub>197</sub> and BCG concomitantly showed significantly higher MenC-PS-specific IgG levels than mice that received saline.

After the second immunization with MenC-CRM<sub>197</sub>, mice primed with MenC-CRM<sub>197</sub> and BCG concomitantly showed the highest MenC-PS-specific IgG levels (Fig. 1a), significantly higher than mice pretreated with BCG 1 day ( $P < 0.001$ ) or 7 days ( $P < 0.0015$ ) before MenC-CRM<sub>197</sub> priming and higher than mice that received only MenC-CRM<sub>197</sub> ( $P = 0.002$ ) or saline ( $P < 0.001$ ). The mice pretreated with BCG 1 day before MenC-CRM<sub>197</sub> priming showed MenC-PS-specific IgG levels that were lower than those of mice that were pretreated with BCG a week before priming ( $P = 0.038$ ), but not different from those of mice that received MenC-CRM<sub>197</sub> only. The bactericidal activity (SBA titer  $\geq 64$ ) and affinity maturation of MenC-PS-specific IgG were detected only after the second MenC-CRM<sub>197</sub> immunization in mice that received MenC-CRM<sub>197</sub> and BCG concomitantly for the neonatal priming (data not shown).

After the second immunization with MenC-CRM<sub>197</sub>, there was a shift in the ratio of IgG subclasses of MenC-PS-specific antibodies in mice primed with MenC-CRM<sub>197</sub> plus BCG concomitantly (Fig. 1b), from a dominant Th2-associated IgG1 to a more mixed IgG1, IgG2a, and IgG2b response. The mice that received MenC-CRM<sub>197</sub> plus BCG concomitantly had significantly higher IgG2a than mice that received BCG 1 day before MenC-CRM<sub>197</sub> priming ( $P = 0.006$ ). IgG2a was undetectable in mice that received BCG 7 days before MenC-CRM<sub>197</sub> and in mice that received no BCG. MenC-PS-specific IgG1 was also increased in mice that received MenC-CRM<sub>197</sub> plus BCG concomitantly compared to mice that received BCG 1 day before MenC-CRM<sub>197</sub> ( $P = 0.005$ ) or no BCG ( $P = 0.015$ ).

**Coadministration of BCG has an adjuvant effect on neonatal priming with MenC-CRM<sub>197</sub>.** In a second set of experiments we studied further if BCG had an adjuvant effect on neonatal priming with MenC-CRM<sub>197</sub>. MenC-CRM<sub>197</sub> was administered s.c. to 1-week-old mice with or without concomitant administration of BCG ( $4$  to  $8 \times 10^5$  CFU) s.c., and the second MenC-CRM<sub>197</sub> immunization was given 16 days later. Sixteen days after only one neonatal immunization, the mice primed

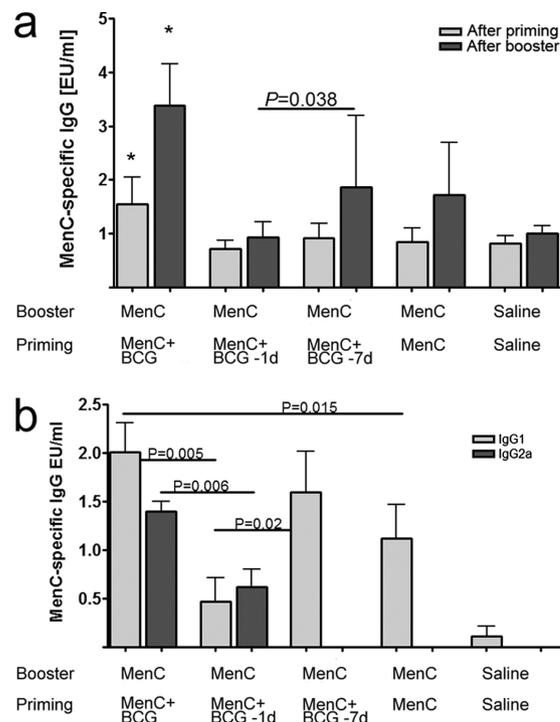


FIG. 1. Antibody response of mice immunized with MenC-CRM<sub>197</sub> at 2 weeks of age with or without BCG treatment and receiving a second immunization with MenC-CRM<sub>197</sub>. Mice received BCG a week before ( $-7$ d), a day before ( $-1$ d), or at the same time that MenC-CRM<sub>197</sub> was given at 2 weeks of age or did not receive BCG. Sixteen days later the mice received a second immunization with MenC-CRM<sub>197</sub>. Control mice received saline at both times. (a) MenC-PS-specific IgG levels 2 weeks after priming (light gray columns) and 2 weeks after the second immunization (dark gray columns). The results are presented as mean + SD for each group ( $n = 8$  per group) for one of two independent experiments showing comparable results. \*,  $P < 0.05$  compared to saline, MenC-CRM<sub>197</sub>, and other groups. (b) MenC-PS-specific IgG1 and IgG2a antibody levels 2 weeks after the second immunization with MenC-CRM<sub>197</sub>. The results are presented as mean + SD for each group ( $n = 8$  per group) for one of two independent experiments showing comparable results.

with MenC-CRM<sub>197</sub> plus BCG showed higher MenC-PS-specific IgG levels than mice that received MenC-CRM<sub>197</sub> ( $P = 0.0015$ ) or saline ( $P < 0.001$ ) (Fig. 2a), demonstrating, already in infancy, the beneficial effect of BCG on antibody levels.

Two weeks after the second immunization with MenC-CRM<sub>197</sub>, mice initially primed with MenC-CRM<sub>197</sub> plus BCG as neonates showed higher MenC-PS-specific IgG levels than mice primed with MenC-CRM<sub>197</sub> alone ( $P < 0.05$ ) and higher levels than control mice that received saline ( $P < 0.001$ ) (Fig. 2a). If an MenC-CRM<sub>197</sub> booster was administered 2 weeks after the second immunization, mice primed as neonates with MenC-CRM<sub>197</sub> plus BCG still showed higher MenC-PS-specific IgG levels than mice primed with MenC-CRM<sub>197</sub> ( $P < 0.05$ ) (data not shown).

**Effects of concomitant administration of BCG on MenC-PS-specific IgG subclasses, affinity maturation, and protective efficacy.** After the second immunization with MenC-CRM<sub>197</sub>, MenC-PS-specific IgG1 levels were higher in mice primed as neonates with MenC-CRM<sub>197</sub> plus BCG than in mice that received MenC-CRM<sub>197</sub> only ( $P < 0.05$ ) (Fig. 3a). MenC-PS-

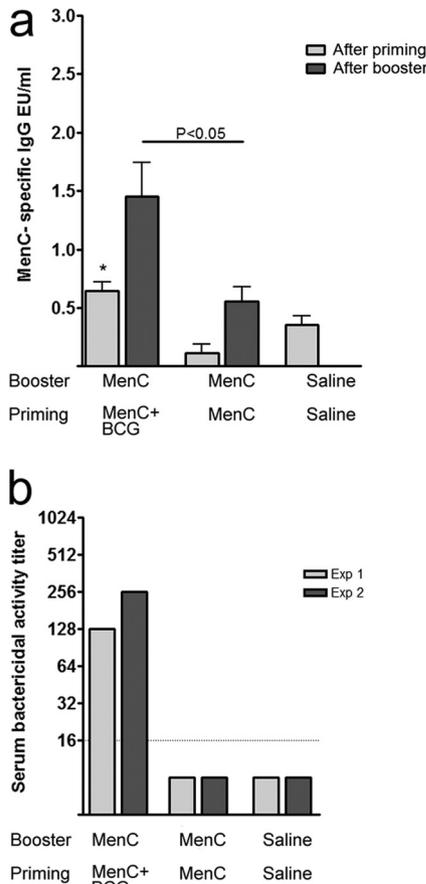


FIG. 2. Effect of BCG on MenC-PS-specific IgG response and protective capacity when administered with MenC-CRM<sub>197</sub> to neonatal mice. Mice received BCG at the same time as the priming with MenC-CRM<sub>197</sub> at 1 week of age. Sixteen days later the mice received a second immunization with MenC-CRM<sub>197</sub> without BCG. (a) MenC-PS-specific IgG levels 2 weeks after priming (light gray columns) and 2 weeks after the second immunization with MenC-CRM<sub>197</sub> (dark gray columns). The results are presented as mean + SD for each group ( $n = 8$  per group) for one of three independent experiments showing comparable results. \*,  $P < 0.05$  compared to saline and MenC-CRM<sub>197</sub> groups. (b) SBA induced by priming neonatal mice with MenC-CRM<sub>197</sub> with or without BCG, 2 weeks after the second immunization with MenC-CRM<sub>197</sub>. Immunizations are indicated at bottom. SBA was measured in pooled sera (equal volumes from each mouse within group;  $n = 8$  per group). SBA detection limit is 16 and is indicated with a dotted line. Samples with undetectable SBA are arbitrarily assigned an SBA titer of 8, corresponding to half the detection limit.

specific IgG2b levels were also higher in mice primed with MenC-CRM<sub>197</sub> plus BCG and reimmunized with MenC-CRM<sub>197</sub> than in mice primed with MenC-CRM<sub>197</sub> only ( $P < 0.05$ ), and IgG2a levels tended to be higher, although the difference was not significant ( $P = 0.06$ ) (Fig. 3a).

Two weeks after the second immunization with MenC-CRM<sub>197</sub>, the affinity maturation of MenC-PS-specific IgG was detected only when neonatal mice were primed with MenC-CRM<sub>197</sub> plus BCG (Fig. 3b). Accordingly, after the MenC-CRM<sub>197</sub> reimmunization, only mice primed with MenC-CRM<sub>197</sub> plus BCG had SBA titers of  $>128$  (Fig. 2b), which was used as a surrogate for protective efficacy (5).

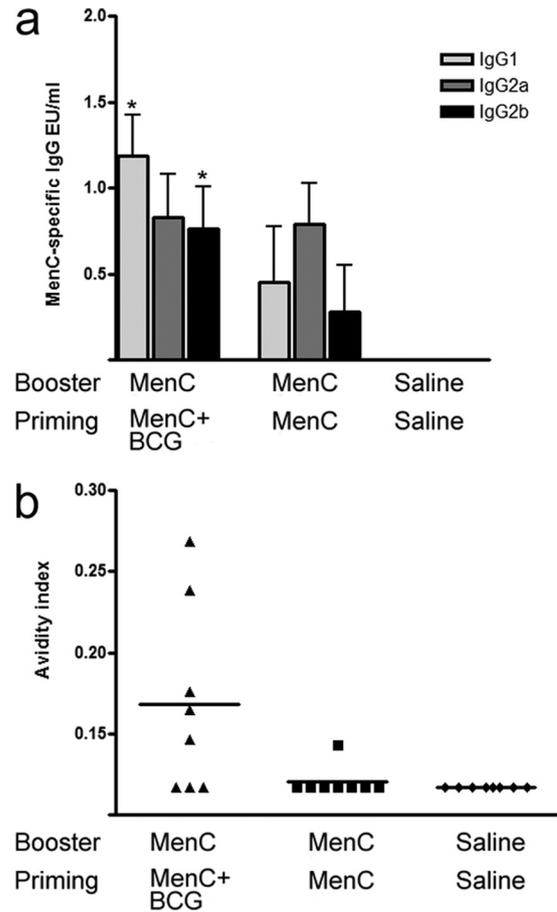


FIG. 3. The effect of BCG on IgG subclasses and avidity of MenC-PS-specific antibodies. (a) IgG subclasses of MenC-PS-specific antibodies 2 weeks after the second immunization with MenC-CRM<sub>197</sub>. Mice received BCG at the same time as the priming with MenC-CRM<sub>197</sub> at 1 week of age. Sixteen days later the mice received a second immunization with MenC-CRM<sub>197</sub> without BCG. Immunizations are indicated at bottom. IgG subclass levels (mean + SD) are presented for each group ( $n = 8$  per group) for one of three independent experiments showing comparable results. \*,  $P < 0.05$  compared to MenC-CRM<sub>197</sub> group. (b) AI of MenC-PS-specific IgG 2 weeks after the second immunization with MenC-CRM<sub>197</sub>. Immunizations are indicated at bottom. AIs (expressed as M KSCN) are shown for individual mice, and a line indicates the mean for each group. Results are presented for one of three independent experiments showing comparable results. The AI detection limit was 0.117 (0.117 M KSCN).

DISCUSSION

BCG vaccination has been shown to induce protective immunity against *M. tuberculosis* in mice, both when given s.c. (26) and intravenously (i.n.) (14), and the BCG vaccine dose in these models was comparable ( $10^6$  and  $10^5$ , respectively) to the dose used to assess adjuvant activity in our experiments. Protection against *M. tuberculosis* is dependent largely on innate immunity and Th1-mediated immunity, and an effect of BCG on antibodies to *M. tuberculosis* or heterologous antigens was not reported (26). In prime-boost protocols for tuberculosis vaccines, BCG has a dual function, as it primes for T-cell responses to antigens shared by BCG and *M. tuberculosis* and acts as an adjuvant for heterologous *M. tuberculosis* antigens not present in BCG (43). The cell wall skeleton of BCG in-

duces the maturation of dendritic cells (DC) through the involvement of Toll-like receptor 2 (TLR-2) and TLR-4 (56). The minimal structural unit of the peptidoglycan cell wall, muramyl dipeptide (MDP), is responsible for the TLR-2- and TLR-4-dependent DC maturation through the MyD88-dependent pathway (57). This adjuvant effect of MDP has been used successfully with hepatitis B virus surface antigen (HBsAg), enhancing the immune responses specific for the antigen (23). Adjuvants and BCG that stimulate dendritic cells to produce interleukin-12 (IL-12) (27), which is limited in neonates (18), may lead to stimulation of IL-21-producing follicular T-helper cells (Tfh) (49), the key helper cells for B cells responding to T-cell-dependent (TD) antigens, which would lead to an increase in all IgG subclasses (reviewed in reference 37).

We assessed whether BCG was able to act as an adjuvant for MenC-CRM<sub>197</sub> by analyzing its effects on MenC-PS-specific IgG levels and subclasses, avidity, and SBA. SBA has long been the accepted correlate of protection when evaluating the protective efficacy of different meningococcal vaccines in humans (17), and since humans are the only natural reservoir of meningococci, SBA is also used in murine models to determine the efficacy of vaccinations. The results demonstrate that BCG has a significant adjuvant effect when given concomitantly with MenC-CRM<sub>197</sub>, but no effect if administered a day or week before. The adjuvant effect of BCG during the priming of neonatal mice with MenC-CRM<sub>197</sub> is still significant after two reimmunizations with MenC-CRM<sub>197</sub>. The lack of adjuvant effect in mice pretreated with BCG a day or a week before MenC-CRM<sub>197</sub> might be due to the activation of dendritic cells by BCG, causing their migration to the lymph nodes and resulting in a suboptimal response at the time of the MenC-CRM<sub>197</sub> immunization. C57BL/6 mice are most often used in studies of immune responses to BCG or *Mycobacterium tuberculosis*, as they are inbred and susceptible to both bacteria (8), whereas higher challenge doses are needed for outbred white mice (12). Thus, the limited adjuvant effects of BCG on the response to MenC-CRM<sub>197</sub> could be the interplay between the BCG dose used and the natural BCG resistance of NMRI mice, leading to suboptimal stimulation of the immune system. Dendritic cells are a reservoir for BCG and IL-12 production by splenic dendritic cells, and induction of primary T-cell responses occurs only in the early phase of BCG infection (27), which is in agreement with our results on the need for concomitant administration of BCG to have significant adjuvant effect.

The second immunization was given 16 days after the priming, since it has been demonstrated that germinal center formation peaks at day 14 in mice primed with tetanus toxoid as neonates and at day 10 in mice primed as adults (40). Furthermore, the immunological maturation of 3-week-old mice corresponds to that of human infants (47, 48, 51). At 3 weeks of age, before the second immunization, the mice primed as neonates with MenC-CRM<sub>197</sub> plus BCG already had enhanced MenC-PS-specific IgG, indicating that the neonatal priming was more efficient than that in the mice that did not receive BCG during the priming. The second immunization in mice primed as neonates with MenC-CRM<sub>197</sub> plus BCG induced a rapid increase in MenC-PS-specific IgG, indicating efficient generation of memory by the neonatal priming compared to that in the mice that did not receive BCG or saline. Importantly,

BCG enhanced not only MenC-PS-specific IgG antibody levels in the mice, but also affinity maturation and functional activity of the antibodies, reflected in an 8-fold increase in SBA titers, to levels similar to those previously shown after two immunizations with MenC-CRM<sub>197</sub> together with the adjuvant LT-K63 or CpG (7). In mice primed as neonates with MenC-CRM<sub>197</sub> plus BCG concomitantly and boosted with MenC-CRM<sub>197</sub>, SBA titers of 128 and 256 were obtained. BCG also increased the avidity of the IgG antibodies and altered the IgG subclass distribution, enhancing IgG2b and IgG2a to a lesser extent, which were not induced by MenC-CRM<sub>197</sub> alone, in addition to enhancing IgG1 antibody levels significantly. The BCG-induced enhancement of IgG1 and IgG2b MenC-PS-specific antibodies suggests that BCG may induce IL-12 production by dendritic cells to stimulate Tfh, which are key inducers of the clonal expansion of B cells, antibody isotype switching, plasma cell differentiation, and the induction of germinal centers (reviewed in reference 37) possibly through enhanced production of IL-21, a fundamental cytokine for Tfh generation (62) and the switching of naive human B cells to IgG subclasses and IgA (2). IgG2a and IgG2b are the most effective complement-fixing isotypes (58), which may together with higher avidity contribute to increased SBA. SBA titers of 4 are considered protective in humans, but baby rabbit complement as used in this study gives a higher SBA than human complement (66), since neither rabbit nor murine factor H binds to the meningococcal surface (19). An SBA titer of  $\geq 128$  is considered protective, since baby rabbit complement was used in the killing assay (5). The increase in avidity and SBA and change in the distribution of the IgG subclasses indicate that the adjuvant effect of BCG is mediated, at least in part, through increased T-cell help which has been modulated from a predominantly Th2-biased to a more mixed Th1/Th2 response.

Studies of human infants have shown that BCG administered at birth enhances the IFN- $\gamma$  responses of CD4<sup>+</sup> cells to unrelated vaccines and increases antibody responses to hepatitis B virus surface antigen (HBsAg) (hepatitis B vaccine) and oral polio vaccine serotype 1 (OPV-1) even when given 2 months after BCG priming (39).

The discrepancy between these results reported for humans and the results from our murine study may be related to the different natures of the vaccines (protein versus polysaccharide-protein conjugate vaccines). Furthermore, in mice the appearance of B cells is delayed compared to that of T cells, the splenic architecture appears at day 6, and germinal centers appear 3 to 4 weeks after birth, whereas fetal thymocytes can respond to mitogens already at day 17 of gestation (22). In contrast, humans have functional B and T cells at birth. For example, neonates primed with an acellular pertussis vaccine at birth responded to a booster at 2 months of age with accelerated immune responses to all pertussis antigens (29), and neonates immunized with a 7-valent pneumococcal conjugate were able to prime antigen-specific T-cell responses (59). On the other hand, marginal zone B cells, which respond to T-cell-independent antigens, like polysaccharides, do not appear until after 2 years of age (65). We have previously shown that neonatal responses to the polysaccharide moiety of a pneumococcal conjugate vaccine are more limited than responses to its protein part, in antibody levels and the generation of memory

B cells and antibody-secreting cells in spleen and bone marrow, but can still be overcome by safe and effective adjuvants (4, 24) (S. P. Bjarnarson, B. C. Adarna, H. Benonisson, G. Del Giudice, and I. Jonsdottir, submitted for publication). Thus, stronger B-cell-stimulating adjuvants may be needed to sufficiently enhance the humoral response to conjugate vaccines.

Taken together, the results of this study show that BCG has adjuvant effects on the priming of neonatal mice, if given concomitantly with MenC-CRM<sub>197</sub>, which are comparable to those of effective adjuvants like CpG and LT-K63. Thus, vaccination with polysaccharide-protein conjugates against meningococcus and other encapsulated bacteria may benefit from concomitant administration with BCG in the neonatal period to accelerate and enhance production of protective antibodies.

#### ACKNOWLEDGMENTS

This study was supported by The Landspítali Research Fund and the Iceland Research Fund.

Giuseppe Del Giudice and Elena Mori are employees of Novartis Vaccines and Diagnostics.

#### REFERENCES

- Andersen, P., and T. M. Doherty. 2005. The success and failure of BCG—implications for a novel tuberculosis vaccine. *Nat. Rev. Microbiol.* **3**:656–662.
- Avery, D. T., V. L. Bryant, C. S. Ma, R. de Waal Malefyt, and S. G. Tangye. 2008. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J. Immunol.* **181**:1767–1779.
- Baudner, B. C., et al. 2002. Enhancement of protective efficacy following intranasal immunization with vaccine plus a nontoxic LTK63 mutant delivered with nanoparticles. *Infect. Immun.* **70**:4785–4790.
- Bjarnarson, S. P., et al. 2005. The advantage of mucosal immunization for polysaccharide-specific memory responses in early life. *Eur. J. Immunol.* **35**:1037–1045.
- Borrow, R., P. Balmer, and E. Miller. 2005. Meningococcal surrogates of protection—serum bactericidal antibody activity. *Vaccine* **23**:2222–2227.
- Brewer, T. F. 2000. Preventing tuberculosis with bacillus Calmette-Guérin vaccine: a meta-analysis of the literature. *Clin. Infect. Dis.* **31**(Suppl. 3):S64–S67.
- Brynjolfsson, S. F., S. P. Bjarnarson, E. Mori, G. Del Giudice, and I. Jonsdottir. 2008. Neonatal immune response and serum bactericidal activity induced by a meningococcal conjugate vaccine is enhanced by LT-K63 and CpG2006. *Vaccine* **26**:4557–4562.
- Chackerian, A. A., and S. M. Behar. 2003. Susceptibility to Mycobacterium tuberculosis: lessons from inbred strains of mice. *Tuberculosis (Edinb.)* **83**: 279–285.
- Clutterbuck, E. A., et al. 2008. Serotype-specific and age-dependent generation of pneumococcal polysaccharide-specific memory B-cell and antibody responses to immunization with a pneumococcal conjugate vaccine. *Clin. Vaccine Immunol.* **15**:182–193.
- Del Giudice, G. 2003. Vaccination strategies. An overview. *Vaccine* **21**(Suppl. 2):S83–S88.
- Dellicour, S., and B. Greenwood. 2007. Systematic review: impact of meningococcal vaccination on pharyngeal carriage of meningococci. *Trop. Med. Int. Health* **12**:1409–1421.
- Freudenstein, H., E. Weinmann, and I. Hill. 1988. Potency testing of BCG vaccines on white mice: influence of variables on survival time, lung findings and vaccine assessment. *Vaccine* **6**:315–327.
- Gheesling, L. L., et al. 1994. Multicenter comparison of Neisseria meningitidis serogroup C anti-capsular polysaccharide antibody levels measured by a standardized enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **32**: 1475–1482.
- Giri, P. K., I. Verma, and G. K. Khuller. 2006. Protective efficacy of intranasal vaccination with Mycobacterium bovis BCG against airway Mycobacterium tuberculosis challenge in mice. *J. Infect.* **53**:350–356.
- Gold, R., M. L. Lepow, I. Goldschneider, T. L. Draper, and E. C. Gotschlich. 1975. Clinical evaluation of group A and group C meningococcal polysaccharide vaccines in infants. *J. Clin. Invest.* **56**:1536–1547.
- Goldblatt, D., L. van Etten, F. J. van Milligen, R. C. Aalberse, and M. W. Turner. 1993. The role of pH in modified ELISA procedures used for the estimation of functional antibody affinity. *J. Immunol. Methods* **166**:281–285.
- Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein. 1969. Human immunity to the meningococcus. I. The role of humoral antibodies. *J. Exp. Med.* **129**:1307–1326.
- Goriely, S., et al. 2001. Deficient IL-12(p35) gene expression by dendritic cells derived from neonatal monocytes. *J. Immunol.* **166**:2141–2146.
- Granoff, D. M., J. A. Welsch, and S. Ram. 2009. Binding of complement factor H (FH) to Neisseria meningitidis is specific for human FH and inhibits complement activation by rat and rabbit sera. *Infect. Immun.* **77**:764–769.
- Hannesdottir, S. G., T. A. Olafsdottir, G. D. Giudice, and I. Jonsdottir. 2008. Adjuvants LT-K63 and CpG enhance the activation of dendritic cells in neonatal mice. *Scand. J. Immunol.* **68**:469–475.
- Harrison, L. H., et al. 2011. The Global Meningococcal Initiative: recommendations for reducing the global burden of meningococcal disease. *Vaccine* **29**:3363–3371.
- Holsapple, M. P., L. J. West, and K. S. Landreth. 2003. Species comparison of anatomical and functional immune system development. *Birth Defects Res. B Dev. Reprod. Toxicol.* **68**:321–334.
- Jain, V., S. P. Vyas, and D. V. Kohli. 2009. Well-defined and potent liposomal hepatitis B vaccines adjuvanted with lipophilic MDP derivatives. *Nanomedicine* **5**:334–344.
- Jakobsen, H., et al. 2002. Intranasal immunization with pneumococcal conjugate vaccines with LT-K63, a nontoxic mutant of heat-labile enterotoxin, as adjuvant rapidly induces protective immunity against lethal pneumococcal infections in neonatal mice. *Infect. Immun.* **70**:1443–1452.
- Jakobsen, H., et al. 2006. Early life T cell responses to pneumococcal conjugates increase with age and determine the polysaccharide-specific antibody response and protective efficacy. *Eur. J. Immunol.* **36**:287–295.
- Jeon, B. Y., et al. 2008. Mycobacterium bovis BCG immunization induces protective immunity against nine different Mycobacterium tuberculosis strains in mice. *Infect. Immun.* **76**:5173–5180.
- Jiao, X., et al. 2002. Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. *J. Immunol.* **168**:1294–1301.
- Kaufmann, S. H., G. Hussey, and P. H. Lambert. 2010. New vaccines for tuberculosis. *Lancet* **375**:2110–2119.
- Knuf, M., et al. 2008. Neonatal vaccination with an acellular pertussis vaccine accelerates the acquisition of pertussis antibodies in infants. *J. Pediatr.* **152**:655–660.
- Kvalsvig, A. J., and D. J. Unsworth. 2003. The immunopathogenesis of meningococcal disease. *J. Clin. Pathol.* **56**:417–422.
- Lambert, P. H., M. Liu, and C. A. Siegrist. 2005. Can successful vaccines teach us how to induce efficient protective immune responses? *Nat. Med.* **11**:S54–S62.
- Leach, A., et al. 1997. Induction of immunologic memory in Gambian children by vaccination in infancy with a group A plus group C meningococcal polysaccharide-protein conjugate vaccine. *J. Infect. Dis.* **175**:200–204.
- MacLennan, J., et al. 2001. Immunologic memory 5 years after meningococcal A/C conjugate vaccination in infancy. *J. Infect. Dis.* **183**:97–104.
- MacLennan, J., et al. 1999. Immune response to revaccination with meningococcal A and C polysaccharides in Gambian children following repeated immunisation during early childhood. *Vaccine* **17**:3086–3093.
- Marchant, A., et al. 1999. Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guérin vaccination. *J. Immunol.* **163**:2249–2255.
- Maslanka, S. E., et al. 1997. Standardization and a multilaboratory comparison of Neisseria meningitidis serogroup A and C serum bactericidal assays. The Multilaboratory Study Group. *Clin. Diagn. Lab Immunol.* **4**:156–167.
- McHeyzer-Williams, L. J., N. Pelletier, L. Mark, N. Fazilleau, and M. G. McHeyzer-Williams. 2009. Follicular helper T cells as cognate regulators of B cell immunity. *Curr. Opin. Immunol.* **21**:266–273.
- Olafsdottir, T. A., K. Lingnau, E. Nagy, and I. Jonsdottir. 2009. IC31, a two-component novel adjuvant mixed with a conjugate vaccine enhances protective immunity against pneumococcal disease in neonatal mice. *Scand. J. Immunol.* **69**:194–202.
- Ota, M. O., et al. 2002. Influence of Mycobacterium bovis bacillus Calmette-Guérin on antibody and cytokine responses to human neonatal vaccination. *J. Immunol.* **168**:919–925.
- Pihlgren, M., et al. 2003. Unresponsiveness to lymphoid-mediated signals at the neonatal follicular dendritic cell precursor level contributes to delayed germinal center induction and limitations of neonatal antibody responses to T-dependent antigens. *J. Immunol.* **170**:2824–2832.
- Poland, G. A. 2010. Prevention of meningococcal disease: current use of polysaccharide and conjugate vaccines. *Clin. Infect. Dis.* **50**(Suppl. 2):S45–S53.
- Pollard, A. J. 2004. Global epidemiology of meningococcal disease and vaccine efficacy. *Pediatr. Infect. Dis. J.* **23**:S274–S279.
- Rahman, M. J., and C. Fernandez. 2009. Neonatal vaccination with Mycobacterium bovis BCG: potential effects as a priming agent shown in a heterologous prime-boost immunization protocol. *Vaccine* **27**:4038–4046.
- Rennels, M., J. King, Jr., R. Ryall, T. Papa, and J. Froeschle. 2004. Dosage escalation, safety and immunogenicity study of four dosages of a tetravalent meningococcal polysaccharide diphtheria toxoid conjugate vaccine in infants. *Pediatr. Infect. Dis. J.* **23**:429–435.
- Richmond, P., et al. 2001. Ability of 3 different meningococcal C conjugate vaccines to induce immunologic memory after a single dose in UK toddlers. *J. Infect. Dis.* **183**:160–163.
- Robbins, J. B., R. Schneerson, P. Anderson, and D. H. Smith. 1996. Preven-

- tion of systemic infections, especially meningitis, caused by *Haemophilus influenzae* type b. *JAMA* **276**:1181–1185.
47. **Roduit, C., et al.** 2002. Immunogenicity and protective efficacy of neonatal vaccination against *Bordetella pertussis* in a murine model: evidence for early control of pertussis. *Infect. Immun.* **70**:3521–3528.
  48. **Sabirov, A., and D. W. Metzger.** 2008. Intranasal vaccination of infant mice induces protective immunity in the absence of nasal-associated lymphoid tissue. *Vaccine* **26**:1566–1576.
  49. **Schmitt, N., et al.** 2009. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity* **31**:158–169.
  50. **Sharip, A., et al.** 2006. Population-based analysis of meningococcal disease mortality in the United States: 1990–2002. *Pediatr. Infect. Dis. J.* **25**:191–194.
  51. **Siegrist, C. A.** 2001. Neonatal and early life vaccinology. *Vaccine* **19**:3331–3346.
  52. **Snape, M. D., et al.** 2008. Immunogenicity of a tetravalent meningococcal glycoconjugate vaccine in infants: a randomized controlled trial. *JAMA* **299**:173–184.
  53. **Tan, L. K., G. M. Carlone, and R. Borrow.** 2010. Advances in the development of vaccines against *Neisseria meningitidis*. *N. Engl. J. Med.* **362**:1511–1520.
  54. **Taunay, A., P. A. Galvao, J. S. de Morais, E. C. Gotschlich, and R. A. Feldman.** 1974. Disease prevention by meningococcal serogroup C polysaccharide vaccine in preschool children: results after eleven months in Sao Paulo, Brazil. *Pediatr. Res.* **8**:429A.
  55. **Trotter, C. L., and M. E. Ramsay.** 2007. Vaccination against meningococcal disease in Europe: review and recommendations for the use of conjugate vaccines. *FEMS Microbiol. Rev.* **31**:101–107.
  56. **Tsuji, S., et al.** 2000. Maturation of human dendritic cells by cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guerin: involvement of Toll-like receptors. *Infect. Immun.* **68**:6883–6890.
  57. **Uehori, J., et al.** 2005. Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation. *J. Immunol.* **174**:7096–7103.
  58. **Unkeless, J. C., E. Scigliano, and V. H. Freedman.** 1988. Structure and function of human and murine receptors for IgG. *Annu. Rev. Immunol.* **6**:251–281.
  59. **van den Biggelaar, A. H., et al.** 2009. Neonatal pneumococcal conjugate vaccine immunization primes T cells for preferential Th2 cytokine expression: a randomized controlled trial in Papua New Guinea. *Vaccine* **27**:1340–1347.
  60. **Veenhoven, R. H., et al.** 2004. Nasopharyngeal pneumococcal carriage after combined pneumococcal conjugate and polysaccharide vaccination in children with a history of recurrent acute otitis media. *Clin. Infect. Dis.* **39**:911–919.
  61. **Vekemans, J., et al.** 2001. Neonatal bacillus Calmette-Guerin vaccination induces adult-like IFN-gamma production by CD4+ T lymphocytes. *Eur. J. Immunol.* **31**:1531–1535.
  62. **Vogelzang, A., et al.** 2008. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* **29**:127–137.
  63. **World Health Organization.** 2001. Epidemics of meningococcal disease African meningitis belt. *Wkly. Epidemiol. Rec.* **76**:281–288.
  64. **World Health Organization.** 2010. Meningitis in Chad, Niger and Nigeria: 2009 epidemic season. *Wkly. Epidemiol. Rec.* **85**:57–68.
  65. **Zandvoort, A., et al.** 2001. CD27 expression in the human splenic marginal zone: the infant marginal zone is populated by naive B cells. *Tissue Antigens* **58**:234–242.
  66. **Zollinger, W. D., and R. E. Mandrell.** 1983. Importance of complement source in bactericidal activity of human antibody and murine monoclonal antibody to meningococcal group B polysaccharide. *Infect. Immun.* **40**:257–264.