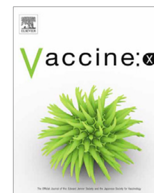
Vaccine: X 4 (2020) 100049

Contents lists available at ScienceDirect

Vaccine: X

journal homepage: www.elsevier.com/locate/jvacx



Early pneumococcal clearance in mice induced by systemic immunization with recombinant BCG PspA-PdT prime and protein boost correlates with cellular and humoral immune response in bronchoalveolar fluids (BALF)

Cibelly Goulart [a,1], Dunia Rodriguez [a], Alex I. Kanno [a], José Lourenço S.C. Silva [a,b], Luciana C.C. Leite [a,*]

[a] Laboratório de Desenvolvimento de Vacinas, Instituto Butantan, Brazil
[b] Programa de Pós-Graduação Interunidades em Biotecnologia, Universidade de São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 16 October 2018
Received in revised form 22 November 2019
Accepted 26 November 2019
Available online 3 December 2019

Keywords:
Streptococcus pneumoniae
PspA
PdT
rBCG
Cytokines
Protection

ABSTRACT

An effective immunological response in the lungs during a pneumococcal infection is a key factor to the bacteria clearance and prevention of sepsis. In order to develop broad-range pneumococcal vaccines several pneumococcal proteins and strong adjuvants have been investigated. Previously, we constructed a recombinant BCG (rBCG) strain expressing a fragment of PspA (Pneumococcal surface protein A) fused to PdT (detoxified form of pneumolysin). Immunization of mice with a priming dose of rBCG PspA-PdT followed by a booster dose of rPspA-PdT fused protein induced a high antibody response in the serum and protected mice against lethal challenge. Here, we investigated the humoral and cellular immune response in the Bronchoalveolar lavage fluid (BALF). Immunization of mice with rBCG PspA-PdT / rPspA-PdT induced rapid clearance of bacteria after challenge, an early control of the cellular influx and reduced inflammatory cytokine levels in the BALF. In addition, rBCG PspA-PdT / rPspA-PdT induced higher lymphocyte recruitment to the lungs at 48 h, showing an increased percentage of $CD4^{+}$ T cells. Furthermore, BALF samples from mice immunized with rBCG PspA-PdT / PspA-PdT showed high binding of IgG2c and enhanced complement deposition on the pneumococcal surface; antibody binding was specific to PspA as no binding was observed to a PspA-knockout strain. Taken together, our results show that the immunization with rBCG PspA-PdT / rPspA-PdT induces humoral and cellular immune responses in the lungs, promotes an early clearance of pneumococci and protects against the systemic dissemination of pneumococci.

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1. Introduction

Pneumococcal proteins have been extensively investigated as alternative pneumococcal vaccines. Whereas polysaccharide-based vaccines induce protection restricted to the serotypes included in the vaccine formulation, well-conserved proteins are able to induce protective responses against pneumococcal strains bearing different capsular types [1]. The selection of the antigen, adjuvant and delivery system are key factors to the development of a successful vaccine. BCG is a live attenuated anti-tuberculosis vaccine, able to induce both humoral and cellular immune responses [2]. Due its adjuvant properties, BCG strains have been evaluated as delivery system for heterologous antigens derived from viruses, bacteria and parasites [3].

The Pneumococcal surface protein A (PspA) is highly immunogenetic and interferes with complement deposition onto the pneumococcal surface, reducing opsonophagocytosis [4]. Pneumolysin (Ply) is a cytolysin that plays an important role in the evasion of the host immune system, forming pores in cholesterol-containing cell membranes and activating the complement system during pneumococcal infections [5,6]. Antibodies generated against recombinant PspA and PdT, a genetically detoxified form of Ply, are able to inhibit their activity and promote an efficient immunological response leading to protection against systemic disease [7,8]. In addition, Langermann and colleagues (1999) demonstrated that rBCG strains expressing PspA fragments protected mice against a lethal pneumococcal challenge [9].

* Corresponding author.
E-mail address: luciana.leite@butantan.gov.br (L.C.C. Leite).
[1] Current affiliation: School of Life Science / Faculty of Science, University of Technology Sydney.

https://doi.org/10.1016/j.jvacx.2019.100049
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We have previously demonstrated the antigenic potential of a fusion protein composed by PspA and PdT (rPspA-PdT) [10]. In order to improve the immunogenicity of this protein we constructed a rBCG strain to deliver PspA-PdT through a live attenuated vector (rBCG PspA-PdT) [11]. Immunization of mice with the rBCG PspA-PdT as a priming dose and a single dose of rPspA-PdT as a booster (rBCG PspA-PdT / rPspA-PdT) induced a high antibody response against both proteins in the sera, promoted a shift from IgG1 to IgG2 antibody isotype and increased the expression of inflammatory cytokines in splenocytes culture. Furthermore, immunization with rBCG PspA-PdT / rPspA-PdT protected mice against pneumococcal aspiration pneumonia/sepsis challenge [11].

Since pneumococcal pneumonia usually precedes the invasion of the bacteria to the bloodstream [12], the immunological responses induced in the lungs are a key factor to prevent disease dissemination. In addition, it is known that neutrophils are quickly recruited to the lungs during a pneumococcal infection, playing an important role in the bacterial clearance [13]. However, an exacerbated inflammatory response characterized by prolonged cellular influx and increased release of inflammatory cytokines is correlated with tissue damage and poor prognosis of the pneumococcal disease [13–15]. Immunization of mice with several pneumococcal antigens including PspA and pneumolysoids has been shown to promote a more rapid clearance of pneumococci, which results in reduced neutrophil recruitment and controled cytokine release in the lungs, leading to protection against tissue damage and increasing the survival after pneumococcal challenge [8,15,16].

In this context, we investigated the role played by humoral and cellular immune responses in the Bronchoalveolar lavage fluid (BALF) induced by subcutaneosly immunization of mice with rBCG PspA-PdT / rPspA-PdT that leads to rapid pneumococcal clearence in the first hours after intranasal challenge.

2. Material and methods

2.1. Pneumococcal strains

S. pneumoniae strains WU2 ($PspA^{+}$) and JY119 ($PspA^{-}$) were grown as previous described [10] and maintained at −80 °C until used.

2.2. Mouse immunization

All animal experiments were approved by the Ethics Committee at Instituto Butantan, São Paulo – SP (CEUAIB), (Permit Number 1360/15). Female C57BL/6 mice (n = 5 mice per time point for each group) from Faculdade de Medicina – Universidade de São Paulo (São Paulo, Brazil) were immunized subcutaneously (s.c.) with 1×10^6 CFU of rBCG PspA-PdT or WT-BCG; mice of the Control group received sterile 0.9% saline solution. rPspA-PdT protein (10 μg) was administered (s.c.) in saline and 100 μg of $Al(OH)_3$ as adjuvant [11], as a single dose (rPspA-PdT group) or as a booster dose 30 days after priming with WT-BCG or rBCG PspA-PdT (WT-BCG / rPspA-PdT and rBCG PspA-PdT / rPspA-PdT groups).

2.3. Intranasal pneumococcal challenge

Immunized mice were anesthetized by i.p. injection of a mixture containing ketamine (100 mg/Kg) and xylazine (10 mg/Kg) 21 days after the last dose, before receiving 1×10^6 CFU of the WU2 pneumococcal strain in 50 μL saline delivered intranasally by aspiration.

2.4. Blood and Bronchoalveolar lavage fluid (BALF) collection and cell count

Blood samples from the *retro*-orbital plexus were collected and maintained in heparinized tubes. Mice were euthanized by i.p. injection with a mixture containing ketamine (300 mg/Kg) and xylazine (30 mg/Kg) before or at the indicated time points after challenge. For BALF collection, the trachea was cannulated using a catheter and lungs were rinsed twice with 0.5 and 1.0 mL of cold PBS and collected samples kept on ice. Cells obtained from the BALF were counted using a hemocytometer and 4×10^4 cells were used to prepare cytospin slides. The cells were stained using the Quick Panoptic Instant Prov (Newprov) staining Kit, according to the manufacturer's instructions, which allows identification of the different cell types by morphology. The number of neutrophils, macrophages and lymphocytes were obtained by differential cell counting of 100 cells through microscopic analysis.

2.5. Recovery of pneumococci from blood and BALF

Individual samples of blood and BALF collected before or at the indicated time points post infection were diluted serially and plated onto blood agar. Colony-forming units (CFU) recovered were counted 18 h after incubation at 37 °C in anaerobic jars.

2.6. Antibody and cytokine analysis

The BALF samples were centrifuged at 1000 × g for 10 min and the supernatant stored at −80 °C for antibody and cytokine analysis. Antibody production against recombinant PspA and PdT was evaluated by ELISA using an IgG standard curve and horseradish peroxidase (HRP) conjugated anti-mouse IgG antibody (Southern Biotechnology). Cytokine production was directly measured in the BALF samples. The granulocyte-colony stimulating factor (G-CSF) and IL-17 were analyzed by ELISA (Peprotec and R&D System) and IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ were determined by Cytometric Bead Array (CBA; BD Bioscience), according to the manufacturers recommendations.

2.7. Lymphocytes flow cytometry immunophenotyping

BALF samples were collected as described above and 1×10^6 cells were stained with APC-CY7 conjugated anti-mouse CD3, PE-CY5 conjugated anti-mouse CD4, PE conjugated anti-mouse CD8 and FITC conjugated anti-mouse B220 (BD Bioscience). The flow cytometric acquisition of 30.000 events was performed using a FACS Canto II (BD, Bioscience) and the data were analyzed using FlowJo version 7.6.5.

2.8. Antibody binding and complement deposition assays

The ability of antibodies from the BALF of immunized mice (before challenge) to bind to the PspA exposed on the surface of the pneumococcal strain WU2 and promote C3 deposition was evaluated as previously described [10]. Briefly, pneumococci were incubated with individual and non-diluted BALF samples followed by incubation with FITC-conjugated anti-mouse IgG antibody (1:500 in PBS – MP Biomedical) or FITC-conjugated anti-mouse IgG1 or IgG2 antibody (1:100 in PBS – Southern Biotech). For the complement deposition assay, after incubation with BALF, pneumococci were washed once with PBS and incubated with 10% normal mouse sera (NMS) diluted in Hank's Balanced Salt Solution (HBSS – GIBCO) containing 0.1% of gelatin (SIGMA). Next, pneumococci were incubated with FITC-conjugated anti-mouse C3 molecule antibody (1:500 in PBS – MP Biomedical). Samples were then analyzed by flow cytometry using a FACS Canto II (BD,

Bioscience) and the data analyzed using FlowJo version 7.6.5. Non-stained pneumococci were used to determine the negative cell population. The percentage of positive cells was used for statistical analysis. Additionally, the antibody binding assay was performed on a parent PspA knock-out strain, JY119, under the same conditions. Geometric mean of fluorescence intensity (Geo MFI) was evaluated in histograms using the Flow Jo 7.6.5 software.

2.9. Statistical analysis

All results are representative of two independent experiments (n = 5 per time point for each group). Collected samples were analyzed individually and shown as means (+SEM). Statistical analyses were performed by one-way ANOVA with a Tukey's Multiple Comparison Test between the groups using GraphPad Prism: Significant results are shown by **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ versus control group or between groups as indicated by the connecting bars.

3. Results

3.1. rBCG PspA-PdT / rPspA-PdT immunization rapidly controls the pneumococcal load in the BALF and prevents systemic dissemination

We evaluated the pneumococcal loads recovered from BALF and blood of mice immunized with rBCG PspA-PdT / rPspA-PdT and controls in the first hours after an aspiration/intrapulmonary challenge using the virulent strain, WU2. All immunized groups showed similar CFU counts ($\sim 10^5$–10^6) in the BALF samples within the first 6 h post infection, as observed in Fig. 1A. Mice immunized with WT-BCG or those that received saline (Control) were unable to limit the infection and the pneumococci counts in the BALF remained high until the final data point at 72 h (Fig. 1A). In addition, high pneumococci count ($\sim 10^3$–10^5) was recovered from blood samples of both groups 24 h post infection (Fig. 1B). BALF samples from mice immunized with a single dose of rPspA-PdT showed reduced bacterial count ($\sim 10^4$) 12 h post infection (Fig. 1A), while WT-BCG / rPspA-PdT immunized mice started to control the infection after 24 h (Fig. 1A). Pneumococci were recovered in the blood samples from these groups 48 h post infection (Fig. 1 B). On the other hand, mice immunized with rBCG PspA-PdT / rPspA-PdT started to control the pneumococcal infection at 12 h, showing the lowest bacterial count in the BALF ($\sim 10^2$) when compared with all other groups at this point; the pneumococcal count continued decreasing and no bacteria was detected 48 h post infection (Fig. 1A). Furthermore, rBCG PspA-PdT / rPspA-PdT immunized group did not show pneumococcal dissemination to the bloodstream (Fig. 1B).

3.2. rBCG PspA-PdT / rPspA-PdT immunization controls the cellular influx 24 h after the challenge and increases the lymphocytes recruitment

The cellular influx in the BALF samples was evaluated at the indicated time-points before or after pneumococcal intranasal challenge. The total cell count increased in all groups 12 h post infection (Fig. 2A), which was largely dependent on the increased neutrophil count at the same time point (Fig. 2B). The increased neutrophil count remained high in the Control and WT-BCG groups until the final time-point at 72 h (Fig. 2B). Mice immunized with WT-BCG / rPspA-PdT or rBCG PspA-PdT / rPspA-PdT started to control the cellular influx 24 h post infection, showing reduced total cell and neutrophil counts when compared with control groups (Fig. 2A and B). Similar results were observed 48 h post infection in mice that received a single dose of rPspA-PdT (Fig. 2B). Additionally, BALF samples from mice immunized with rBCG PspA-PdT / rPspA-PdT showed a higher number of lymphocytes 48 h post infection (Fig. 2D) when compared with Control or WT-BCG groups (Fig. 2D). At the 72-h data point, BALF samples from mice immunized with rPspA-PdT, WT-BCG / rPspA-PdT or rBCG PspA-PdT / rPspA-PdT showed reduced total cell counts (Fig. 2A) and the predominance of macrophages over neutrophils (Fig. 2C and B). No significant eosinophil counts were observed in any of the groups tested (data not shown).

3.3. rBCG PspA-PdT / rPspA-PdT immunization controls inflammatory cytokine production in the BALF

Cytokine production was measured in the BALF samples before or at the indicated time points post infection. A peak of IL-6 and G-CSF was observed in all groups at 12 h post infection (Fig. 3A and B). BALF samples from mice immunized with rPspA-PdT, WT-BCG / rPspA-PdT or rBCG PspA-PdT / rPspA-PdT showed lower levels of IL-6 at 48 and 72 h when compared with the Control and WT-BCG groups (Fig. 3A). In addition, WT-BCG / rPspA-PdT and rBCG PspA-PdT / rPspA-PdT immunized groups showed reduced level of G-CSF at 24 h and 48 h (Fig. 3B). TNF-α production was controlled in BALF samples from mice immunized with rPspA-PdT, WT-BCG / rPspA-PdT or rBCG PspA-PdT / rPspA-PdT 12 h post infection (Fig. 3C). In these groups, TNF-α level decreases gradually and is almost undetectable at the 72-h data point, whereas it remains higher in the Control and WT-BCG groups (Fig. 3C). While rPspA-PdT, WT-BCG / rPspA-PdT or rBCG PspA-PdT / rPspA-PdT groups maintain low levels of IFN-γ, this cytokine increases progressively in samples from the Control group or mice receiving only WT-BCG (Fig. 3D). No significant differences in IL-10 secretion were observed, IL-2 and IL-4 were not detected in any of the groups tested (data not shown).

3.4. rBCG PspA-PdT / rPspA-PdT immunization induces an early CD4$^+$ T cell response

Since a high lymphocyte count was observed in BALF samples from mice immunized with rBCG PspA-PdT / rPspA-PdT at 48 h post infection, we investigated the presence of CD4$^+$, CD8$^+$ or B220$^+$ cells using flow cytometry. Lymphocyte population was gated as shown in Supplementary Fig. S1. BALF samples from mice immunized with rPspA-PdT, WT-BCG / rPspA-PdT or rBCG PspA-PdT / rPspA-PdT showed increased percentage of CD4$^+$ and CD8$^+$ T cells at 48 h post infection when compared with the Control group (Fig. 4 A). Interestingly, a higher percentage of CD4$^+$ T cells was observed in BALF samples from mice immunized with rBCG PspA-PdT / rPspA-PdT when compared with mice receiving WT-BCG or a single dose of rPspA-PdT (Fig. 4A). Furthermore, at 72 h post infection the percentage of CD4$^+$ T cells increased in all groups that received a dose of recombinant protein (Fig. 4B), while the percentage of CD8$^+$ T cells and B220$^+$ cells were significantly higher in samples from mice immunized with rPspA-PdT or rBCG PspA-PdT / rPspA-PdT (Fig. 4B).

3.5. BALF from mice immunized with rBCG PspA-PdT / rPspA-PdT showed increased binding of IgG2 and C3 deposition on the pneumococcal surface

BALF samples were analyzed for the presence of anti-rPspA and anti-rPdT antibodies by ELISA. The presence of anti-PspA IgG was observed in BALF from all groups that received a dose of rPspA-PdT. The group primed with rBCG PspA-PdT and boosted with rPspA-PdT showed a higher amount of anti-PspA IgG than mice receiving a single dose of rPspA-PdT (Fig. 5A), no significant levels of anti-rPdT antibodies were detected in the BALF samples of the

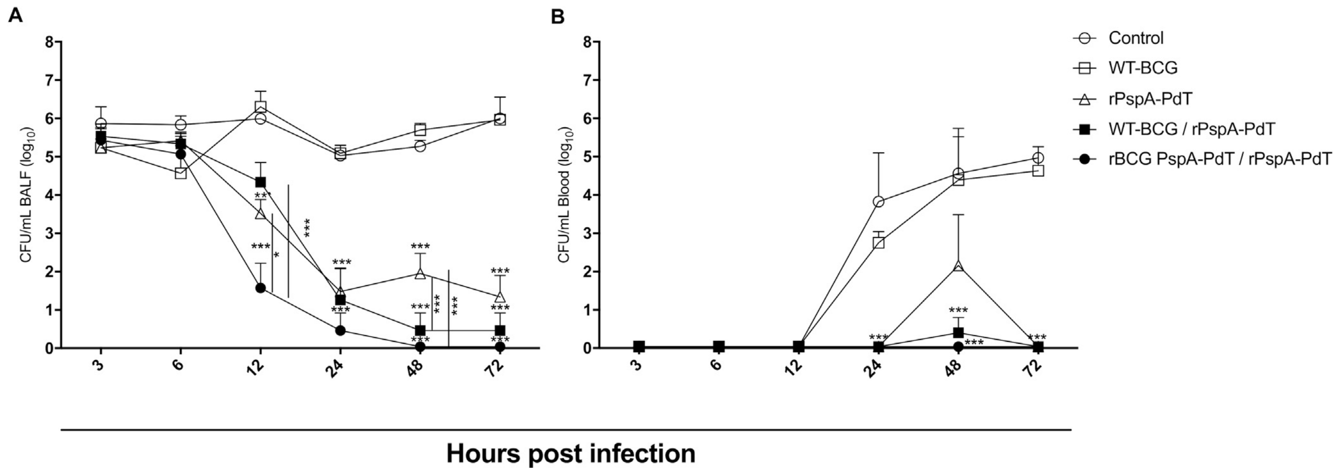


Fig. 1. Immunization of mice with rBCG PspA-PdT / rPspA-PdT controls the pneumococcal loads in the BALF 12 h after the challenge and prevents systemic dissemination. BALF and blood samples were collected from immunized mice after intranasal challenge with 10^6 CFU of pneumococcal strain WU2 at the indicated time points: (A) Pneumococcal count in BALF samples (B) Pneumococcal count in blood samples. Samples were plated on blood agar and the CFU recovered counted after overnight incubation. Results are representative of two independent experiments (n = 5 per time point for each group) and shown as means (+SEM). Statistical analyses were performed by one-way ANOVA with a Tukey's Multiple Comparison Test between the groups using GraphPad Prism: Significant results are shown by *** p < 0.001, ** p < 0.01 and * p < 0.05 versus the Control group or between groups as indicated by connecting bars.

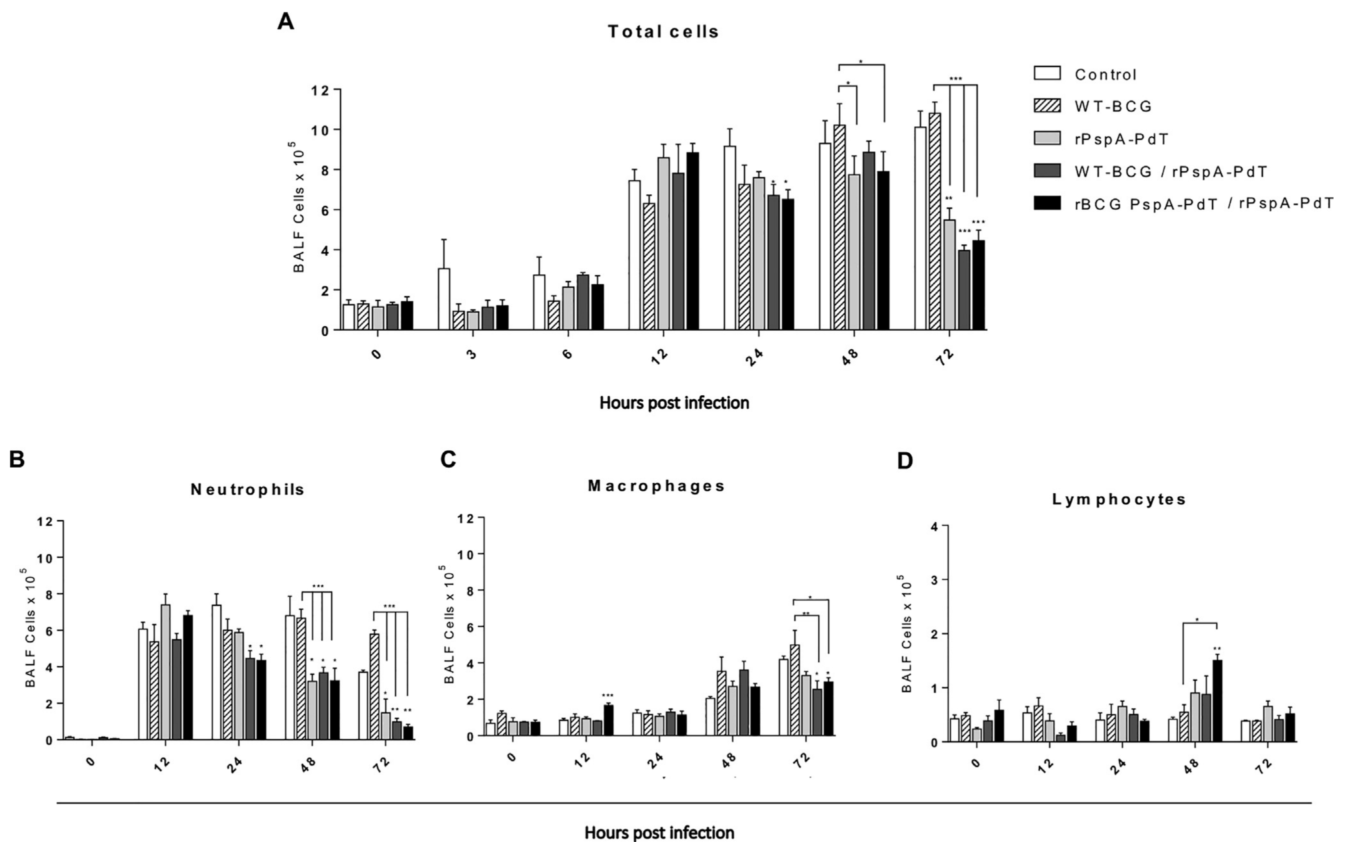


Fig. 2. rBCG PspA-PdT / rPspA-PdT immunization controls the cellular influx 24 h after the challenge and increases lymphocyte recruitment. BALF samples were collected from immunized and challenged mice: (A) Number of total infiltrated cells, (B) Neutrophils, (C) Macrophages and (D) Lymphocytes. Total cells were counted using a hemocytometer. Slides were prepared by cytospin, stained with Instant Prov (Newprov) and 100 cells were differentially counted. Results are representative of two independent experiments (n = 5 per time point for each group) and shown as means (+SEM). Statistical analyses were performed by one-way ANOVA with a Tukey's Multiple Comparison Test between the groups using GraphPad Prism: Significant results are shown by *** p < 0.001, ** p < 0.01 and * p < 0.05 versus the Control group or versus WT-BCG group as indicated by connecting bars.

tested groups (data not shown). Next, antibodies present in BALF samples were evaluated for their ability to bind to PspA exposed on the pneumococcal surface. Significantly higher total IgG binding was observed when the pneumococci were incubated with BALF samples from mice receiving rBCG PspA-PdT / rPspA-PdT (~67% of positive cells), followed by rPspA-PdT (~52% of positive cells) and WT-BCG / rPspA-PdT (~51% of positive cells) (Fig. 5B). Pneumococci incubated with BALF samples from the Control or WT-BCG

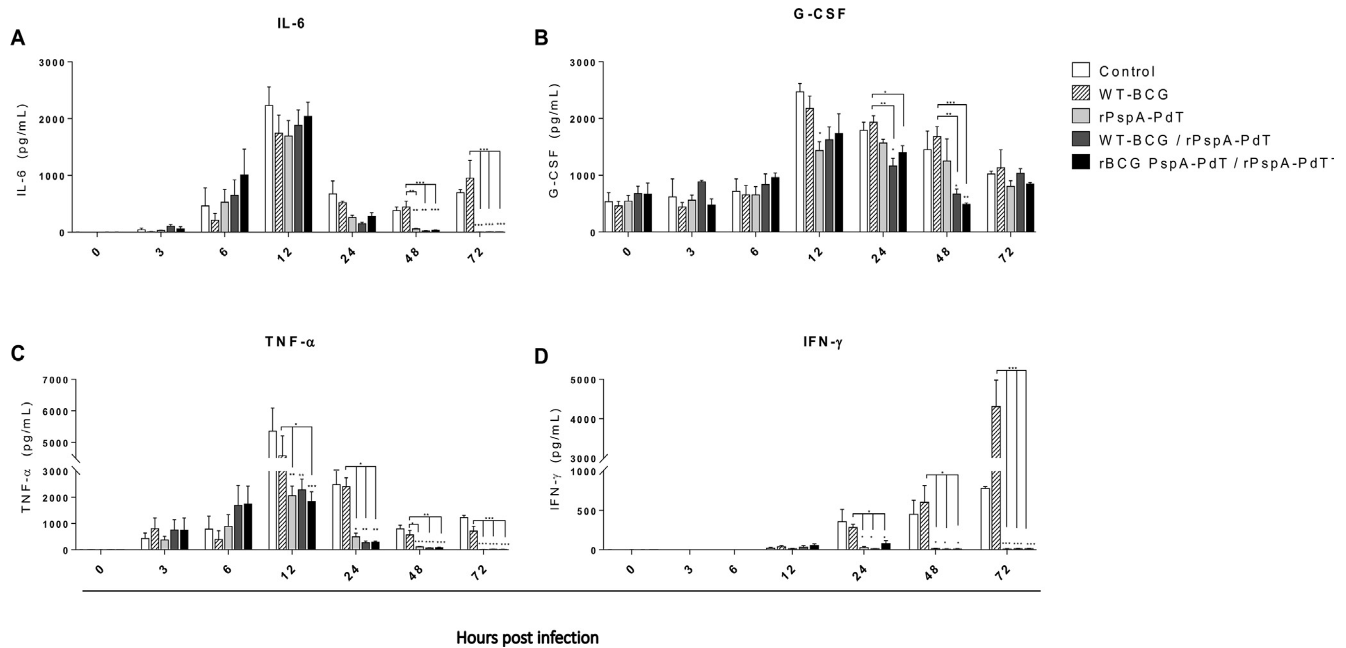


Fig. 3. rBCG PspA-PdT / rPspA-PdT immunization controls cytokines production in the BALF. BALF samples were collected from mice at the indicated time after intranasal challenge and used for cytokine measurement with Th1/Th2 CBA kit or ELISA: A) IL-6, B) G-CSF, C) TNF-α and D) IFN-γ. Results are representative of two independent experiments (n = 5 per time point for each group) and shown as means (+SEM). Statistical analyses were performed by one-way ANOVA with a Tukey's Multiple Comparison Test between the groups using GraphPad Prism: Significant results are shown by *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ versus the Control group or versus WT-BCG group as indicated by connecting bars.

groups showed reduced binding of IgG (~15% of positive cells). In addition, high binding of IgG1 (~67% of positive cells) and especially IgG2c (~48% of positive cells) were observed for BALF samples from mice immunized with rBCG PspA-PdT / rPspA-PdT (Fig. 5C and D), whereas samples from mice receiving WT-BCG / rPspA-PdT or rPspA-PdT showed only binding of IgG1. Likewise, BALF from rBCG PspA-PdT / rPspA-PdT immunized group promoted significantly higher C3 deposition (~48% of positive cells) on the pneumococcal surface than WT-BCG, rPspA-PdT or WT-BCG / rPspA-PdT groups (~38, 42 and 43% of positive cells, respectively) (Fig. 5D).

In order to investigate the specificity of binding of the BALF antibodies to the pneumococcal surface, the same samples were incubated with a strain of pneumococci lacking PspA. Low antibody binding was observed on the surface of JY119, a PspA-knockout pneumococcal strain when compared to the wild-type WU2 pneumococcal strain (Fig. 6).

4. Discussion

Pneumococcal infections trigger rapid neutrophil recruitment to the lungs, which can either be essential for bacterial clearance or lead to an uncontrolled inflammation associated with lung injury [13]. In this study, a pronounced neutrophil influx was observed in all groups 12 h post infection. This influx remains high for the Control and WT-BCG groups, while mice receiving rBCG PspA-PdT / rPspA-PdT or WT-BCG / rPspA-PdT reduced the cellular influx 24 h post infection, showing lower airway neutrophilia. The ability of pneumococcal antigens to induce a controlled inflammatory response in the lungs after infection has been correlated with effective protection in mice [14–16]. Likewise, an earlier pneumococcal clearance was observed in BALF samples from rBCG PspA-PdT / rPspA-PdT, WT-BCG / rPspA-PdT and rPspA-PdT immunized groups, while rBCG PspA-PdT / rPspA-PdT also prevented pneumococcal dissemination to the bloodstream.

After the intranasal pneumococcal challenge, IL-6 levels were increased, peaking at 12 h in all groups, while a decrease was only observed in rBCG PspA-PdT / rPspA-PdT, WT-BCG / rPspA-PdT and rPspA-PdT at 24 and 48 h. G-CSF also peaks at 12 h, however immunization with rBCG PspA-PdT / rPspA-PdT and WT-BCG / rPspA-PdT reduced the levels of G-CSF 24 h and 48 h post infection. It has been demonstrated that these cytokines can play an important role in the protection at the early stages of pneumococcal pneumonia [14,16–18]. IL-6 deficient mice showed high pneumococcal loads in the lungs 40 h post infection and died earlier than wild-type mice [17], while the administration of G-CSF increased the survival of splenectomized mice after a pneumococcal challenge by rising the number of circulating neutrophils and improving lung clearance [19]. On the other hand, the ability to control the expression of IL-6 and G-CSF after the first 12 h followed by reduced production of TNF-α and IFN-γ during all the infection can be correlated with protection against tissue damage and increased survival, as previously demonstrated [14,16].

Mice immunized with rBCG PspA-PdT / rPspA-PdT exhibited a greater CD4 + T cell recruitment to the lungs 48 h post infection as compared to mice that received a single dose of rPspA-PdT. Similar percentages of $CD4^+$, $CD8^+$ and $B220^+$ were observed between rBCG PspA-PdT / rPspA-PdT and rPspA-PdT groups only after 72 h. The role of T and B lymphocytes in the prevention of pneumococcal lung infection and sepsis are still unclear. $CD4^+$ T cells expressing IL-17 were described as essential to protect against pneumococcal colonization [21], while $CD8^{-/-}$ mice were shown to be more susceptible to serotype 3 pneumococcal infection [22]. However, the depletion of $CD4^+$, $CD8^+$ T cells or B lymphocytes did not abrogate the protection induced by mice immunized with PspA [14].

We have previously demonstrated that the s.c. immunization of mice with WT-BCG / rPspA-PdT or rBCG PspA-PdT / rPspA-PdT induced a higher IgG antibody response in the serum than a single dose of rPspA [11], while only the rBCG PspA-PdT / rPspA-PdT immunization promoted IgG class switching from IgG1 to IgG2c.

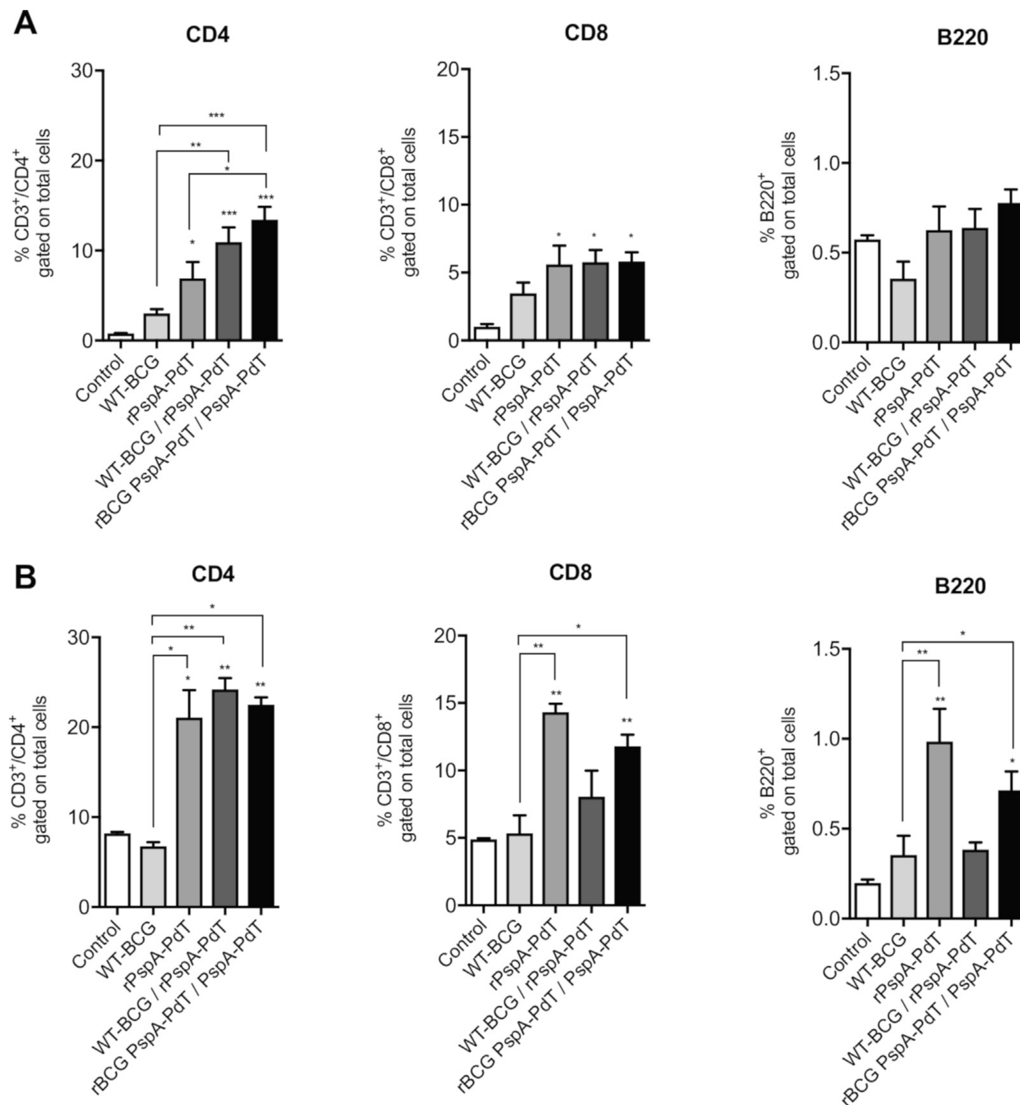


Fig. 4. rBCG PspA-PdT / rPspA-PdT immunization induces an early CD4+ T cell response. BALF samples were collected from mice at (A) 48 h or (B) 72 h after intranasal challenge and stained with APC-CY7 conjugated anti-mouse CD3, PE-CY5 conjugated anti-mouse CD4, PE conjugated anti-mouse CD8 or FITC conjugated anti-mouse B220. Flow cytometry analysis was performed using FACS Canto II and 30,000 gated events were recorded; data was analyzed using FlowJo. Results are representative of two independent experiments (n = 5 per time point for each group) and shown as means (+SEM). Statistical analyses were performed by one-way ANOVA with a Tukey's Multiple Comparison Test between the groups using GraphPad Prism: Significant results are shown by *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ versus the Control group or between groups as indicated by connecting bars.

Here we observed that these s.c. immunizations also induce an effective immune response in the lungs, characterized by the high antibody response in the BALF. Immunization with rBCG PspA-PdT / rPspA-PdT induced higher anti-PspA IgG levels in the BALF than a single dose of rPspA-PdT. Furthermore, whereas all tested groups promoted high binding of IgG1 to the pneumococcal surface, only BALF from mice receiving rBCG PspA-PdT / rPspA-PdT showed binding of IgG2c antibody to the pneumococci and promoted high complement deposition. The IgG antibody binding was specific to the PspA molecule, since no binding was observed in a PspA-KO strain of pneumococci. The IgG2 isotype has been described to efficiently bind to the pneumococcal surface, enhancing complement deposition and phagocytosis [11,14,23]. We have previously shown that rBCG PspA-PdT / rPspA-PdT immunization induced increased production of IFN-γ before challenge, which is required for production of IgG2c [11]. Here we show the reduction in IFN-γ after challenge, at a timepoint where IgG2c has already been produced. While the production of IFN-γ by T cells is required to promote antibody isotype switching to IgG2c [20] as observed after immunization of mice with rBCG PspA-PdT / PspA-PdT [11], the control of IFN-γ secretion in the lungs after pneumococcal challenge has been shown to be important for a favorable prognostic [14,16].

Priming with either WT-BCG or rBCG PspA-PdT induced similar humoral and cellular immune responses after the booster dose with rPspA-PdT. This phenomenon is due to a non-specific effect of BCG, which has been termed "trained immunity" [24]. This "trained immune" response induced by WT-BCG immunization is relatively short-lived and does not induce a memory response, as we have demonstrated by the reduced expression of the CD69 molecule on splenic CD4+ T cells of WT-BCG / rPspA immunized mice [11]. Furthermore, we did not detect anti-PdT antibodies in the BALF, perhaps reflecting the lower immunogenicity of this antigen in comparison to PspA, or dilution of the antibodies in the total

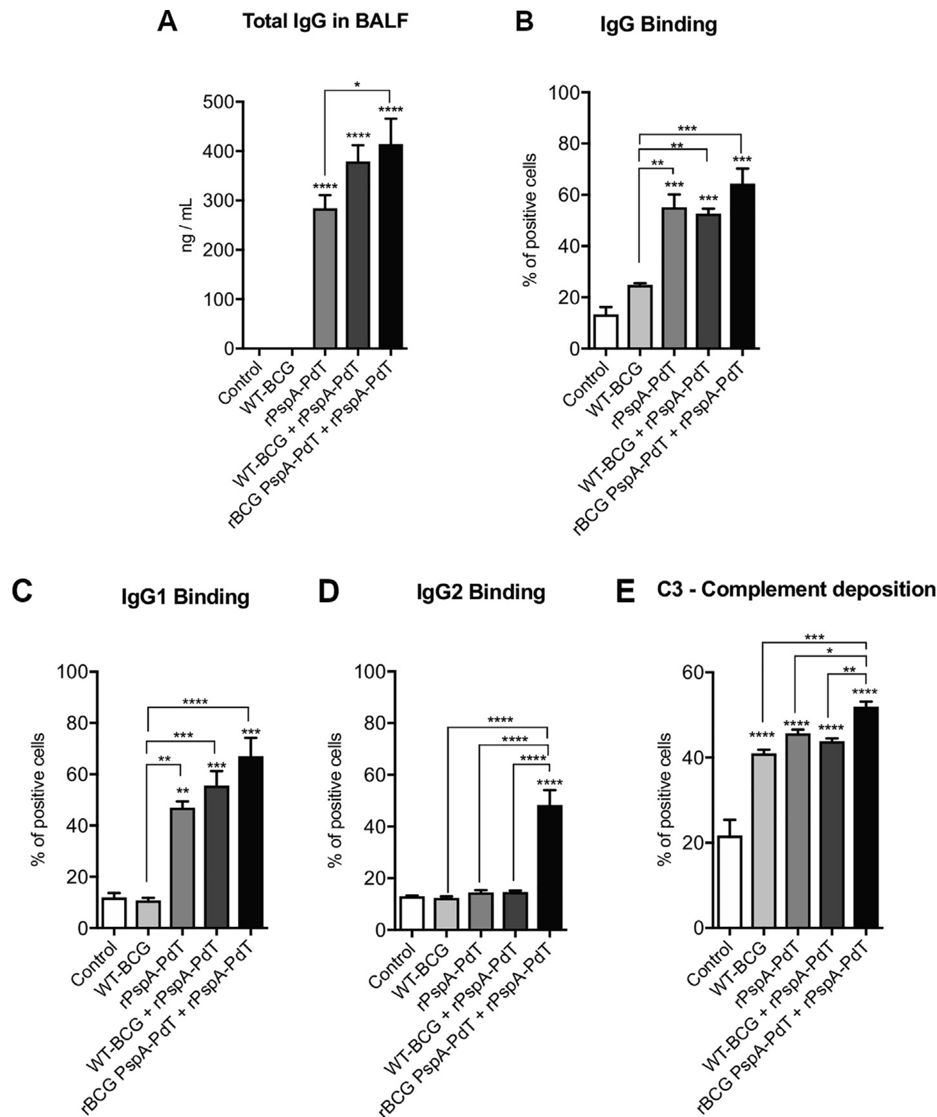


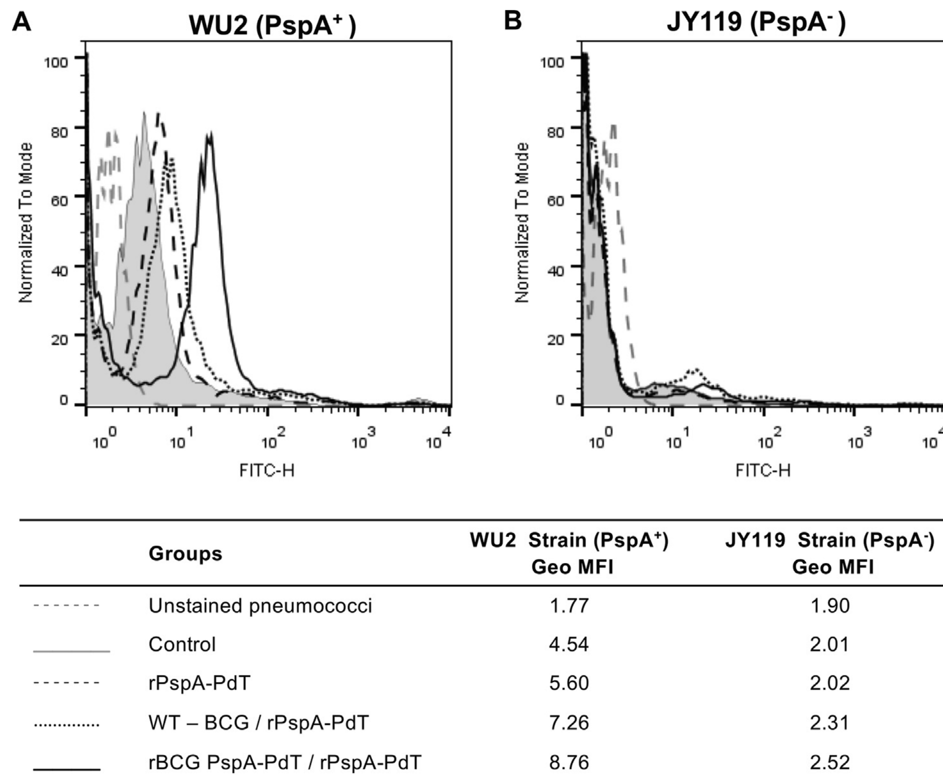
Fig. 5. BALF from mice immunized with rBCG PspA-PdT / rPspA-PdT showed increased IgG2 binding and C3 deposition on the pneumococcal surface. BALF samples were collected from mice before the pneumococcal challenge. (A) The anti-PspA antibody production was evaluated by ELISA. Pneumococcal WU2 strain was incubated with BALF samples from mice immunized with WT-BCG, rPspA-PdT, WT-BCG /rPspA-PdT, rBCG PspA-PdT / rPspA-PdT or the Control group, followed by incubation with FITC conjugated mouse antibodies (B) IgG, (C) IgG1, (D) IgG2 or (E) C3. For the C3 deposition assay, 10% of NMS was added. Flow cytometry analysis was conducted using FACS Canto II and 20,000 gated events were recorded and the data analyzed using FlowJo version 7.6.5. Non-stained pneumococci sample was used to determine the negative cell population. Results are representative of two independent experiments (n = 5 per time point for each group) and shown as means (+SEM). Statistical analyses were performed by one-way ANOVA with a Tukey's Multiple Comparison Test between the groups using GraphPad Prism: Significant results are shown by **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ versus the Control group or between groups as indicated by connecting bars.

volume of BALF samples. Indeed, we have previously demonstrated that immunization with rBCG PspA-PdT / rPspA-PdT induces an antibody response in the serum able to inhibit the hemolytic activity of Ply in red blood cells [11], suggesting an important role of anti-PdT antibodies in the protection against systemic infection. Similarly, the production of IgA against PspA and PdT was only detected in the antisera of immunized mice (data not shown).

Wilson, et al., demonstrated that naturally acquired protective immunity to pneumococcal lung infection requires both humoral and cell-mediated immune responses [25]. In fact, using the rBCG PspA-PdT with a booster dose of rPspA-PdT we observed the presence of high antibody levels in the lungs followed by a controlled cellular influx with increased lymphocyte recruitment. Taken together, our results suggest that immunization with rBCG PspA-PdT prime/ rPspA-PdT boost induces humoral and cellular immune responses in the lungs, and the early clearance of pneumococci in the BALF protects against the systemic dissemination of the pneumococcal disease.

CRediT authorship contribution statement

Cibelly Goulart: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Dunia Rodriguez:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - review & editing. **Alex I. Kanno:** Formal analysis, Investigation, Methodology, Writing - review & editing. **José Lourenço S.C. Silva:** Formal analysis, Investigation, Methodology. **Luciana C.C. Leite:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.



	Groups	WU2 Strain (PspA+) Geo MFI	JY119 Strain (PspA-) Geo MFI
- - - - - - -	Unstained pneumococci	1.77	1.90
———	Control	4.54	2.01
- - - - - - -	rPspA-PdT	5.60	2.02
....	WT – BCG / rPspA-PdT	7.26	2.31
———	rBCG PspA-PdT / rPspA-PdT	8.76	2.52

Fig. 6. IgG antibodies induced by immunization with rBCG PspA-PdT / rPspA-PdT bind onto the surface of PspA+ but not PspA- pneumocococal strain. Pneumococci were incubated with non-diluted BALF samples followed by incubation with FITC-conjugated anti-mouse IgG antibody (1:500 in PBS – MP Biomedical). Samples were then analyzed by flow cytometry using a FACS Canto II (BD, Bioscience). Geometric mean of fluorescence intensity (Geo MFI) was evaluated in histograms using the Flow Jo 7.6.5 software. Results are representative of two independent experiments using pooled BALF samples.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We would like to thank Dr. David E. Briles for providing the pneumococcal strains.

Funding

This work was supported by FAPESP (Grants number: 2009/17030-9 and 2017/24832-6) and Fundação Butantan.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2019.100049.

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