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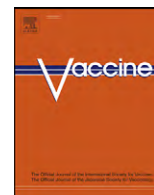

Mucosal priming of newborn mice with *S.* Typhi Ty21a expressing anthrax protective antigen (PA) followed by parenteral PA-boost induces B and T cell-mediated immunity that protects against infection bypassing maternal antibodies

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ABSTRACT

The currently licensed anthrax vaccine has several limitations and its efficacy has been proven only in adults. Effective immunization of newborns and infants requires adequate stimulation of their immune system, which is competent but not fully activated. We explored the use of the licensed live attenuated *S.* Typhi vaccine strain Ty21a expressing *Bacillus anthracis* protective antigen [Ty21a(PA)] followed PA-alum as a strategy for immunizing the pediatric population. Newborn mice primed with a single dose of Ty21a(PA) exhibited high frequencies of mucosal IgA-secreting B cells and IFN-γ-secreting T cells during the neonatal period, none of which was detected in newborns immunized with a single dose of PA-alum. Priming with Ty21a(PA) followed by PA-boost resulted in high levels of PA-specific IgG, toxin neutralizing and opsonophagocytic antibodies and increased frequency of bone marrow IgG plasma cells and memory B cells compared with repeated immunization with PA-alum alone. Robust B and T cell responses developed even in the presence of maternal antibodies. The prime-boost protected against systemic and respiratory infection. Mucosal priming with a safe and effective *S.* Typhi-based anthrax vaccine followed by PA-boost could serve as a practical and effective prophylactic approach to prevent anthrax early in life.

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1. Introduction

Concern over the illicit use of the bacterium *Bacillus anthracis*, a potential bioterror agent and the causative agent of anthrax, has heightened in recent years. The deliberate dissemination of anthrax spores through the US postal service in 2001 and the resulting harm and disruption it caused illustrated our vulnerability to such an attack and the need to develop effective and safe diagnostic, therapeutic, and prophylactic tools [1].

The anthrax vaccine currently available for use in humans in the U.S. consists of a cell-free culture filtrate containing *B. anthracis* protective antigen (PA) adsorbed to aluminum hydroxide (AVA-BioThrax®). PA is the non-toxic cell-binding component of the organism's tripartite toxin and the pathogen's major virulence factor. A similar cell-free vaccine consisting of alum-precipitated culture filtrate containing PA (AVP) is available in the U.K. [2].

While animals studies support the immunogenicity and protective efficacy of AVA, the extent to which this vaccine prevents disease in humans has been less clear. The immunization schedule is lengthy, consisting of five intramuscular injections over a period of 18 months followed by yearly boosters [3]. Local adverse reactions can occur that intensify with successive injections, and most importantly data demonstrating the ability of AVA to protect human against inhalational anthrax is lacking [reviewed in [4,5]]. In addition, the vaccine is perceived by the public (including high-risk groups) as unsafe and ineffective [6–8], and as a consequence its use has been limited to military personnel who have received it reluctantly [9].

There is indeed a pressing need to develop vaccines and immunization strategies capable of inducing rapid and effective protection, which can be safely given to all members of the population including vulnerable high-risk groups such as infants and young children who are particularly susceptible to bacterial infection. Anthrax has a rapid onset and progression in young children and severe complications have been described [10,11].

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Furthermore, infants and young children cannot be easily treated with antibiotics, let alone the aggressive and prolonged antibiotic therapy needed to effectively treat inhalational anthrax [2,11]. Even if alternative therapeutic antimicrobials become available in the near future, the rapid course of infection suggests that post-exposure therapy alone would be insufficient to prevent mortality [12,13]. Thus, safe and effective prophylactic vaccines capable of protecting the pediatric population against biological warfare are urgently needed.

A successful immunization strategy for infants will have to overcome several major obstacles, including: (1) the low levels of activation or "inexperience" of the neonatal/infant immune system, (2) a bias towards Th2-type responses, and (3) the presence of maternal antibodies that can oftentimes interfere with successful immunization. An ideal vaccine for this age group would be capable of inducing long-lasting protective levels of anthrax toxin neutralizing antibodies and robust mucosal and cell-mediated immunity following minimal dosing via a user-friendly route of immunization.

Our group was the first to demonstrate that attenuated strains of *Salmonella enterica* serovars Typhi and Typhimurium expressing a foreign vaccine antigen could prime robust immune responses in newborn mice following mucosal delivery despite the presence of high levels of maternal antibodies [14]. In subsequent studies we showed that unlike conventional subunit vaccines, live attenuated *Salmonella* has the capacity to enhance the activation and maturation of neonatal DCs in vivo, thus favoring more efficient T cell priming and ensuing adaptive immunity [15]. We also found that neonatal responses can be further enhanced by employing a heterologous prime-boost regimen; newborn mice primed with *S.* Typhi expressing *Yersinia pestis* F1 and boosted (as infants) with F1-alum developed protective immunity against systemic plague infection [15].

In this study, we examined the immune responses and protective efficacy afforded by neonatal mucosal priming using the licensed live attenuated typhoid vaccine strain Ty21a expressing *B. anthracis* PA followed by a parenteral PA-alum boost. Ty21a was chosen in preference to other strains because of its excellent record of safety, tolerability and immunogenicity in humans including school-age children [16,17], toddlers [18–20] and infants [18]. We also examined the ability of the neonatal *S.* Typhi priming-PA-boost immunization strategy to elicit B and T cell responses in the presence of maternal antibodies.

2. Materials and methods

2.1. S. Typhi Ty21a expressing B. anthracis PA

Plasmid pSEC91-83 encoding PA83 from *B. anthracis* [21] was electroporated into *S.* Typhi vaccine strain Ty21a. Transformants were recovered on Luria–Bertani (LB) agar plates containing kanamycin (10 μg/ml). PA expression was assessed by SDS-PAGE and immunoblot as described previously [15] using an anti-PA monoclonal antibody (Abcam, Cambridge, MA) followed by HRP-labeled goat anti-mouse IgG (Roche, Indianapolis, IN) and ECL Plus detection system (Amersham Biosciences, Buckinghamshire, UK). Master and working cell banks were produced from single colonies and stored at −70 °C. The vaccine strain *S.* Typhi Ty21a(pSEC91-83) is henceforth referred to as Ty21a(PA). Ty21a(PA) and Ty21a used for immunization were grown at 37 °C in LB broth supplemented with kanamycin as required. The immunizing dose was verified by plating serial dilutions onto LB agar with and without antibiotic. In vivo vaccine distribution following immunization was examined in a similar manner, by plating serial dilutions of homogenized tissue.

2.2. Immunoflourescence

Ty21a(PA) and Ty21a were grown overnight as described above and incubated with anti-PA monoclonal antibody (Abcam) in PBS, 0.1% BSA and 0.01% NaN_3 for 1 h at room temperature followed by FITC anti-mouse IgG (Invitrogen, Carlsbad, CA) as previously described [15]. Stained bacteria were visualized using a Nikon Eclipse 2000-E UV fluorescent microscope.

2.3. Mice and immunizations

BALB/c (Charles River Laboratories, Wilmington, MA) and A/J mice (The Jackson Laboratories, Bar Harbor, ME) were bred as previously described [14]. Newborns were primed intranasally (i.n.) with one or two doses of Ty21a(PA) or Ty21a (1×10^9 CFU in 5 μl volume) administered on day 7 or on days 7 and 15 after birth. All animals were boosted intramuscularly (i.m.) on day 22 after birth with 2 μg PA (List Biologicals, Campbell, CA) adsorbed to 0.5% aluminum hydroxide (Alhydrogel®, Brenntag Biosector, Frederikssund, Denmark), hereafter referred to as "alum". Control groups received PBS or PA i.m. on day 7 after birth followed by PA-boost on day 22. One-week-old mice were used in these studies as they best reflect the maturity of the immune system in human newborns (1–28 days of age) and their responses to vaccines [22]. Blood was collected at different time points as previously described [15]. To study interference by maternal antibodies, female breeders were immunized twice, 14 days apart, with 2 μg of PA-alum via i.m.; the last dose was administered 2 weeks before mating. Blood was collected before immunization, at the time of breeding and immediately after delivery. Litters from immune mothers were cross-fostered to naive surrogate females immediately after birth as previously described [14]. All animal studies described were approved by the University of Maryland Institutional Animal Care and Use Committee.

2.4. PA-specific IgG, IgG subclasses and IgG avidity

PA-specific serum IgG, IgG1 and IgG2a were measured by ELISA as previously described [23] using HRP-labeled anti-mouse IgG, IgG1, and IgG2a (Roche) conjugates and TMB Microwell Peroxidase substrate (KPL, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). End-point titers were calculated as the inverse of the serum dilutions that produced an absorbance (A_{450}) value of 0.2 above the blank [15]. IgG avidity was measured by ELISA using 6 M urea as a chaotropic agent as previously described [24].

2.5. Antibody-secreting cells (ASC) and memory B cells (B_{Mem})

PA-specific ASCs were measured in the nasal-associated lymphoid tissue (NALT), lung, and bone marrow (BM) by ELISPOT as previously described [14,24]. Briefly, fresh cells resuspended in complete RPMI [RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), 200 mM glutamine, and gentamicin 50 μg/ml, all from Invitrogen-Gibco, Gand Island, NY] were added in serial two-fold dilutions (5×10^5–6.25×10^4) to Immulon II plates previously coated with PA (5 μg/ml) and incubated overnight at 37 °C, 5% CO_2. HRP-labeled goat anti-mouse IgA (Zymed Laboratories, San Francisco, CA) and IgG (Roche) were used as conjugates, followed by True Blue substrate (KPL) in agarose overlay [15]. BM PA-specific and IgG^+ B_{Mem} lymphocytes were measured by ELISPOT after polyclonal expansion and overnight antigen recall as previously described [15].

2.6. Toxin neutralization and opsonophagocytic assays (OPA)

Anthrax toxin neutralizing antibodies were measured in individual samples using the method developed by Quinn [25,26].

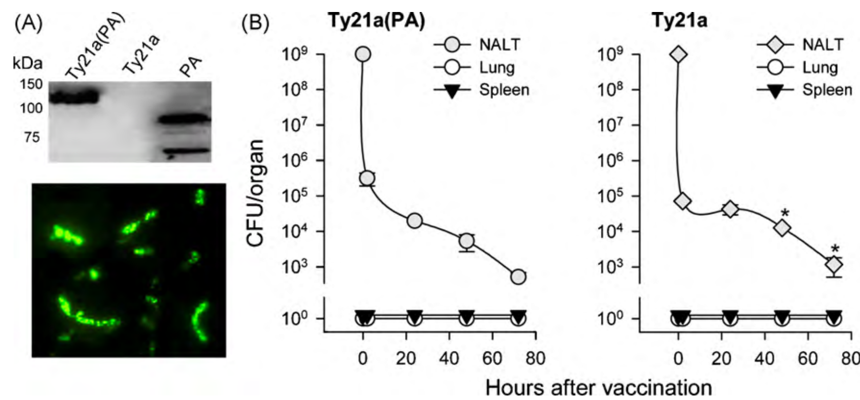

Fig. 1. Antigen expression and in vivo distribution of vaccine organisms. (A) PA expression by Ty21a(PA) determined by western blot and immunofluorescence. (B) Colonization and persistence of vaccine strains in neonatal mouse tissue. Ty21a(PA) or Ty21a was administered i.n. to newborn mice (12 per group). NALT, lungs and spleen were harvested at different time points, homogenized and cultured to determine bacterial counts. The data are presented as mean CFU ± SD. Time 0 indicates the vaccine inocula. Significant differences from Ty21a(PA) (* $p < 0.05$) are indicated.

Titers were calculated as the reciprocal of the serum dilution that resulted in 50% neutralization of toxin-mediated cytotoxicity (ED_{50}) through a four-parameter logistic-log fit curve using an end-point algorithm (Taylor method) developed by the U.S. Center for Disease Control. Opsonophagocytic antibodies were measured as previously described [27] with minor modifications. Spores from Sterne strain 34F2 (Colorado Serum Corporation, Denver, CO) were cultured in sporulation medium [28] for 5–12 days at 30 °C, washed and heated at 60–65 °C for 1 h to kill remaining vegetative cells. Pre- and post-vaccination serum samples were incubated in a 1:10 dilution with a spore suspension of 6×10^6 CFU/ml for 30 min at 4 °C, washed with PBS and added to a monolayer of J774A.1 macrophage cells at a multiplicity of infection of 3:1 spores per cell. Cultures were incubated for 30 min (37 °C, 5% CO_2), washed, and incubated again for another 30 min with fresh complete RPMI. Cells were then lysed, heat inactivated at 60–65 °C for 30 min, and plated onto LB agar for viable colony counts. The opsonization index was calculated as the net increase in spore counts measured after vaccination. Counts in the absence of serum were subtracted from experimental wells.

2.7. IFN-γ ELISPOT and T cell proliferation

IFN-γ-secreting cells were measured incubating serially diluted fresh splenocytes with PA (5 μg/ml) in nitrocellulose plates coated with anti-IFN-γ antibodies as previously described [15]. Cells stimulated with Con A (2 μg/ml; Sigma–Aldrich, St. Louis, MO) or with RPMI 1640 alone served as positive and negative controls, respectively. T cell proliferation was measured by incorporation of [^{3}H] thymidine into spleen cells incubated with PA (5 μg/ml) for 6 days as previously described [14,15].

2.8. Lethal toxin (LeTx) and i.n. spore challenge

BALB/c mice were injected through the tail vein with a mixture of 36 μg of PA (Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases, Manassas, VA) and 15 μg of lethal factor (List Biologicals) equivalent to 2 MLD_{50} of LeTx [29]. A/J mice were anesthetized with isoflurane and challenged i.n. with 7.5×10^8 Sterne spores (100 MLD) in a 40 μl volume. Both challenges were performed 63 days after birth. Mice were carefully monitored for 14 days after infection; health assessment was performed daily using a 1–4 scoring system [15]. A score of 1 (normal) was assigned to healthy mice with normal posture and no signs of dehydration; score 2 to early stage of piloerection with normal posture and mild dehydration; score 3 for mild piloerection, dull, hunched and moderate dehydration; and score 4 for severe piloerection, dull, hunched and squinting with severe tenting. Very sick or moribund animals were promptly euthanized. All surviving mice were euthanized on day 15.

2.9. Statistical analysis

All measurements were compared using Student's *t* test or the Mann–Whitney *U* test if normality failed. Kruskal–Wallis nonparametric ANOVA followed by Dunn's test were used for multiple comparisons among groups. Associations between PA-IgG and TNA titers were examined by Pearson's correlation. Survival curves were compared using the log-rank and Wilcoxon tests. Differences with $p < 0.05$ were considered significant. Statistical analysis was performed using SigmaStat 3.5 (Systat Software, San Jose, CA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Antigen expression and in vivo distribution of vaccine organisms

The licensed live oral *S.* Typhi vaccine strain Ty21a was adapted to express *B. anthracis* PA83 fused to the *Salmonella* ClyA export protein encoded by plasmid pSEC91-83. Expression of the ClyA–PA fusion protein was confirmed by immunoblot; a single band of ∼116 kDa recognized by anti-PA monoclonal antibody was present in the Ty21a(PA) lysates (Fig. 1(A), top). PA was also abundantly expressed on the bacterial surface, as shown by immunofluorescent staining (Fig. 1(A), bottom). Since our immunogenicity model requires antigen delivery by live organisms, we first examined in vivo the distribution and persistence of Ty21a(PA) in mucosal and systemic tissues, including Ty21a as a control. A single dose (1×10^9 CFU) of vaccine was given to 7 day-old newborn mice and nasal-associated lymphoid tissue (NALT), lung and spleens were harvested 0–72 h later. Ty21a(PA) was recovered from the NALT up to 3 days after immunization (Fig. 1(B)), but not from any other tissue studied, at any time point. No colonies were recovered from unvaccinated controls. The counts of Ty21(PA) were somewhat lower than those of Ty21a, yet the difference was only significant at later time points, i.e. 2 and 3 days after immunization (Fig. 1(B)).

3.2. PA IgG responses in newborns primed with Ty21a(PA) and boosted with PA-alum

Recombinant *Salmonella* vaccine strains carrying foreign antigens have an extraordinary capacity for immunological priming that leads to fast and potent anamnestic immune responses after

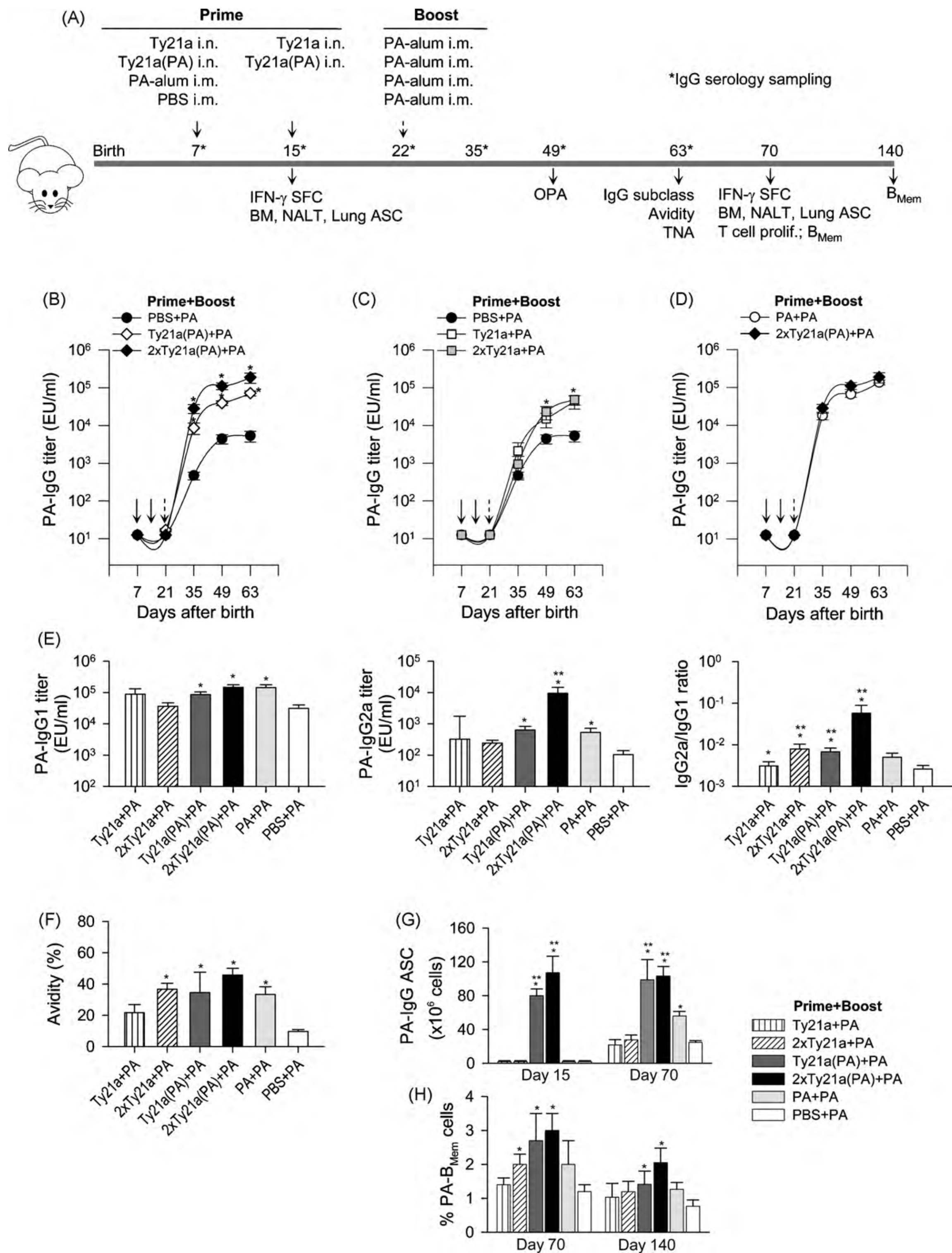

Fig. 2. Antibody and memory B cell (B_{Mem}) responses in mice primed as newborns with Ty21a(PA) and boosted with PA-alum (aluminum hydroxide). (A) Timeline for immunization and immunological measurements. Newborn mice (three litters per group) were administered one or two doses of Ty21a(PA) or Ty21a on day 7 or days 7 and 15 after birth (solid arrows). Additional groups received PBS or PA-alum on day 7. All animals were boosted with PA-alum on day 22 (dashed arrows). (B–D) PA-specific serum IgG. (E) IgG subclass distribution. (F) PA IgG avidity. Mean antibody titers, IgG avidity indices and IgG2a/IgG1 ratios ± SEM are shown. (G) PA-specific BM IgG-secreting cells; data represent mean number of IgG ASCs per 10^6 cells ± SD. (H) PA-specific BM B_{Mem}; the percentage of %PA-IgG ASCs per total IgG^+ B_{Mem} cells ± SD is shown. Significant differences from controls that received PBS followed by PA-boost (*$p < 0.05$) or PA priming followed by PA-boost (**$p < 0.05$) are indicated.

a subsequent parenteral protein boost [15,21,30]. We examined the capacity of Ty21a(PA) to prime the immune system during the neonatal period for an enhanced response following a parenteral boost with PA-alum. Newborn mice were administered one or two i.n. priming doses of Ty21a(PA), on day 7 or on days 7 and 15 after birth. A group primed with PA-alum on day 7 was included to compare the heterologous prime-boost regimen with PA-alum immunization. Unprimed controls received PBS or Ty21a. All animals were boosted on day 22 with PA-alum (2 μg). A summary of the groups, immunizations and immunological readouts is shown

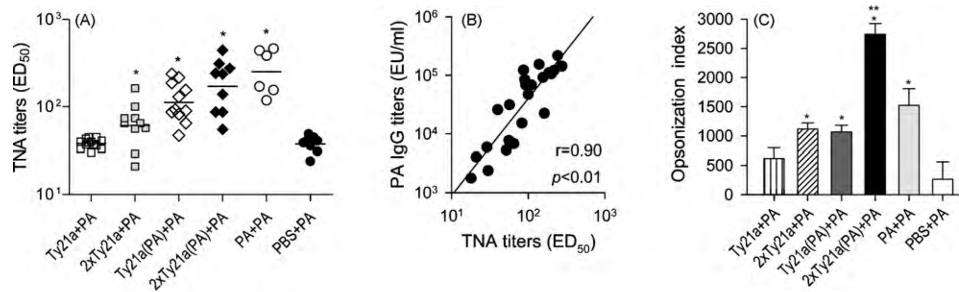

Fig. 3. Toxin neutralizing and opsonophagocytic antibodies. Newborns were immunized as described in Fig. 2(A). (A) TNA titers; the symbols represent individual titers measured on day 63 after birth, the line indicates geometric mean titers. (B) Correlation between TNA and PA IgG titers (data include all mice, day 63). (C) Opsonophagocytic titers measured on day 49 after birth; results represent the net increase in anthrax spore uptake by macrophages incubated with post-immunization sera ± SEM from three independent experiments. Significant differences from the PBS-PA-boost (* $p < 0.05$) and PA prime-PA-boost (** $p < 0.001$) control groups are indicated.

in Fig. 2(A). The kinetics of PA serum IgG responses are shown in Fig. 2(B–D). Newborns primed with one or two doses of Ty21a(PA) had a prompt response to the boost, producing high levels of PA-specific serum IgG antibodies. These responses largely surpassed those of unprimed controls that received PBS or Ty21a (Fig. 2(B) and (C), $p < 0.03$, day 35). The PA IgG titers were higher in mice that received two priming doses of Ty21(PA) as opposed to a single priming immunization (Fig. 2(B), $p < 0.001$, days 35–63).

An interesting finding was that higher PA IgG responses developed after the boost in newborns that had received either one or two doses of Ty21a (Fig. 2(C)) as compared with those that received PBS ($p < 0.02$, days 49 and 63). The improvement in antibody responses was independent of the number of priming immunizations with Ty21a preceding the boost and it was seen at a later time point (i.e., 1 month after the boost), in contrast with the increase in antibodies seen in Ty21a(PA)-primed mice, which was fast and proportional to the frequency of priming.

When comparing Ty21a(PA)-priming followed by PA-boost to priming and boosting with PA-alum, we found that both regimens produced similar levels of antibodies (Fig. 2(D)), although these antibodies had different functional properties, as indicated below. Neither Ty21a(PA) nor PA-alum primed newborns had detectable antibody responses after the priming, but both groups showed a sharp increase in PA IgG soon after the boost.

We further examined the PA IgG antibodies induced by the prime-boost immunization by studying their subclass distribution. While all groups produced high levels of IgG1, mice that had been primed as newborns with Ty21a(PA) produced the highest levels of IgG2a. Those who had been primed twice with Ty21(PA) had the highest IgG2a/IgG1 ratio of all treatments (Fig. 2(E)). Higher IgG2a/IgG1 ratios were associated with live vector priming, which in all cases surpassed those of unprimed PBS controls. Neonatal priming with Ty21a also improved the avidity of PA serum IgG antibodies. Newborns primed twice with Ty21a(PA) had the highest avidity indices of all treatments, surpassing those of unprimed PBS controls (Fig. 2(F)).

3.3. *PA IgG-secreting plasma cells and B_{Mem} cells*

To study the capacity of the neonatal heterologous prime-boost regimen to generate long-term protection we examined the presence of IgG plasma cells and B_{Mem} cells in the BM. High frequencies of PA-specific IgG ASCs were found 1 week after vaccination in newborns that received a single priming dose of Ty21a(PA) (Fig. 2(G)). In contrast, no ASCs were detected in newborns that had been primed with one dose of PA-alum or in unprimed controls that received Ty21a or PBS. High frequencies of PA IgG ASCs were still present on day 70 after birth (∼7 weeks after the boost) in Ty21a(PA)-primed mice, largely exceeding the numbers found in unprimed controls that received Ty21a or PBS. Mice primed and boosted with PA-alum had detectable PA IgG ASC responses on day 70, albeit markedly lower than those of the Ty21(PA)-prime PA-boost group ($p < 0.02$). Consistent with these observations, the highest frequencies of BM PA-specific B_{Mem} cells were detected up to 140 days after birth in mice primed as newborns with Ty21a(PA), reaching levels that surpassed those of unprimed controls that received PBS (Fig. 2(H); $p < 0.002$).

3.4. *Toxin neutralizing and opsonophagocytic capacity of PA antibodies*

In an attempt to determine the functional activity of the antibodies produced by the neonatal heterologous prime-boost immunization, we investigated their capacity to neutralize anthrax lethal toxin (LeTx) in vitro and to facilitate the phagocytosis of anthrax spores immediately before challenge. Mice that had been primed as newborns with one or two doses of Ty21a(PA) exhibited TNA responses that exceeded those of unprimed mice that had received PBS (Fig. 3(A)) Similarly to what had been seen for PA IgG, unprimed newborns that received Ty21a (not expressing PA) developed higher TNA titers after the PA-boost than those that received PBS. The increase in TNA response, however, was significant when mice received two doses of Ty21a as opposed to a single immunization, which was sufficient to enhance the levels of IgG. There was no difference between the mean TNA titers of mice that had been primed twice with Ty21a(PA) and boosted with PA and those of mice primed and boosted with PA. Furthermore, there was a strong positive correlation between PA IgG and TNA titer produced by neonatal immunization (Fig. 3(B)).

The capacity of PA antibodies to promote opsonophagocytic clearance of anthrax spores was examined using an in vitro macrophage-based assay. Newborns primed twice with Ty21a(PA) had the highest opsonophagocytic titers of all treatments (Fig. 3(C)). Similar to what was observed for toxin neutralization, the opsonophagocytic capacity of antibodies improved in mice exposed to Ty21a; higher OPA titers were seen in the group that received two doses of Ty21a followed by PA compared to those that received PBS.

3.5. *Ty21a(PA) priming induces mucosal antibody-secreting cells*

To assess the capacity of the prime-boost immunization to induce mucosal immunity, we measured the frequencies of IgA and IgG ASCs in the NALT and lungs. PA-specific ASCs were detected in both tissues only in mice that had been primed as newborns with Ty21a(PA) (Fig. 4(A)) High frequencies of IgA ASCs were observed 1 week after priming (day 15), and these further increased after the boost (day 70 after birth), particularly in the lungs. IgG ASC responses, although of a lower magnitude, were also detected in both tissues on day 70. The magnitudes of the IgG and IgA ASC

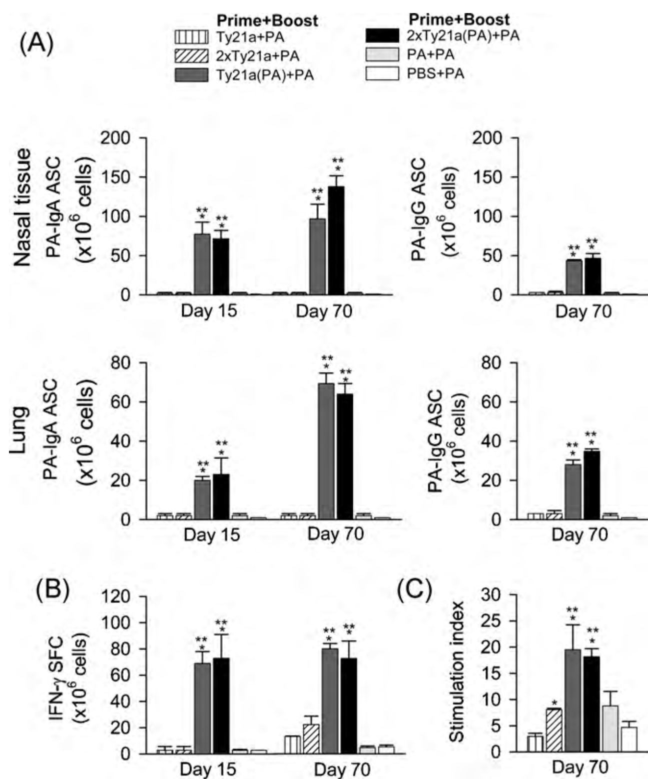

Fig. 4. Mucosal and cell-mediated immune responses induced by the Ty21a(PA) prime-PA-boost regimen. Newborn mice were immunized as described in Fig. 2(A). (A) PA-specific IgA and IgG ASCs in nasal tissue and lungs. The mean IgA or IgG ASCs per 10^6 cells ± SD of replicate wells are shown. (B) Frequencies of PA-specific IFN-γ secreting T cells stimulated ex vivo with PA; mean spot-forming cells (SFCs) per 10^6 cells ± SD of replicate cultures from two independent experiments are presented. (C) PA-specific T cell proliferation of spleen cells measured by [^{3}H] thymidine incorporation; mean stimulation index ± SD from replicate cultures are shown. Significant differences from the PBS-PA-boost ($^*p < 0.04$) and PA prime-PA-boost ($^{**}p < 0.015$) control groups are indicated.

responses were independent of the number of priming immunizations. In contrast, newborns that received PA-alum either as a prime, as a prime and boost, or only as a boost, failed to induce mucosal immune responses; no ASC response could be detected in the NALT or in the lungs at any time point.

3.6. *Newborns primed with Ty21a(PA) develop Th1-type cell-mediated immunity*

A single priming dose of Ty21a(PA) elicited vigorous PA-specific T cell-mediated IFN-γ responses as early as 15 days after birth, and these responses were still present on day 70. We did not observe IFN-γ production in mice primed or primed and boosted with PA (Fig. 4(B)). Proliferation of PA-specific T cells was seen in spleens on day 70, the highest responders being newborns primed with Ty21a(PA). Responses in this group surpassed those of unprimed controls that received Ty21a or PBS and those of mice that had been primed and boosted with PA (Fig. 4(C)). Unlike the humoral response, neither the frequency of IFN-γ producing cells nor the proliferative T cell responses increased in proportion with the number of priming doses of Ty21a(PA).

3.7. *Neonatal priming with Ty21a(PA) followed by a PA-boost protects against toxin challenge*

Protection afforded by the Ty21a(PA) prime-PA-boost regimen was examined using a systemic anthrax toxin lethal challenge (Fig. 5). The attack rate for unvaccinated (naive) mice was 100%. The

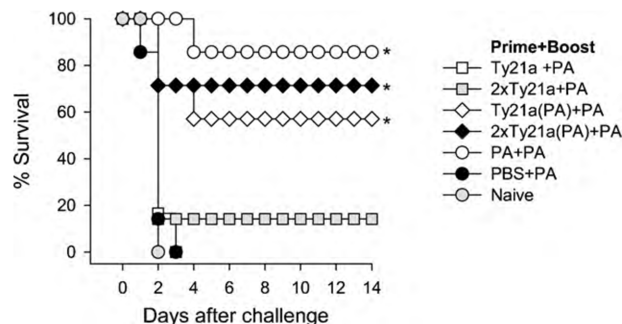

Fig. 5. Protection against systemic anthrax LeTx challenge. Newborns were immunized as described in Fig. 2(A) and challenged 63 days after birth with anthrax LeTx ($2MLD_{50}$). All naive mice died within 24 h of challenge. $^*p < 0.015$, indicate significant differences compared with the PBS-PA control group.

vaccine efficacy (VE) was 72% for mice that had been primed twice with Ty21a(PA) and boosted with PA and 85% for those primed and boosted with PA; there was no significant difference in the level of protection between these two groups. Mice primed once with Ty21a(PA) had a VE of 57%. All unprimed controls that received PBS prior to the boost, as well as those that received a single dose of Ty21a, succumbed to infection. Mice that received two doses of Ty21a prior to the boost had 14% VE.

3.8. *Neonatal priming with Ty21a(PA) followed by a PA-boost protects against spore challenge*

We next examined the protective efficacy of the prime-boost regimen against respiratory anthrax infection through pulmonary spore challenge in susceptible A/J mice. In this experiment, we included selected treatments: (1) the heterologous prime-boost regimen that produced the best immune responses in previous experiments, i.e., two priming doses of Ty21a(PA) followed by PA-boost; (2) priming controls, i.e. 2xTy21a(PA) and PBS, both followed by PA-boost, (3) PA prime-PA-boost for comparison and (4) naive controls. The PA IgG and TNA titers measured immediately before challenge are shown in Fig. 6(A). Mice primed twice with Ty21a(PA) as well as mice primed with PA had the highest PA IgG titers after the boost, rising above those of unprimed controls that received Ty21a or PBS. The same response profile was observed for the TNA antibodies. While the trend of antibody responses was similar in both mouse strains, the TNA titers in the A/J mice were found to be higher (mice had been immunized under identical conditions and the same time point was compared). We also noticed a poor correlation between PA IgG and TNA titers produced in this mouse strain versus those of BALB/c mice.

Our first task for assessing the protective efficacy of the prime-boost vaccine regimen against pulmonary infection was to establish the adequate spore challenge dose for age-matched A/J mice. Increasing numbers of spores correlated with severity of disease and lethality (Fig. 6(B)); 7.5×10^6 spores/40 μl was defined as 1 Mouse Lethal Dose (1 MLD) in our working conditions. Vaccinated animals were challenged via the respiratory route on day 63 after birth with 100 MLD of anthrax spores, and they were monitored over 14 days for signs of disease and survival. The unvaccinated mice died by day 4 after infection, whereas all vaccinated mice survived (Fig. 6(C), left panel). Nonetheless, there were clear differences in the severity of disease of among these animals following infection (Fig. 6(C), right panel). Mice primed twice with Ty21a(PA) as well as those primed with PA exhibited only mild signs of infection and all recovered by day 9. In contrast, unprimed mice that received PBS developed more severe signs of disease that included piloerection and moderate dehydration.

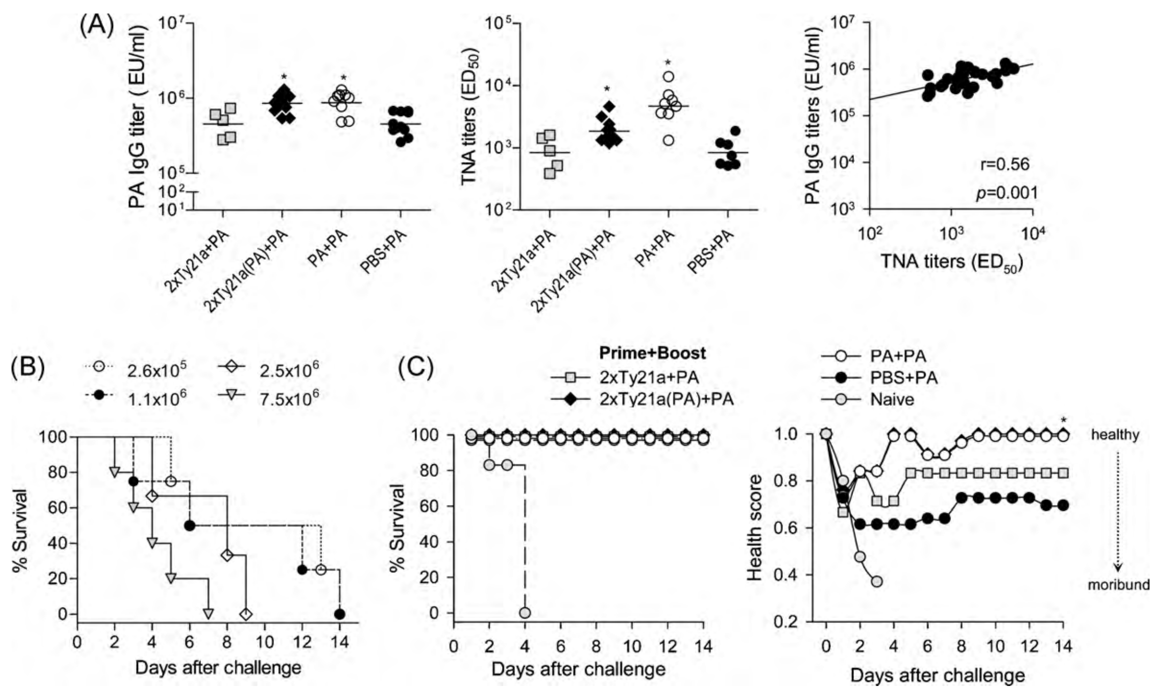

Fig. 6. Neonatal mucosal priming with Ty21a(PA) followed by PA-boost protects against respiratory anthrax spore challenge. A/J newborn mice were immunized as described in Fig. 2(A). (A) PA IgG and TNA titers measured on day 63 after birth. The symbols represent individual titers, the line indicates geometric mean titers. The right panel shows the correlation between the PA IgG and TNA titers. Significant differences compared with the PBS-PA control group (* $p \leq 0.03$) are indicated. (B) Pulmonary spore challenge lethal dose analysis. A/J mice (the same age as vaccinated mice) were given increasing CFUs of Sterne spores in a 40-μl volume (to reach the lungs). Survival curves from five mice per group are presented. (C) Protective efficacy following vaccination. A/J newborns immunized as described above were challenged i.n. on day 63 after birth with 100 MLD (7.5×10^8 CFU) of Sterne spores. The graphs show percent survival and mean health scores (inverse values) during the 14-day monitoring period.

3.9. Immune responses induced by Ty21a(PA) priming followed by PA-boost in the presence of maternal antibodies

Lastly, we examined the capacity of the Ty21a(PA)-prime-PA-boost regimen to induce immune responses in the face of pre-existing antibodies of maternal origin. Newborns were bred from naive and immune mothers. Immediately after birth, litters from immune mothers were transferred to naive surrogates to avoid additional transfer of maternal antibodies present in milk, which are transported across the intestinal epithelium into the neonatal circulation in rodents but not in humans [31]. Fig. 7(A) shows the kinetics of PA IgG and TNA antibodies in pups born to naive- and PA-vaccinated mothers. The first panel shows the normal decline of placentally transferred PA IgG after birth. As observed in our earlier experiments, neonatal priming with either Ty21a(PA) or PA enhanced the PA IgG and TNA responses after the boost; the superior responses in these groups over the priming controls (PBS and Ty21a) were especially noticeable on day 35. The presence of maternal antibodies did not affect the production of PA IgG in newborns primed with either Ty21a(PA) or PA. In fact, the TNA and IgG curves in the naive and immune groups were superimposable from day 35 after birth through day 63. It was noted, however, that despite the presence of high levels of PA IgG in immune pups at early time points, these antibodies did not exhibit the same neutralizing capacity as those measured at a later time point (days 49 and 63). A marked inflection point is seen in the TNA but not in the PA IgG curves on day 35. Based on the TNA titers, both the Ty21(PA) and PA-primed groups from either background had developed protective immunity ($\geq$100 ED_{50}) by day 49, one month after the PA-boost.

We also evaluated the influence of maternal antibodies on the development of mucosal immune responses. Newborns primed with Ty21a(PA) but not those primed with PA had a high frequency of IgA ASC in the NALT and lung on day 15 (Fig. 7(B)), and these responses increased after the boost as measured on day 70. There were no differences between the responses of newborns from naive versus immune mothers.

Additionally, we examined induction memory by measuring PA-specific plasma cells and B_{Mem} cells in the BM in the face of maternal antibodies. IgG plasma cells were detected on day 15 in newborns primed with a single dose of Ty21a(PA) at a frequency that largely exceeded that of PA-primed mice (Fig. 7(C)). These IgG ASC responses increased on day 70. Post-boost IgG ASC responses were seen in unprimed controls that received Ty21a or PBS, albeit lower than those of Ty21a(PA) primed mice. The B_{Mem} cell responses measured on day 70 followed a very similar pattern. Neither of these responses was altered by the presence of maternal antibodies.

Finally, we investigated the presence of cell-mediated immunity, which has been shown to remain largely unaffected by placentally transferred antibodies. PA-specific proliferative responses and IFN-γ production were detected on days 15 and 70 (Fig. 7(D)). Mice primed with Ty21a(PA) were the highest responders. No differences were seen between newborns born to naive versus PA-immune mothers.

4. Discussion

In this study we have demonstrated that an immunization strategy encompassing mucosal neonatal priming with the licensed oral typhoid vaccine Ty21a expressing *B. anthracis* PA followed by a parenteral boost with PA-alum was well tolerated and successfully protected mice against systemic and pulmonary anthrax infection. Furthermore, this strategy was found to induce robust mucosal and systemic antibody and T cell-mediated immune responses even in the presence of high levels of maternal antibodies.

Ty21a(PA) expressed high levels of PA on the bacterial surface, and a large proportion of the vaccine organisms recovered from immunized animals retained the antigen-encoding plasmid.

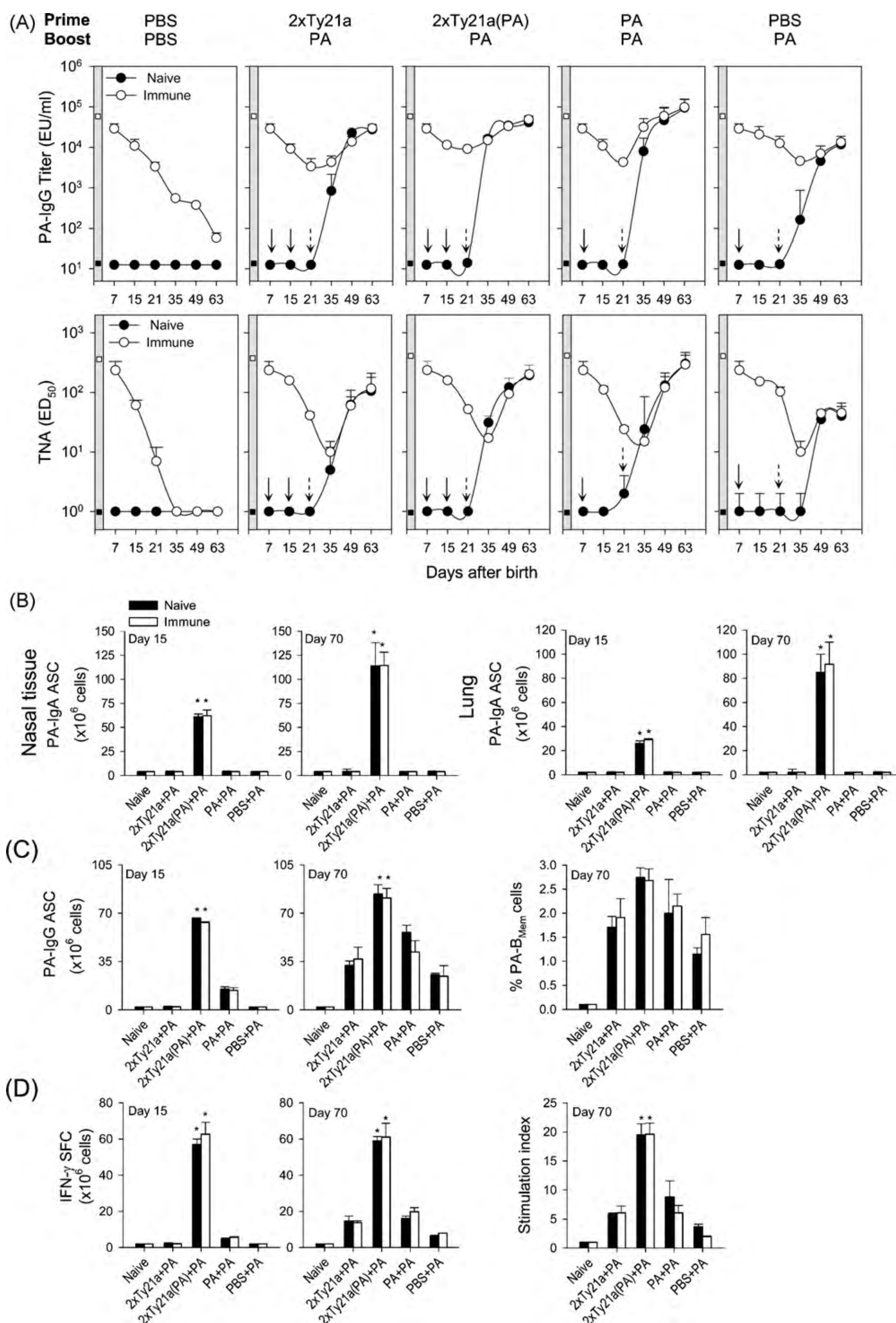

Fig. 7. Antibody, mucosal immune responses and cell-mediated immunity in newborn mice primed with Ty21a(PA) and boosted with PA-alum in the presence of maternal antibodies. BALB/c newborns from naive and PA-immune mothers were immunized as described in Fig. 2(A). (A) Kinetics of PA IgG and TNA responses in naive and immune pups. The maternal PA IgG and TNA levels at the time of delivery are shown in the grey area on the *y*-axis (naive mothers: black squares, immune mothers white squares). Arrows indicate each immunization. The data are presented as geometric mean titers for each group ± SEM. (B) PA-specific IgA ASCs in NALT and lungs measured on days 15 and 70 after birth. (C) PA-specific IgG ASCs and PA-specific B_{Mem} cells measured in BM on days 15 and/or 70. Results are expressed as mean ASCs per 10^6 cells ± SD of replicate cultures or %PA-IgG ASCs per total IgG⁺ B_{Mem} cells ± SD. (D) Frequencies of PA-specific IFN-γ secreting T cells and in vitro PA-specific T cell proliferation measured on days 15 and/or 70 after birth. Results are presented as mean SFCs per 10^6 cells ± SD of replicate cultures or fold increase in proliferation over background (stimulation index). Significant differences ($^*p \leq 0.002$) from the PA prime-PA-boost group are indicated.

The vaccine actively colonized the nasal epithelium and this tissue likely plays a key role in antigen sampling and distribution to local lymph nodes.

Since *B. anthracis* PA is probably the most advanced anthrax vaccine candidate pursued [reviewed in [2,4]], it was deemed important to compare our neonatal Ty21a(PA) prime-PA-boost strategy to that of repeated parenteral immunization with PA-alum. Regardless of which vaccine was used as the priming agent, PA or Ty21a(PA), detectable levels of serum antibodies were only seen after the PA-boost, although it was clear that both vaccines had an excellent priming capacity as evidenced by the rapid increase in PA IgG titers post-boost. Newborns primed with Ty21a(PA) achieved antibody levels that were similar and even slightly higher than those of newborns primed with PA-alum. Their IgG and TNA

levels increased in proportion to the number of priming immunizations, and they produced a more balanced Th1/Th2 response (with higher levels of IgG2a) and antibodies of higher avidity and superior functional capacity (i.e., opsonophagocytosis) compared with antibodies produced by repeat immunization with PA-alum. Likewise, the breadth of the responses was notably expanded. A single nasal immunization with Ty21a(PA) was sufficient to stimulate sizeable numbers of IgA and IgG ASCs in the nasal tissue and lungs, IgG plasma cells in the BM, and IFN-γ secreting T cells in the spleen. None of these responses was detected in newborns primed with PA-alum.

The demonstration of mucosal PA-specific ASCs in newborns primed with Ty21a(PA) was particularly relevant. Mucosal antibodies alone might may not be able to protect against aerosolized organisms [32], but they can still provide a second layer of defense by clearing spores from the respiratory airways [33–35]. Not only can PA antibodies neutralize LeTx, they can also inhibit spore germination extracellularly [36], enhance spore phagocytosis by alveolar [37] and peritoneal macrophages [36] and promote intracellular killing of germinated organisms [27,38]. Inducing both systemic as well as mucosal immunity would be a valuable feature of any new vaccine, as in the event of an intentional release, individuals are likely to be exposed to the organism via aerosol/mucosal routes.

The capacity of the Ty21a(PA)-prime-PA-boost strategy to induce robust cell-mediated immunity, including IFN-γ-secreting T cells, is particularly advantageous for our target population (e.g. young infants) due to their increased susceptibility to intracellular pathogens. IFN-γ produced by $CD4^+$ T cells is also believed to contribute to protection against anthrax by augmenting innate immunity and facilitating phagocytosis and killing via activated macrophages [39]. In contrast, neonatal priming with PA-alum followed by PA-boost stimulated comparatively poor T cell responses; we failed to detect vaccine-induced IFN-γ-responses, and proliferation of PA-specific T cells in vitro was modest at best.

The overall superior responses engendered in newborns primed with Ty21a(PA) likely reflect the Th1-type adjuvant properties of *S.* Typhi, which through activation of neonatal APC, can trigger the cascade of events (including B and T cell activation) necessary to initiate an adaptive immune response. These differences in T cell responses may also reflect more efficient antigen processing and presentation when PA is delivered through a bacterial vector. Similarly, robust T cell responses to PA were reported in naturally infected individuals but not in volunteers immunized with the UK AVP vaccine [40]. Overall, these results suggest that repeated immunization with PA-alum may not be the best approach to stimulate long-lasting $CD4^+$-mediated T cell responses either in adults or in the very young ones.

An additional advantage of the proposed Ty21a(PA)-prime-PA-boost regimen was its capacity to induce balanced Th1/Th2 responses as opposed to further biasing the Th2-type environment typical of early life. Several studies reported Th2-type predominance in adult mice immunized with PA-alum [41] or BioThrax® [42]. The repeated use of alum-adjuvanted vaccines during the first months of life promotes a heavy bias to Th2-type responses that has been associated with increased risk of allergic reactions and asthma [43].

In addition to stimulating robust T cell responses, Ty21a(PA) priming also favored the activation and differentiation of neonatal B cells into PA-specific long-lived plasma cells and memory B cells, which are necessary to support long-term protection. Indeed, a sizable pool of BM plasma blasts was produced by newborns primed with Ty21a(PA) but not by those primed with PA-alum. The latter group also exhibited a much lower frequency of memory B cells following the boost. The ability to induce long-lasting, high affinity memory B cells has been proposed as a key attribute of any next generation anthrax vaccine [44]. The results of this study demonstrate that the Ty21a(PA)-prime-PA-boost is an effective means of stimulating long-term immune memory and protection, and it thus has the potential to reduce the need for multiple immunizations as is currently required for AVA.

It was interesting that newborns primed with Ty21a (not expressing PA) mounted PA IgG responses superior not only in magnitude but also in quality (i.e., toxin neutralizing and opsonophagocytic capacity) to those of PBS-primed controls. The improvement was not limited to antibodies but extended to memory B cells and cell-mediated immunity. This non-specific immune stimulation could be attributed to a Ty21a-driven activation and maturation of neonatal immune cells [15] that enables them to respond more efficiently to a vaccine antigen given later in life. Similarly, more efficient responses to routine vaccines (Hep B and oral polio) were reported in human newborns who received BCG, and this was attributed to its influence on the maturation of DCs [45].

While characterization of the quality of the immune response provides useful clues as to how a vaccine stimulates the immune system of the host, the ultimate aim of a vaccine is to confer protection. Neonatal priming with Ty21a(PA) followed by PA-boost stimulated a PA-specific response that protected BALB/c mice from a lethal injected dose of anthrax toxin and A/J mice from a lethal i.n. spore challenge. In both cases, Ty21a(PA)-primed mice developed high levels of PA antibodies, a significant proportion of which possessed toxin neutralizing activity (a known correlate of protection against anthrax [reviewed in [12]]) and opsonophagocytic activity. Although the trend of the antibody responses was similar in both mouse strains, the TNA titers were markedly higher in the A/J mice, but there was a poor correlation between PA IgG and TNA titers in this strain. Studies in the literature that examined antibody responses to PA both in A/J and BALB/c showed conflicting results. While our results agree with those of Kolla et al. [46] who reported higher PA-specific IgG titers in adult A/J mice immunized with PA-alum compared with BALB/c mice, the opposite was reported by Abboud and Casadevall [47]. The later study, however, used a different adjuvant (complete and incomplete Freund), dosing schedule and different sampling time points. The high titers in our immunized A/J mice likely account for the protection observed in all the vaccinated groups. Of importance, our protective efficacy results agree with those of Osorio et al. [48] and Stokes et al. [28] who showed that adult A/J mice immunized i.n. or orally with *S.* Typhi or *S.* Typhimurium expressing PA were fully protected against lethal aerosol spore challenge.

In addition to demonstrating the ability of mucosally delivered Ty21a(PA) to prime a protective immune response in newborn mice, our study also addressed the effect that high levels of maternally derived PA-specific antibodies would have on immune priming. It is known that placentally transferred maternal antibodies present at the time of immunization (even residual levels) can interfere with the successful immunization of newborns and infants creating a “window of susceptibility” to infection [49,50]. This effect has been well described for several routine vaccines such as measles, poliomyelitis, tetanus, diphtheria, pertussis and *Haemophilus influenzae* type b (Hib) [50]. Thus, we could envisage a scenario in which women from high-risk groups (i.e., military, health care professionals) who had received the anthrax vaccine could transfer to their babies antibodies that can prevent them from mounting a protective immune response following immunization. In 1998, the Department of Defense began the Anthrax Vaccine Immunization Program for designated US Armed Forces Personnel, and as of 2007, 5.5 million doses of BioThrax were administered to more than 1.5 million civilians and military personnel [51]. A single study identified at least 51,000 infants born to military women vaccinated against anthrax (some of them during pregnancy) between 1998 and 2004 [8].

Reassuringly, our results suggest that the presence of maternal antibodies does not adversely affect the ability of a Ty21a(PA)-prime-PA-boost to elicit a protective immune response. Indeed, pups born to immune mothers who were subsequently primed with Ty21a(PA) and boosted with PA mounted a full range of PA-specific responses including robust IgG and TNA responses, mucosal IgG- and IgA-secreting cells, BM IgG-secreting plasma cells, memory B cells and cell-mediated immunity. Even if some of the vaccine antigen (secreted from Ty21a or given as a protein in combination with alum) was blocked by maternal antibodies, there was sufficient "free material" to stimulate an efficient B cell and T cell response. Unlike other antigens, *B. anthracis* PA is uniquely immunogenic, and low amounts might be sufficient to prime an immune response. As expected, T cell responses in the presence of maternal antibodies remained unaffected, as either free or antibody-bound antigen can be equally taken up by DCs for subsequent T cell stimulation [52].

An original contribution of this work is the demonstration that heterologous mucosal priming with Ty21a(PA) followed by a PA-boost elicits long-lasting protective immunity when given early in life. To our knowledge, this is also the first study to explore maternal antibody inhibition of immune responses induced by an anthrax vaccine candidate. A Ty21a-based anthrax vaccine employed in a prime-boost strategy could represent a practical and effective prophylactic tool to prevent anthrax in pediatric and general populations. Indeed, an orally delivered, self-administered attenuated *S.* Typhi-based vaccine capable of stimulating protection following minimal dosing in a large segment of the population would be an extremely attractive prospect for authorities seeking to protect at-risk civilian groups. A further attractive feature of the Ty21a platform is the ability to engineer the bacterium to deliver multiple antigens and thus achieve optimal protection from a single construct. Research efforts to develop more effective human *S.* Typhi vaccines currently include the evaluation of rationally defined delayed attenuating mutant strains [53], non-antibiotic resistance markers [54] and improved antigen expression/secretion systems.

In conclusion, the mucosal live vector prime-parenteral protein boost approach described here is highly promising and warrants further evaluation in clinical trials.

Conflicts of interest

The authors have no conflicting financial interest.

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