In accordance with this, it has been suggested that gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) are key cytokines in the early innate immune response to this pathogen. These cytokines play a crucial role in recruiting immune cells to the site of infection and activating them to mount an effective immune response.

However, the importance of Th1 and Th2 responses in the immune response to pneumococcal infections is still a matter of debate. Th1 responses are characterized by the production of interferon-γ (IFN-γ), tumor necrosis factor (TNF-α), and other pro-inflammatory cytokines, which are thought to be important in combating bacterial infections. On the other hand, Th2 responses are characterized by the production of interleukin-4 (IL-4) and IL-13, which are thought to be important in promoting humoral immunity and antibody production.

Recent studies have suggested that a balanced Th1/Th2 response is crucial for optimal immunity against pneumococcal infections. This is because different stages of the infection require different types of immune response. For example, the early innate immune response is dominated by Th1 cytokines, while the later adaptive immune response is dominated by Th2 cytokines. A balanced Th1/Th2 response is likely to be more effective in controlling the infection than an unbalanced response.

This is supported by several studies that have shown that immunization with recombinant proteins can elicit an antibody response that is biased towards Th1 or Th2 responses. For example, immunization with PspA (pneumococcal surface protein A) DNA is able to elicit a balanced Th1/Th2 response compared to immunization with PspA protein (with alum as adjuvant), even though the antibody levels elicited by DNA immunization are lower than those elicited by immunization with the protein. This shows that DNA immunization can be a promising candidate for the development of a cost-effective protein-based vaccine.

Although immunity to pneumococcal diseases has long been assumed to depend mainly on the humoral immune response, Malley and collaborators have recently shown antibody-independent immunity mediated by CD4+ T cells in a murine model of pneumococcal disease (16). This is consistent with the idea that both humoral and cellular immunity are important in protecting against pneumococcal infections.

In conclusion, the immune response to pneumococcal infections is complex and involves both humoral and cellular components. A balanced Th1/Th2 response is likely to be more effective in controlling the infection than an unbalanced response. Therefore, developing vaccines that can elicit a balanced Th1/Th2 response is likely to be important in the future development of vaccines against pneumococcal infections.
Our previous results showed that protective Th1 immune responses against pneumococcal infection in a mouse model can be achieved successfully by DNA vaccines using vectors expressing PspA (8, 17). The PspA fragment used in this study comprises the N-terminal region through the proline-rich region without the signal sequence from a clade 3 family 2 PspA and is expressed in a secreted form by a DNA vaccine vector (pSec-pspA3NS). A previous study by our group showed that immunization of mice with the same fragment as a recombinant protein was able to induce anti-PspA antibodies that increased C3 deposition only on the pneumococci with PspA from the same family (family 2). On the other hand, PspA hybrids containing fused portions of both families (families 1 and 2) were able to increase C3 deposition on pneumococci bearing PspA fragments from both families (5).

The present study aims at characterizing the induction and functionality of the antibodies induced by immunization against S. pneumoniae, using PspA as a DNA vaccine and a recombinant protein in wild-type BALB/c mice. IL-4 KO mice were also used in order to alter the polarization of the immune response. Furthermore, mice immunized with DNA vaccine and recombinant protein were also analyzed in terms of cytokine release in response to an intraperitoneal pneumococcal challenge.

**Materials and Methods**

**Immunization of mice.** Five- to 7-week-old female BALB/c WT or IL-4 KO mice from ICB-USP (Universidade de São Paulo, São Paulo, Brazil) were supplied with food and water ad libitum. Animal experimental protocols were approved by the ethics committee of Instituto Butantan (São Paulo, Brazil).

Groups of four to six animals were lightly anesthetized and inoculated intramuscularly (i.m.) with 50 g of rPspA3NS, using 50 g of Al(OH)3 as adjuvant, on days 1 and 2. Alum-immunized mice or animals injected with the empty vector (pSec2a) were used as controls.

**Detection of anti-PspA-specific antibodies through ELISA.** Mice were bled individually from the retroorbital plexus 2 weeks after the last immunization for detection of antibodies through enzyme-linked immunosorbent assay (ELISA) in plates coated with rPspA3NS. The assay was performed using goat anti-mouse IgG, anti-mouse IgG1, anti-mouse IgG2a, and anti-goat IgG conjugated with horseradish peroxidase (Southern Biotech). Standard curves were generated using mouse IgG, IgG1, and IgG2a.

**Bacterial strains and growth conditions.** S. pneumoniae strains P-30 (serotype 6B; PspA clade 3), from Universidade Federal de Goiás (Goiânia, Brazil), and St 679/99 (serotype 6B; PspA clade 3), from Instituto Adolfo Lutz (São Paulo, Brazil), were cultured, aliquoted, and stored at -80°C in Todd-Hewitt 0.5% yeast extract containing 20% glycerol. To estimate the challenge dose and viability of bacterial stocks, groups of nonimmune mice were challenged with serial dilutions of the standard inoculum.

**i.p. challenge.** Immunized mice were challenged by intraperitoneal (i.p.) injection of 500 CFU of St 679/99 in 0.5 ml of saline 2 weeks after the final immunization. Animals were then observed for 1 week, and inactive, sick animals were euthanized.

**Antibody binding and complement deposition.** Antibody binding and complement deposition assays were performed as previously described (23). Briefly, S. pneumoniae P-30 frozen stocks were plated on blood agar overnight, grown in Todd-Hewitt 0.5% yeast extract to an optical density of 600 nm of 0.4 to 0.5 (~108 CFU/ml), and harvested by centrifugation. Bacteria were washed and resuspended in phosphate-buffered saline (PBS) and then incubated with 25% pooled sera for 30 min at 37°C. For the antibody binding assay, bacteria were then washed with PBS and resuspended in fluorescein isothiocyanate-conjugated goat anti-mouse IgG (MP Biomedicals). For complement deposition, after another wash with PBS, bacteria were resuspended in gelatin-Veronal buffer (Sigma) and incubated for 30 min at 37°C with 10% normal mouse serum as a source of complement. The bacteria were then washed, resuspended in fluorescein isothiocyanate-conjugated goat anti-mouse complement C3 (MP Biomedicals), and incubated for 30 min at 4°C in the dark. Finally, in both assays, bacteria were washed twice with PBS, resuspended in 1% formaldehyde, and analyzed using FACSCalibur flow cytometry (BD Biosciences).

**ELISPOT assay for detection of IFN-γ, IL-4, or TNF-producing spleen cells.** Pooled spleen cells for each group were obtained from immunized BALB/c mice 2 weeks after the final immunization (before challenge or 16 h after i.p. challenge with St 679/99), and cytokine-secreting cells were detected using IFN-γ, IL-4, and TNF enzyme-linked immunospot (ELISPOT) sets (BD Biosciences). Spleenocytes were incubated for 20 h in the presence of rPspA3NS (5 μg/ml). Preparation of spleen cells was performed as described previously (21), and the medium was supplemented with polyoxymlin B (10 μg/ml) to avoid nonspecific stimulation from lipopolysaccharide present in rPspA. An antibiotic-antimycotic solution (Gibco) containing penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (250 ng/ml) was added to the medium to eliminate live pneumococci from spleen cells obtained from infected mice. Mean numbers of spot-forming cells (SFCs) were calculated from triplicate wells.

**ELISA detection of IFN-γ or TNF-α in the supernatants of spleen cell cultures.** Cytokines in the supernatants of spleen cell cultures stimulated for 72 h with rPspA3NS (5 μg/ml) were assayed by sandwich ELISA (Peprotech).

**Statistical analysis.** Differences in anti-PspA antibody concentrations between groups were analyzed by Student’s t test, and those in the overall survival rate were analyzed by the Fisher exact test (P ≤ 0.05).

**Results**

**Humoral immune response elicited by DNA vaccine or recombinant protein.** Groups of BALB/c WT and IL-4 KO mice were immunized i.m. with the DNA vaccine (pSec-pspA3NS) or subcutaneously with recombinant protein, using alum as an adjuvant (rPspA3NS). As shown in Fig. 1A, total IgG antibody levels were higher in mice immunized with rPspA3NS than in those immunized with the DNA vaccine, with a statistical difference for IL-4 KO animals (P = 0.02). The levels of antibody to PspA elicited by both protein and DNA immunization were higher in the IL-4 KO mice than in the WT mice, with a statistical difference for mice immunized with the recombinant protein (P = 0.01). When the IgG isotypes of the anti-PspA antibodies elicited in the WT and IL-4 KO mice by the protein and DNA immunizations were determined, it was observed that only WT mice immunized with recombinant protein showed a marked predominance of IgG1 antibodies (Fig. 1B). Balanced amounts of IgG1 and IgG2a antibodies were observed for the other immunized groups.

**Antibody binding and complement deposition.** In order to investigate whether anti-PspA antibodies detected by ELISA would be able to bind to the surfaces of intact pneumococci (Fab region adequacy) and to mediate C3 deposition (Fc region activity), sera from mice immunized with pSec-pspA3NS or rPspA3NS were incubated with pneumococcal isolate P-30, and the amounts of bound antibody and complement C3 were determined (Fig. 2). Sera from alum-immunized mice (control group) are represented by the gray area in each graph. Sera from WT and IL-4 KO mice immunized with rPspA3NS (dotted lines) showed higher binding capacities than did sera from pSec-pspA3NS-immunized mice (solid lines) (Fig. 2A), indicating a strict correlation between total anti-PspA IgG concentration and antibody binding to the bacterial surface. However, when the ability of the immune sera to mediate C3 deposition onto the pneumococcal surface was analyzed (Fig. 2B), sera...
from WT animals injected with pSec-pspA3NS demonstrated a more pronounced increase in C3 deposition than did those from rPspA3NS-immunized mice, despite a lower binding capacity. Furthermore, sera from IL-4 KO mice immunized with both recombinant protein and DNA vaccines showed similar abilities to mediate complement deposition. A direct comparison between the data on complement deposition obtained for WT and IL-4 KO mice would not be reliable, since background C3 deposition mediated by the sera of control animals differed significantly between the two strains of animals.

Cytokine secretion elicited by DNA vaccine or recombinant protein. In order to investigate the role of cellular immune responses, we next immunized BALB/c WT mice with pSec-pspA3NS or rPspA3NS and, 2 weeks after the last immunization, isolated splenocytes 16 h after challenge with S. pneumoniae St 679/99. In the ELISPOT assay, the number of SFCs after in vitro stimulation with rPspA was determined, and results are shown in Fig. 3. After pneumococcal challenge, we observed a more pronounced increase in SFCs secreting IFN-γ in the pSec-pspA3NS-immunized group than in the control groups (alum and pSec2a) and the rPspA3NS-immunized group (Fig. 3A); very similar results were obtained for TNF secretion for the same immunized groups of mice (Fig. 3B). In contrast to the results with IFN-γ, a fourfold increase in IL-4 SFCs was observed in rPspA3NS-immunized mice compared with the number after pSec-pspA3NS immunization (Fig. 3C).

The presence of IFN-γ in the supernatant of splenocytes was also determined by ELISA before and after pneumococcal challenge (Fig. 4). Cells obtained from mice immunized with pSec-pspA3NS and restimulated in vitro showed a considerable improvement in IFN-γ secretion after challenge, which was more pronounced than that for immunization with rPspA3NS, corroborating the ELISPOT data. Secretion of IFN-γ was not detected in any of the groups before the challenge (Fig. 4A). We also looked for TNF-α, and interestingly, we observed that all groups showed larger amounts of this cytokine before than after the challenge, except for the pSec-pspA3NS-immunized group, which showed a trend toward an enhancement of TNF-α secretion after challenge with pneumococci (Fig. 4B).

Protection elicited by DNA vaccine or recombinant protein. Immunized BALB/c mice were then challenged i.p. with St 679/99, and survival was analyzed (Table 1). Both formulations with PspA elicited statistically significant protection in WT mice at similar levels, which is in accordance with previous results published by our group. IL-4 KO mice also showed enhancement of survival with both the recombinant protein and DNA vaccines, but rPspA protection had borderline significance. Both strategies thus showed a capacity to elicit protection against pneumococcal challenge.

DISCUSSION

Genetic immunization with vectors expressing PspA has previously been shown to be protective against pneumococcal challenge (3, 8, 17, 19). Our previous results showed that this
immunization elicited a more balanced anti-PspA IgG1/IgG2a response that protected mice against i.p. pneumococcal challenge at levels similar to those for recombinant protein immunization, despite significantly lower total anti-PspA IgG production (8). In this work, we show that recombinant protein immunization elicited higher levels of anti-PspA antibodies than did DNA immunization in WT and IL-4 KO mice. We also observed an increment in total anti-PspA IgG in IL-4 KO mice immunized with either rPspA3NS or pSec-pspA3NS compared to WT animals. Higher antibody responses to PspA have previously been observed in IL-4 KO mice than in WT mice, and this is assumed to be due to the lack of suppression of the IgG response by IL-4 (14). In our present studies, we also analyzed the IgG1/IgG2a ratio, and only WT mice immunized with recombinant protein showed a marked predominance of IgG1 (IgG1/IgG2a ratio = 1.024), while balanced IgG1/IgG2a ratios were observed for the other groups (ratio of 4 for pSec-pspA3NS-immunized WT mice, 2 for rPspA3NS-immunized IL-4 KO mice, and 0.1 for pSec-pspA3NS-immunized IL-4 KO mice).

We evaluated the ability of antibodies elicited in sera from mice immunized with rPspA and pSec-pspA3NS to bind to the pneumococcal surface and to mediate complement deposition. In the case of both WT and IL-4 KO mice, antibody to PspA in sera from mice immunized with rPspA showed much more binding to pneumococci than did antibody in sera from mice immunized with pSec-pspA3NS. These results confirm the functionality of the antibodies detected by ELISA and indicate a direct correlation between larger amounts of total anti-PspA IgG and an increase in antibody binding to the surfaces of intact pneumococci. On the other hand, an increase in antibody binding to pneumococci did not show a correlation with an enhancement of complement deposition. A more accurate correlate of complement deposition seemed to be the IgG1/IgG2a ratio: a more balanced response elicited by pSec-pspA3NS (ratio = 4) correlated with a much more robust deposition of complement than that for the Th2-biased response elicited by rPspA3NS (ratio = 1.024) in WT mice. This hypothesis is further reinforced by the data obtained with IL-4 KO mice, with balanced responses (ratio of 2 for rPspA3NS and 0.1 for pSec-pspA3NS) mediating similar depositions of complement.

Since IgG2a is the isotype with the greatest capacity to mediate complement deposition onto the pneumococcal surface (1), it could be reasonable to correlate a higher total IgG2a concentration with an increase in complement deposition. We propose, though, that large amounts of IgG1 elicited by the recombinant protein could block the binding of anti-PspA IgG2a antibodies, thus compromising complement deposition. This competition between anti-PspA IgG1 and IgG2a binding to the pneumococcal surface might be the reason why a balanced IgG1/IgG2a response might be beneficial in medi-

FIG. 2. Binding of anti-PspA antibodies and complement deposition on the pneumococcal surface. Pooled sera from BALB/c WT and IL-4 KO mice immunized with a recombinant protein or DNA vaccine were tested for the abilities to bind to the pneumococcal surface (A) and to mediate complement deposition (B). S. pneumoniae strain P-30 was incubated with 25% serum from mice immunized with DNA vaccine vector pSec-pspA3NS (solid lines), recombinant PspA3NS (dotted lines), or alum-immunized animals (gray areas). The median fluorescence of the bacteria is shown for each sample. Results are representative of two independent experiments.
Ig isotype to prevent the effector activity by another isotype has been described previously for *Neisseria meningitidis* (9).

Although passive protection experiments have shown that antibodies against PspA are sufficient to induce protection against fatal infection in mice (4), the increasing evidence for the role of cellular immune responses in pneumococcal disease prompted us to further characterize the profiles of cytokines induced in animals immunized with DNA vaccine and recombinant protein after challenge with *S. pneumoniae*. Splenocytes from mice immunized with the DNA vaccine showed secretion of IFN-γ and TNF-α, whereas animals injected with recombinant protein showed induction of IL-4 when restimulated in vitro with rPspA. These results corroborate the data on the IgG isotype ratio induced by immunization, with recombinant protein inducing a Th2-biased response with a high IgG1/IgG2a ratio and secretion of IL-4. In contrast, immunization with the DNA vaccine showed a trend toward a Th1 response, with a balanced IgG1/IgG2a ratio and secretion of IFN-γ and TNF-α. The role of IFN-γ in the resolution of pneumococcal infection is well described for different mouse models (24, 27), but the role of TNF-α is controversial.

A previous study reported that after intranasal pneumococcal infection, TNF-α receptor KO mice exhibit elevated bacteremia and progress more rapidly to a fatal outcome (12). Although studies showed that TNF-α may be dispensable in pulmonary responses, it appears to be a necessary beneficial regulator of systemic organ protection in pneumococcal disease (15). The role of TNF-α may be a double-edged sword, since TNF-α is important in the early stages of infection but its levels need to be controlled later to avoid an exacerbated inflammatory response (12).

A recent study reported that Toll-like receptor 9 (TLR9)-deficient mice, but not TLR1-, TLR2-, TLR4-, or TLR6-deficient mice, are more susceptible to respiratory bacterial infection caused by pneumococci and that pneumococcal infection triggers a TLR9- and MyD88-dependent activation of phagocytic activity from resident macrophages, leading to an early clearance of bacteria from the lower respiratory tract (2). It is known that TLR9 induces an inflammatory response by rec-
ognition of unmethylated CpG present in prokaryotic DNA. We thus postulate that DNA vaccines could induce an immune response similar to the one elicited by bacterial infection, which would be much more suitable than the response elicited by immunization with recombinant protein with alum as an adjuvant in terms of the profile of both humoral and cellular responses. Although it is clear from our data that immunizations with both recombinant protein and DNA vaccines elicit immune responses which are sufficient for protection against lethal challenge with *S. pneumoniae*, immunization with DNA vaccines showed a response that is qualitatively superior, with the induction of a balanced IgG1/IgG2a anti-PspA antibody response that correlated with a more efficient mediation of complement deposition as well as a specific cellular immune response characterized by the secretion of proinflammatory cytokines. We thus propose that a vaccine based on PspA should be administered with adjuvants that induce a more balanced Th1/Th2 response, such as unmethylated CpG oligodeoxynucleotides.

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![FIG. 4. Cytokine responses in spleen cells induced by DNA vaccine and recombinant protein. Splenocytes isolated from immunized BALB/c WT mice before or 16 h after i.p. challenge with pneumococci were incubated for 72 h with PspA (5 μg/ml) or medium only as a control (nonstimulated). IFN-γ (A) and TNF-α (B) levels in the supernatants were detected through sandwich ELISA. Results are representative of two independent experiments.](image)

**TABLE 1. Survival of BALB/c mice after challenge with *S. pneumoniae* 679/99**

| Vaccine         | WT mice | IL-4 KO mice |
|-----------------|---------|--------------|
|                 | No. of survivors/ total no. of mice | % Survival | P value | No. of survivors/ total no. of mice | % Survival | P value |
| Alum            | 0/5     | 0            |          | 2/5     | 40          |          |
| rPspA3NS        | 4/5     | 80           | 0.02a    | 5/5     | 100         | 0.08     |
| pSec2a          | 0/5     | 0            |          | 0/5     | 0           |          |
| pSec-pspA3NS    | 5/5     | 100          | 0.004b   | 4/4     | 100         | 0.009b   |

a Statistically different from animals injected with alum (P ≤ 0.05).
b Statistically different from animals injected with pSec2a (P ≤ 0.05).
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