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VACCINES

A Combination of Recombinant *Mycobacterium bovis* BCG Strains Expressing Pneumococcal Proteins Induces Cellular and Humoral Immune Responses and Protects against Pneumococcal Colonization and Sepsis

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ABSTRACT Pneumococcal diseases remain a substantial cause of mortality in young children in developing countries. The development of potentially serotype-transcending vaccines has been extensively studied; ideally, such a vaccine should include antigens that are able to induce protection against colonization (likely mediated by interleukin-17A [IL-17A]) and invasive disease (likely mediated by antibody). The use of strong adjuvants or alternative delivery systems that are able to improve the immunological response of recombinant proteins has been proposed but poses potential safety and practical concerns in children. We have previously constructed a recombinant *Mycobacterium bovis* BCG strain expressing a pneumococcal surface protein A (PspA)-PdT fusion protein (rBCG PspA-PdT) that was able to induce an effective immune response and protection against sepsis in a prime-boost strategy. Here, we constructed two new rBCG strains expressing the pneumococcal proteins SP 0148 and SP 2108, which confer IL-17A-dependent protection against pneumococcal colonization in mouse models. Immunization of mice with rBCG 0148 or rBCG 2108 in a prime-boost strategy induced IL-17A and gamma interferon (IFN-γ) production. The combination of these rBCG strains with rBCG PspA-PdT (rBCG Mix), followed by a booster dose of the combined recombinant proteins (rMix) induced an IL-17A response against SP 0148 and SP 2108 and a humoral response characterized by increased levels of IgG2c against PspA and functional antibodies against pneumolysin. Furthermore, immunization with the rBCG Mix prime/rMix booster (rBCG Mix/rMix) provides protection against pneumococcal colonization and sepsis. These results suggest the use of combined rBCG strains as a potentially serotype-transcending pneumococcal vaccine in a prime-boost strategy, which could provide protection against pneumococcal colonization and sepsis.

KEYWORDS protection, *Streptococcus pneumoniae*, cytokines, recombinant BCG

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Nasopharynx colonization is the first step in the establishment of pneumococcal disease. This colonization process, which affects virtually 100% of young children, can either remain asymptomatic or lead to pathogenic conditions, such as otitis, sinusitis, pneumonia, meningitis, or sepsis (1). Indeed, epidemiologic evidence suggests that in response to pneumococcal carriage, both serotype-specific and -independent immunity to subsequent carriage develop (2, 3).

Pneumococcal vaccines available are based on pure polysaccharides or polysaccha-

rides conjugated to carrier proteins (pneumococcal conjugate vaccine [PCV]). Ten-valent PCV (PCV10) and PCV13, specially recommended for children younger than 2 years of age, induce a high antibody response against included serotypes, protect against invasive diseases, and reduce pneumococcal carriage (4, 5). One of the main limitations of these serotype-specific vaccines is the "serotype replacement" effect. After only 5 years of the introduction of PCV7, a replacement of pneumococcal infections from serotypes not contained in PCV7 was observed, and pneumococcal disease rates caused by non-PCV7 serotypes rose, in part due to the emergence of serotype 19A (6). Although the most recently licensed formulation, PCV13, includes serotype 19A, there has since been an increase in colonization and invasive disease due to other serotypes (7). These concerns thus support the development of pneumococcal vaccines based on conserved antigens able to induce wide cross-protection against colonization and invasive disease, regardless of serotype.

Pneumococcal surface protein A (PspA) and detoxified forms of pneumolysin (Ply), such as PdT, are proteins that have been extensively studied as pneumococcal vaccine candidates (8). PspA has the ability to inhibit complement deposition onto the pneumococcal surface, thus reducing opsonophagocytosis (9, 10). Ply, a cholesterol-dependent cytolysin, is an important factor in pneumococcal pathogenicity, interfering in the cellular immune response and in the activation of the complement system during pneumococcal infection (11, 12). PspA and pneumolysoids have been studied in preclinical and clinical trials, showing an ability to induce a protective antibody-dependent response (13–17).

We have previously demonstrated that intranasal immunization with live pneumococcus or a killed nonencapsulated pneumococcal whole-cell vaccine (WCV) induces antibody-independent protection against colonization (18). We also demonstrated that this protective immune response is mediated by Th17 $CD4^+$ T cells (19, 20). Supporting these findings, another group found that transferred $CD4^+$ T cells from mice that resolved a prior pneumococcal lung infection promote a strong Th17 $CD4^+$ recall response and protect mice against pulmonary challenge with heterologous pneumococcal strains (21). These data suggest the potential use of conserved pneumococcal proteins able to induce a Th17 response that could induce protection against colonization and cooperate against invasive disease.

Moffitt et al. screened a panel of pneumococcal proteins able to induce a Th17 response *in vitro* when used to stimulate cells from mice immunized or cells from healthy human donors with the whole-cell antigen (WCA) (22). SP 0148, an ABC transporter protein, and SP 2108, a maltose binding protein, were among the most immunogenic antigens. As an intranasal vaccine, these two proteins were able to induce protection in mice against pneumococcal colonization in a $CD4^+$ T cell/interleukin-17 (IL-17)-dependent manner (22). Furthermore, both SP 0148 and SP 2108 are lipidated proteins that activate Toll-like receptor 2 (TLR2) and enhance the IL-17A immune response (23). A three-protein combination (GEN-004) of SP 0148, SP 2108, and SP 1912 (a nonlipidated pneumococcal protein) was recently evaluated as a vaccine candidate in phase I and II clinical trials. The results showed that this vaccine was safe and well tolerated in adults. While the results did not reach statistical significance, vaccine recipients showed a reduced tendency to acquire pneumococcal colonization and showed a lower density of carriage following intentional nasopharyngeal challenge with pneumococcus than the controls (http://www.genocea.com/platform-pipeline/pipeline/gen004-for-pneumococcus/).

In order to improve the immunogenicity of recombinant proteins, strong adjuvants or alternative delivery platforms are good prospects. Since only a few adjuvants are approved for human use, the idea of using already-approved live vaccines as vectors to deliver antigens is very attractive. *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) is a live vaccine against tuberculosis and is distributed worldwide, with an excellent safety track record. It is estimated that approximately 4 billion people have received BCG since its first human application almost a century ago (http://www.who.int/immunization/research/development/tuberculosis/en/). Recombinant BCG strains have

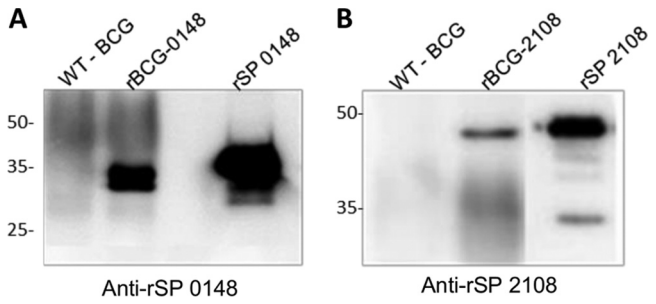

FIG 1 Immunoblotting analyses of SP 0148 and SP 2108 pneumococcal protein expression in rBCG. The expression of pneumococcal proteins in rBCG was evaluated by immunoblotting using specific antisera. (A) Protein extracts (~30 μg each) of WT-BCG (negative control) or rBCG 0148 incubated with anti-rSP 0148 antisera (1:500). (B) Protein extracts (30 μg each) of WT-BCG (negative control) or rBCG 2108 incubated with anti-rSP 2108 antisera. The recombinant proteins rSP 0148 and rSP 2108 (~200 ng) were used as positive controls. Molecular mass markers (in kilodaltons) are indicated on the left side of each gel.

been developed with a few antigens, demonstrating immunogenicity and protective efficacy against viruses, bacteria, and parasites (24–28).

Previously, we selected a PspA molecule able to induce antibodies with broad cross-reactivity (29) and demonstrated the potential use of this PspA molecule in fusion with PdT (30). Recently, in order to improve the immunogenicity of this fusion protein, we constructed a recombinant BCG (rBCG) strain expressing the PspA-PdT fusion protein (rBCG PspA-PdT) (31). Immunization in a prime-boost strategy with rBCG PspA-PdT and rPspA-PdT induced a high antibody response, promoted the IgG1/IgG2 antibody isotype shift, increased complement deposition onto the pneumococcal surface, and protected mice against a lethal challenge (31).

Here, we investigated the use of recombinant BCG as a delivery system for SP 0148 and SP 2108 antigens and evaluated the immune response induced in mice and protection against colonization. In addition, we combined these rBCG 0148 and rBCG 2108 strains with the previously generated rBCG PspA-PdT in a prime-boost strategy to evaluate the ability of this combination vaccine to protect mice against pneumococcal colonization and sepsis.

RESULTS

Expression of pneumococcal proteins in rBCG. Soluble extracts from rBCG 0148 or rBCG 2108 samples were evaluated for pneumococcal protein expression by immunoblotting. Antisera generated against recombinant SP 0148 (rSP 0148) showed two reactive bands at the expected molecular mass (~30 kDa) (Fig. 1A), while a single band was observed at the expected molecular mass when probing with anti-SP 2108 serum (~46 kDa) (Fig. 1B). Wild-type BCG (WT-BCG) was used as a negative control, and rSP 0148 or recombinant SP 2108 (rSP 2108) was used as a positive control, as indicated in Fig. 1. Since we did not observe cross-reactivity against WT-BCG using both antisera, we can conclude that the two bands observed in the rBCG 0148 construct are SP 0148 specific, perhaps reflecting truncated expression or protein degradation.

Immunization with rBCG 0148 and rBCG 2108 in a prime-boost strategy induces IL-17A and IFN-γ responses. Mice were immunized as described in Table 1, and 3 weeks after the last dose, splenocytes were isolated and cultured and cytokine production was measured in the supernatant. Splenocytes from mice immunized with rBCG 0148 prime and rSP 0148 boost (rBCG 0148/rSP 0148) produced robust amounts of IL-17A and gamma interferon (IFN-γ) after *in vitro* stimulation with rSP 0148 compared with all other groups (Fig. 2A and B). Likewise, the group immunized with rBCG 2108/rSP 2108 showed increased levels of IL-17A and IFN-γ after stimulation with rSP 2108 compared to the control group (Fig. 2C and D). However, no significant difference was observed between rBCG 2108/rSP 2108 and WT-BCG/rSP 2108 vaccines. The immunized group that received all three rBCG strains combined (rBCG Mix) showed

TABLE 1 Immunization groups

Group	Priming dose (s.c.)[a]	Booster dose (s.c.)[a]
Saline	Saline	Saline + $Al(OH)_3$
WT-BGC	1×10^6 CFU of WT-BCG	Saline + $Al(OH)_3$
rBCG Mix	3.3×10^5 CFU of each rBCG (rBCG 0148, rBCG 2108, and rBCG PspA-PdT)	Saline + $Al(OH)_3$
WT-BCG/rMix	1×10^6 CFU of WT-BCG	rMix (1 μg rSP 0148 + 3 μg rSP 2108 + 10 μg rPspA-PdT + $Al(OH)_3$)
rBCG 0148/rSP 0148	1×10^6 CFU of rBCG 0148	1 μg rSP 0148 + $Al(OH)_3$
rBCG 2108/rSP 2108	1×10^6 CFU of rBCG 2108	3 μg rSP 2108 + $Al(OH)_3$
rBCG PspA-PdT/rPspA-PdT	1×10^6 CFU of rBCG PspA-PdT	10 μg rPspA-PdT + $Al(OH)_3$
rBCG Mix/rMix	3.3×10^5 CFU of each rBCG (rBCG 0148, rBCG 2108, and rBCG PspA-PdT)	rMix (1 μg rSP 0148, + 3 μg rSP 2108 + 10 μg rPspA-PdT + $Al(OH)_3$

[a]s.c., subcutaneous.

reduced amounts of IL-17A and IFN-γ after rSP 0148 stimulation compared with rBCG 0148/rSP 0148, whereas there was no reduction in cytokine responses with respect to rSP 2108.

Immunization with rBCG Mix/rMix induces an antibody response against PspA and PdT and changes the IgG1/IgG2 isotype profile. Sera from immunized mice

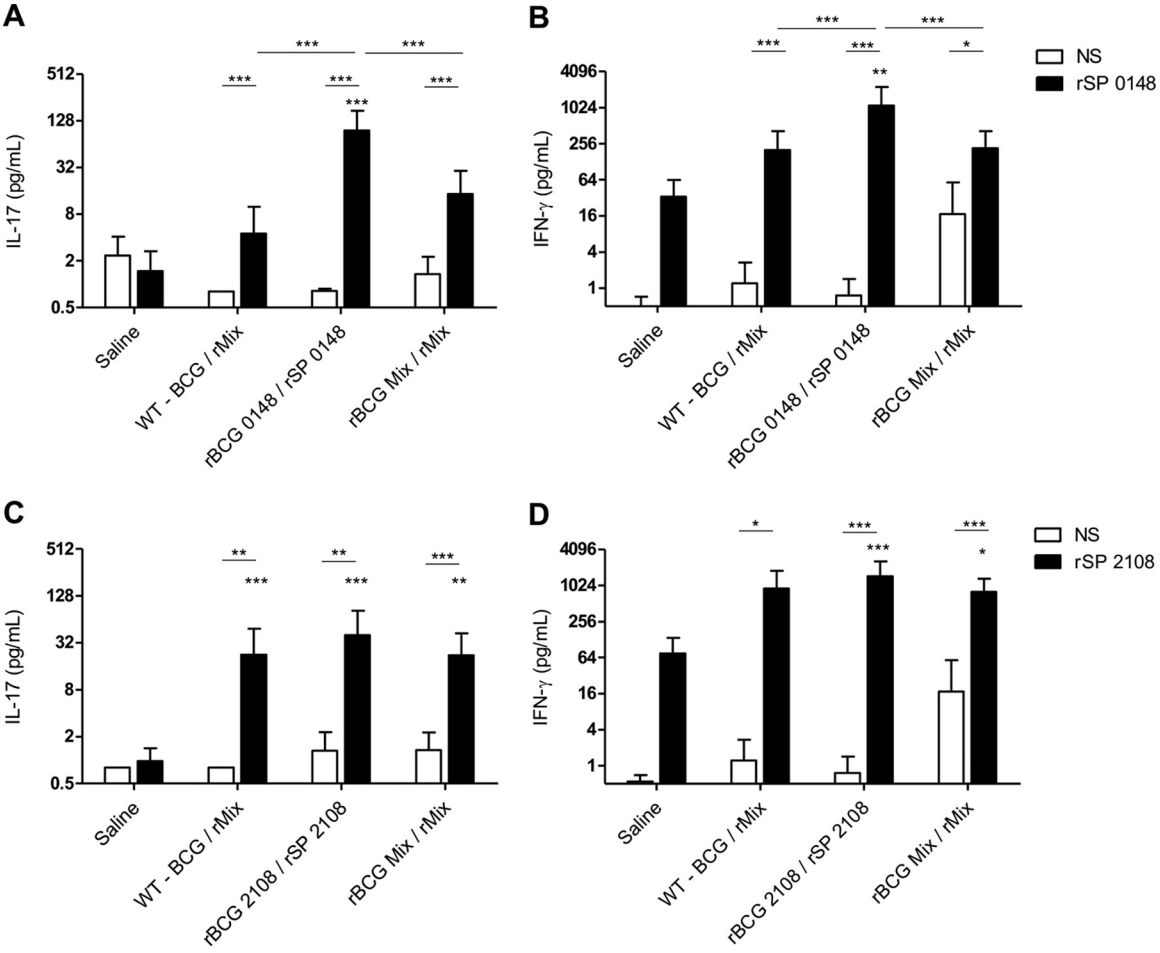

FIG 2 Immunization with rBCG 0148 or rBCG 2108 in a prime-boost strategy induces IL-17 and IFN-γ production. Immunized mice were euthanized 21 days after the booster dose, and splenocytes were stimulated *ex vivo* for 48 h. Cytokine production was evaluated by cytometric bead array (CBA) measuring IL-17 (A) and IFN-γ (B) levels in splenocyte culture stimulated with 5 μg of rSP 0148 (black bars), and IL-17 (C) and IFN-γ (D) levels in splenocyte culture stimulated with 5 μg of rSP 2108 (black bars). NS, nonstimulated (white bars). Results are represented as the means (+ standard errors of the means [SEM]) from 5 animals per group. Statistical analyses were performed by one-way ANOVA with a Tukey's multiple-comparison test: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ for immunized groups versus saline group or between samples, as indicated. Results are representative of two independent experiments.

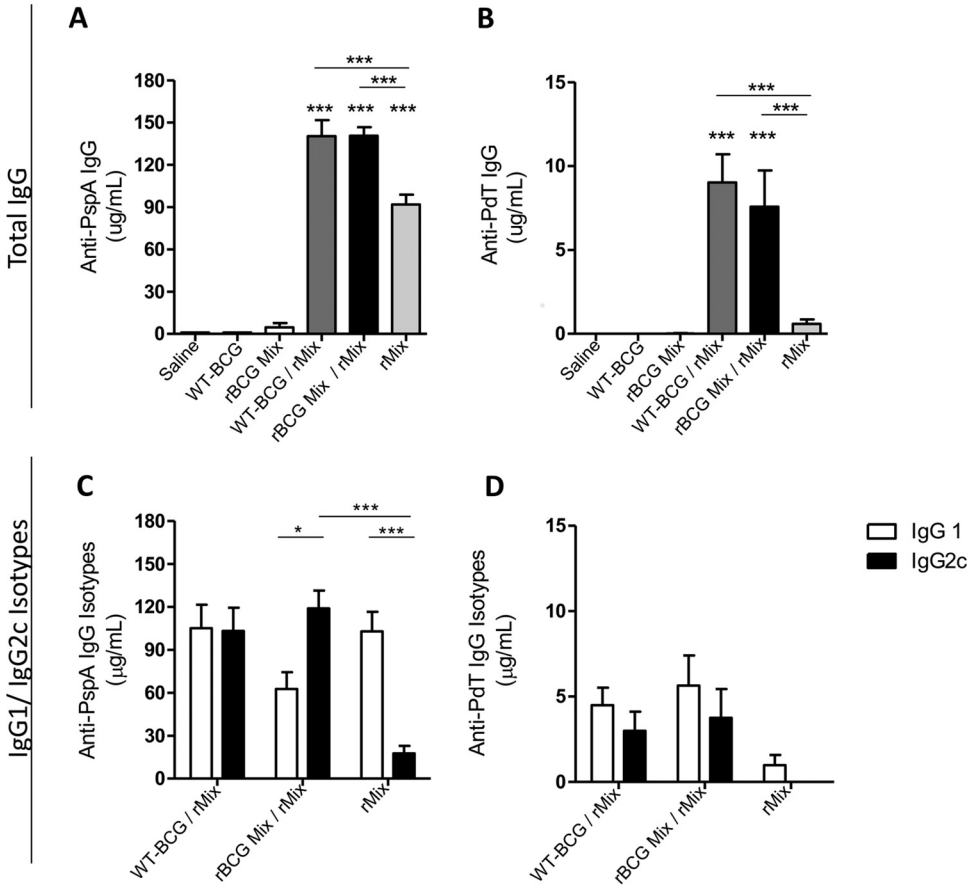

FIG 3 Immunization with rBCG Mix/rMix induces an antibody response against PspA and PdT and changes the IgG1/IgG2 isotype profile. Mice were immunized with a single dose of WT-BCG or rBCG Mix followed, or not, by a booster dose of rMix 30 days later. The rMix group received a single dose of recombinant proteins. The antibodies induced against rPspA or rPdT were evaluated by ELISA. (A and B) Total IgG against rPspA and rPdT, respectively. (C and D) IgG1 and IgG2c subclass antibodies against rPspA and rPdT, respectively. Results are represented as the means (+SEM) from 10 animals per group. Statistical analyses were performed by one-way ANOVA with a Tukey's multiple-comparison test: ***, $P < 0.001$; *, $P < 0.05$ for immunized groups versus saline group or between samples, as indicated. Results are representative of two independent experiments.

were evaluated for antibody concentrations against rPspA or rPdT. Only the groups that received a booster dose of the combined recombinant proteins had detectable antibody concentrations against rPspA or rPdT. The groups of mice primed with rBCG Mix or WT-BCG and boosted with combined recombinant proteins (rMix) showed an increased IgG concentration against rPspA or rPdT compared with the group that was immunized only with a single dose of rMix (Fig. 3A and B). We also analyzed the IgG1 and IgG2c isotype antibodies in the groups where a total IgG response was detected. Antisera of mice immunized with a single dose of rMix showed a predominantly IgG1 isotype antibody response against rPspA (IgG1/IgG2c ratio, 5.80) (Fig. 3C and Table 2). A balanced profile of IgG1/IgG2c against rPspA was observed in the sera of mice primed

TABLE 2 Anti-rPspA and anti-rPdT antibody isotypes in the sera of immunized mice

Antibody	Group	IgG1 (μg/ml)	IgG2c (μg/ml)	IgG1/IgG2c ratio
Anti-PspA	WT-BCG/rMix	120.0 ± 7.7	112.3 ± 15.3	1.17
	rBCG Mix/rMix	62.6 ± 11.7	118.8 ± 12.3	0.53
	rMix	102.8 ± 13.6	17.7 ± 5.1	5.80
Anti-PdT	WT-BCG/rMix	4.5 ± 1.0	3.0 ± 1.1	1.50
	rBCG Mix/rMix	5.6 ± 1.8	3.8 ± 1.7	1.47
	rMix	1.00 ± 0.6		

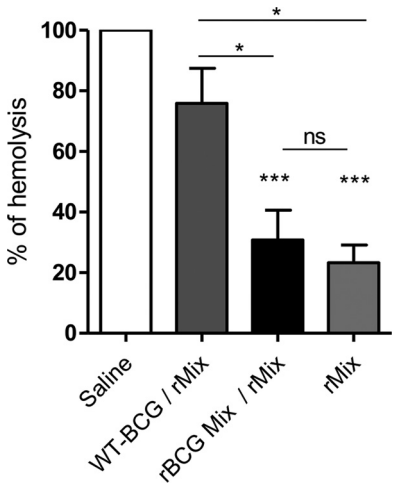

FIG 4 Antisera generated by rBCG Mix/rMix immunization inhibits pneumolysin activity. Ply was incubated with antisera from immunized mice (1:80) and sheep red blood cells. The absorbance of the supernatant at 540 nm was measured. Results are shown as percentages of the hemolytic activity in the presence of sera from mice receiving saline and $Al(OH)_3$ (control). Results are represented as the means (+SEM) of triplicate samples. Statistical analysis was performed by one-way ANOVA with a Tukey's multiple-comparison test. ***, $P < 0.001$; *, $P < 0.05$ for groups versus saline group or between samples, as indicated. Results are representative of two independent experiments using different pooled sera. ns, not significant.

with WT-BCG and boosted with rMix (IgG1/IgG2c ratio, 1.17) (Fig. 3C and Table 2). On the other hand, sera of mice that received a prime dose of rBCG Mix followed by a booster dose with rMix showed predominantly an IgG2c isotype subclass (IgG1/IgG2c ratio, 0.53) response against rPspA (Fig. 3C and Table 2). Immunization with WT-BCG or rBCG Mix followed by a booster dose also increased the IgG2 level against PdT; however, these results were not statistically significant (Fig. 3D and Table 2).

Antisera generated by rBCG Mix/rMix immunization inhibits Ply activity. Antisera obtained after immunization of mice were evaluated for their ability to inhibit the cytolytic Ply activity on sheep red blood cells. It was observed that sera produced against rBCG Mix/rMix reduced the cytolytic activity of Ply by approximately 70% compared with sera from the control group (Fig. 4). This result is similar to those observed using sera from mice immunized with the recombinant proteins mixed (rMix). Antisera from mice immunized with WT-BCG/rMix reduced the Ply activity only by 25%, a reduction that did not meet statistical significance (Fig. 4).

rBCG Mix/rMix protects mice against lethal pneumococcal challenge. We also evaluated the ability of combined rBCG pneumococcal vaccines to protect mice against invasive disease. Mice immunized with WT-BCG or rBCG Mix with or without a booster dose of rMix, rBCG 0148/rSP 0148, or rBCG 2108/rSP 2108 were challenged by intranasal inoculation with the virulent serotype 3 pneumococcal strain WU2 (32) while the mice were under anesthesia. Mice that received saline and $Al(OH)_3$ were used as a control. In this model, mice aspirate the inoculum into the lungs and develop illness by the third day after infection. Immunization with rBCG Mix/rMix or WT-BCG/rMix was able to protect 90% of mice compared to 100% mortality in the saline group (Fig. 5A). Immunization of mice with WT-BCG or rBCG Mix without a booster dose or with rBCG 0148 or rBCG 2108 individually followed by a booster dose of the respective recombinant proteins did not protect against lethal pneumococcal challenge (Fig. 5A).

rBCG Mix/rMix reduces density of pneumococcal colonization. The effect of rBCG vaccines against pneumococcal colonization was evaluated 3 weeks after the booster dose. Mice immunized in a prime/boost scheme were challenged by the intranasal route using the 6B pneumococcal strain St 603 (33). A retrograde nasal wash was performed 7 days later, and the washes were plated on blood agar for counting of the CFU recovered. Immunization of mice with rBCG 0148/rSP 0148 and rBCG 2108/rSP

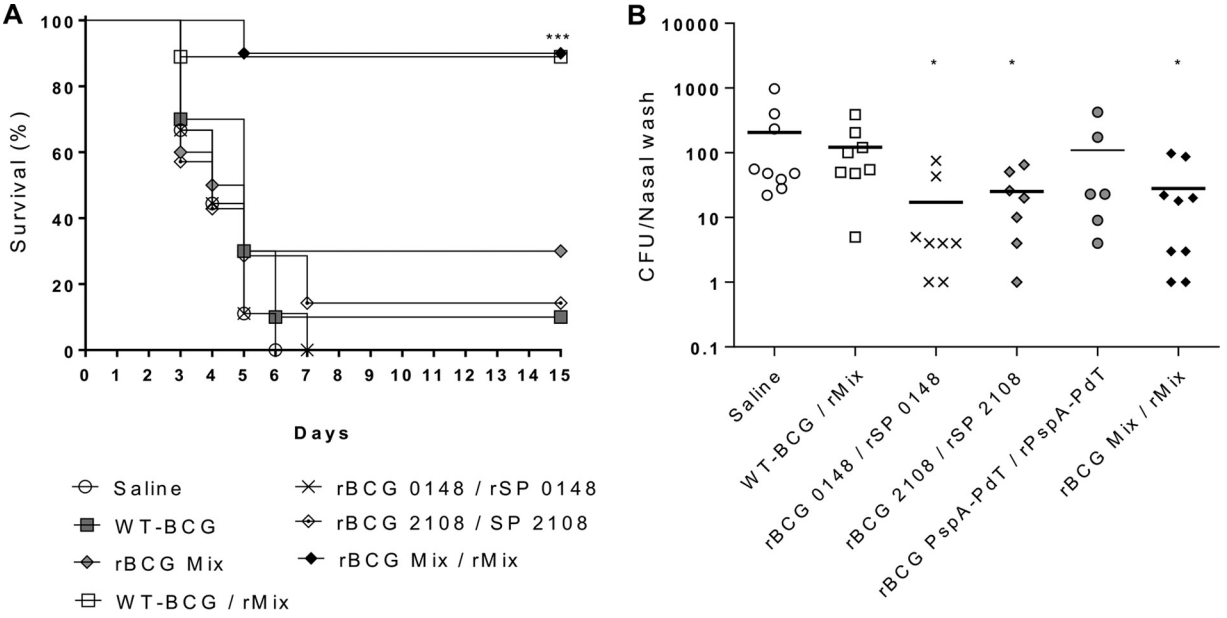

FIG 5 rBCG Mix/rMix immunization protects mice against pneumococcal aspiration pneumonia/sepsis challenge and pneumococcal colonization. C57BL/6 mice were immunized with WT-BCG or with each strain of rBCG, separately or combined (rBCG Mix), and boosted with a dose of the respective recombinant proteins. The challenges were performed 3 weeks after the last dose. (A) Mice were challenged by lung aspiration with 1×10^7 CFU of the WU2 pneumococcal strain and monitored for 15 days. Survival rates in each group were analyzed by Kaplan-Meier test; ***, $P < 0.001$ versus saline or WT-BCG. (B) Mice were challenged by the intranasal route with 1×10^6 CFU of the St 603 pneumococcal strain. The nasal wash was performed 7 days later and plated on blood agar. CFU recovered were counted after overnight incubation. The horizontal lines represent the mean of each group. Statistical analysis was performed by one-way ANOVA with a Tukey's multiple-comparison test. *, $P < 0.05$ versus saline or WT-BCG groups. Results are representative of two independent experiments.

2108 reduced pneumococcal colonization compared to the saline and WT-BCG/rMix groups (Fig. 5B), whereas immunization with rBCG PspA-PdT/rPspA-PdT had no significant effect (Fig. 5B). Importantly, the combination of all three rBCG strains (rBCG Mix) followed by a booster dose of recombinant proteins (rMix) retained the ability to protect mice against pneumococcal colonization (Fig. 5B).

DISCUSSION

The emergence of diseases by nonvaccine pneumococcal serotypes has encouraged the investigation of new vaccines based on serotype-independent antigens. Here, we evaluated the use of BCG strains, which have known adjuvant proprieties, to express the SP 0148 and SP 2108 pneumococcal antigens previously described by their ability to induce an IL-17A response (22).

The pneumococcal antigens SP 0148 and SP 2108 were successfully expressed in rBCG. The ability to induce IL-17A production has been described as a key factor to reduce pneumococcal colonization in mice (19). Therefore, we evaluated cytokine production in the supernatants of cultured splenocytes from immunized mice. We observed that splenocytes of mice receiving rBCG 0148 or rBCG 2108 boosted with the respective recombinant proteins produced significant levels of IL-17A and IFN-γ after *in vitro* stimulation compared with the control group. In accordance with these results, immunization of mice with rBCG 0148/rSP0148 or rBCG 2108/rSP2108 reduced pneumococcal colonization. Although immunization with WT-BCG followed by a single dose of rMix increased IL-17A production in the splenocyte culture after rSP 2108 stimulus, this IL-17A response did not reduce the nasopharyngeal load of pneumococci, suggesting a nonspecific *in vitro* response induced by immunization with WT-BCG. The nonspecific effect of BCG has been described as a "trained immunity response," where immunization with BCG induces a strong and short-lived immune response against heterologous infections; this effect appears to decline after 1 year (34, 35).

We have recently demonstrated that the use of rBCG PspA-PdT followed by a

booster dose of recombinant protein is able to induce an effective immune response and to protect mice against lethal challenge (31). In the current study, we evaluated whether the combination of rBCG PspA-PdT with rBCG 0148 and rBCG 2108 would affect the immunological response against rPspA and rPdT. Immunization of mice with rBCG Mix/rMix showed a higher level of total IgG against rPspA and rPdT than a single dose of the recombinant proteins. Furthermore, rBCG Mix/rMix maintained the ability to promote antibody isotype shift against PspA from IgG1 to IgG2c, which was first demonstrated using rBCG PspA-PdT/rPspA-PdT (31). The increased level of IgG2c against PspA may in theory enhance the complement deposition onto the pneumococcal surface (31, 36, 37), enhancing opsonophagocytosis (36).

Immunization of mice with WT-BCG or rBCG Mix and boosted with rMix induced greater amounts of anti-PdT IgG antibodies than with a single dose of rMix. Interestingly, when we evaluated the functionality of these antibodies, we observed that only sera generated against rBCG Mix/rMix or rMix were able to inhibit the hemolytic activity of Ply on sheep red blood cells. Indeed, the *in vivo* neutralization of Ply can be correlated with protection against tissue damage during a pneumococcal infection (38). The result obtained using sera from mice immunized with WT-BCG/rMix is in accordance with our previous study in which sera from mice receiving a priming dose of WT-BCG and boosted with rPspA-PdT showed poor ability to inhibit the cytolytic activity of Ply (31). These results suggest that immunization with WT-BCG does not prime adequately the immune system to produce high-quality antibodies against Ply; elucidating the mechanism of this effect deserves further investigation.

We also evaluated the ability of rBCG strains to protect mice against pneumococcal lethal challenge or reduce nasopharyngeal colonization. Prime-boost immunization of mice with rBCG 0148/rSP0148 or rBCG 2108/rSP2108 promoted significant protection against colonization; however, they failed to protect against lethal challenge. We previously showed that immunization with rBCG PspA-PdT/rPspA-PdT was able to elicit protection against lethal challenge (31), but this immunization strategy is not effective against pneumococcal colonization. In the attempt to extend protection to both models, we sought to combine the effective immune response of rBCG 0148/rSP0148 and rBCG 2108/rSP2108 against colonization with the protective role of rBCG PspA-PdT/rPspA-PdT for invasive diseases. The combined rBCG mix group in the prime-boost scheme was able to protect against both pneumococcal sepsis and significantly reduce colonization. In the lethal challenge, 90% protection was observed in WT- or rBCG Mix-primed groups, consistent with an anti-PspA/PdT antibody-dependent protection. In fact, passive transfer of sera from immunization of mice with rPspA has demonstrated a high ability to protect naive mice against lethal challenge (13). In the colonization model where the IL-17A-mediated immune response has a protective role, the involvement of SP0148 and SP2108 is clear (22).

Given the impressive impact of conjugate vaccines on invasive disease and colonization, a major challenge in the development of protein-based pneumococcal vaccine is the selection of antigens that can induce protective antibody and T-cell responses. As our study shows, using the rBCG as an approach to deliver pneumococcal proteins, antigens able to induce IL-17A responses and protection against colonization are not necessarily able to protect against invasive disease; conversely, antigens able to induce antibody-mediated protection against sepsis may not necessarily protect against pneumococcal colonization.

BCG vaccine can be administered at birth (http://www.who.int/immunization/research/development/tuberculosis/en/). The use of BCG as a vector to deliver heterologous antigens is aimed at inducing an immune response in the early stages of life and reducing the number of booster doses required to promote effective protection. In this study, we demonstrate that a prime immunization with rBCG 0148 or rBCG 2108 followed by a booster with a single dose of the respective proteins is able to induce a specific immune response and protection against colonization. The prime-boost strategy thus appears to provide protection in mice similar to that with injection with multiple doses of the respective recombinant proteins (22), whereas no protection was

observed when WT-BCG was used as the prime. The combination of rBCG strains delivering PspA-PdT, SP 0148, and SP 2108 in a prime-boost strategy induced an effective immune response mediated by antibodies against both PspA and PdT and IL-17A production against SP 0148 and SP 2108. In addition, the anti-PdT antisera showed the ability to inhibit the hemolytic activity of Ply *in vitro*. The rBCG Mix/rMix protected mice against a lethal challenge and pneumococcal colonization. This formulation comprises a promising serotype-transcending vaccine strategy potentially able to protect in the early stages of pneumococcal nasopharynx colonization and against invasive diseases and deserves further investigation.

MATERIALS AND METHODS

Pneumococcal strains. Pneumococcal strains were grown in Todd-Hewitt broth (Difco, Detroit, MI, USA) supplemented with 0.5% yeast extract (THY) at 37°C, without shaking. The bacteria were plated in blood agar and grown overnight at 37°C before inoculation in THY. The stocks were maintained at −80°C in THY containing 10% glycerol.

Construction of rBCG vaccines. All cloning steps were performed as previously described (31). The gene fragments encoding SP 0148 and SP 2108 were amplified from the TIGR4 pneumococcal strain and inserted into the pMIP12 vector, an *Escherichia coli-Mycobacterium* shuttle vector; this episomal vector contains a P_{BlaF^*} promoter derived from *Mycobacterium fortuitum*, an optimized Shine-Dalgarno sequence (Mega SD), and a kanamycin resistance gene, as described by Le Dantec et al. (39). BCG was transformed by electroporation, as previously described (40), with the pMIP12-0148 or pMIP12-2108 expression vector and plated onto Middlebrook 7H10 medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) and 20 μg/ml kanamycin. To prepare wild-type BCG (WT-BCG) or rBCG vaccines, mid-log-phase liquid cultures of selected clones were centrifuged at 4,000 $\times$ *g*, washed twice in 10% glycerol–phosphate-buffered saline (PBS), resuspended in the same buffer, and maintained in aliquots at −80°C.

Immunoblotting. Immunoblotting was performed as previously described (31). Briefly, BCG cells washed and resuspended in PBS were disrupted by sonication, and ~30 mg of the soluble fraction of each sample was separated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel; WT-BCG was used as a negative control and ~200 ng of the respective SP 0148 or SP 2108 recombinant proteins as a positive control. The membranes were incubated with pooled mice antisera against rSP 0148 or rSP 2108 (diluted 1:500), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000; Sigma).

Animals and immunization. All animal experiments were approved by the ethics committee at Instituto Butantan, São Paulo (CEUAIB) (permit no. 1360/15) or Boston Children's Hospital (protocol 13-11-2561R). Female C57BL/6 mice from Faculdade de Medicina-Universidade de São Paulo (São Paulo, Brazil) or from Jackson Laboratories (Bar Harbor, ME, USA) were immunized subcutaneously with WT-BCG or rBCG strains, as described in Table 1. A booster dose of recombinant protein was administered 30 days after the priming dose using 100 μg of $Al(OH)_3$ as adjuvant (Table 1).

Spleen cell culture and cytokine detection. Three weeks after the last immunization, mice were euthanized and the spleens collected aseptically. Splenocyte culture was performed as previously described (31). Cells were stimulated with 5 μg/ml rSP 0148 or rSP 2108. Culture supernatants were collected after 48 h, and the cytokine levels were measured by a mouse Th1/Th2/Th17 cytometric bead array (CBA) kit (BD Biosciences) using the flow cytometer FACSCanto II (BD Biosciences).

Antibody measurement. Two weeks after the last immunization, the animals were bled by retro-orbital puncture, and total IgG, IgG1, and IgG2 antibody production against rPspA or rPdT was evaluated by enzyme-linked immunosorbent assay (ELISA) using an IgG, IgG1, or IgG2c standard curve and horseradish peroxidase (HRP)-conjugated antibody anti-mouse IgG, IgG1 or IgG2c (Southern Biotechnology).

Hemolysis inhibition assay. To evaluate the neutralizing capacity of antisera containing anti-Ply antibodies, a hemolysis inhibition assay was performed as described previously (30). Briefly, pooled antisera (1:80) were incubated with Ply, and 2% sheep red blood cells were added. After incubation, the samples were harvested, and the absorbance of the supernatant at 540 nm was determined.

Pneumococcal aspiration pneumonia/sepsis and colonization challenge. The lethal aspiration challenge was performed 3 weeks after the last dose; anesthetized mice received 1×10^7 of the WU2 pneumococcal strain in 50 μl of saline delivered intranasally, using the protocol described by Malley et al. (41), with slight modifications. The animals were monitored for 15 days. Moribund mice or animals that developed any evidence of illness were immediately and humanely euthanized. For the colonization challenge, the animals received 1×10^6 CFU of the St 603 pneumococcal strain in 10 μl of saline by the intranasal route. The nasal wash was performed 7 days after the challenge, as described by Malley et al. (33). Briefly, the mice were euthanized, and the wash was done by instilling sterile PBS from transected trachea and collecting the first 6 drops (about 0.1 ml) from the nostrils. The washes were plated on blood agar containing 2.5 mg of gentamicin/ml and incubated at 37°C with 5% CO_2, and the recovered pneumococcal CFU were enumerated after 18 h.

Statistical analysis. Statistical analyses on CBA, ELISA, hemolysis assays, and colonization challenge were performed using one-way analysis of variance (ANOVA) with a Tukey's multiple-comparison test. Survival rates were analyzed by a Kaplan-Meier test.

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