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Abstract

Streptococcus pneumoniae is a leading cause of community-acquired pneumonia, yet the immune mechanisms required for protection against invasive pulmonary infection remain incompletely understood. Using a murine model of homologous protection against invasive pneumococcal pneumonia, we investigated the relative contribution of humoral and cellular immunity through adoptive serum transfer, immune cell depletion, and lung transcriptional profiling. Passive transfer of immune serum conferred robust protection, whereas neutrophil depletion impaired bacterial control, demonstrating that antibodies and neutrophils are key mediators of protection. In contrast, depletion of $CD4^+$ T cells or NK cells did not compromise survival. Although IL-17A has been widely implicated in host defense against pneumococcal infection, IL-17A- deficient mice remained protected, albeit with delayed clearance and reduced early antibody responses. This delay was associated with compensatory upregulation of IL-17F and increased expression of Th1-associated genes in the lung. Together, these findings indicate that IL-17A is not essential for protection and support a model in which coordinated Th1 and Th17-related cytokine responses collectively promote neutrophil recruitment and effective antibody-mediated defense. These results highlight functional redundancy within the IL-17 cytokine axis and suggest that integrated cytokine networks, rather than individual mediators, underpin protective immunity to pneumococcal pneumonia, with implications for next-generation vaccine design.

Keywords: *Streptococcus pneumoniae*; Th17 immunity; IL-17A; pneumococcal pneumonia; neutrophils; antibody-mediated protection

1. Introduction

Streptococcus pneumoniae (Pn) stands as a leading cause of community-acquired pneumonia, and remains as a major global health concern, particularly among vulnerable populations such as children under five years of age [1–3]. The World Health Organization (who) has classified *S pneumoniae* as one of the twelve priority pathogens [4], and pneumococcal infections account for more than 1 million deaths annually worldwide. Pneumococcal diseases encompass a wide spectrum of clinical manifestations, including pneumonia, meningitis, septicemia, and otitis media [5].

S. pneumoniae commonly colonizes the human nasopharynx, particularly in children. In most cases, asymptomatic colonization is cleared by the host immune response within weeks in adults or months in children [6]. However, under certain conditions, such as viral infections or immunosuppression, bacteria may disseminate from the nasopharynx to other anatomical sites. This dissemination may lead to pneumococcal pneumonia when bacteria invade the lungs or, in some cases, to meningitis following spread to the central nervous system [7,8].

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The introduction of pneumococcal conjugate vaccines (PCVs), which target capsular polysaccharides, has substantially reduced the incidence of invasive pneumococcal disease (IPD) caused by vaccine serotypes [9]. Although vaccine-induced protection has traditionally been attributed to serotype-specific opsonizing antibodies, increasing evidence indicates that $CD4^+$ T cell-mediated immunity also contributes to protection against pneumococcal infection, particularly at mucosal sites such as the lung [10].

Historically, protective immunity against pneumococcus has been largely explained by antibody-mediated opsonophagocytosis. While this mechanism is essential for clearing bacteriemia, it may be insufficient to fully control infection at mucosal surfaces, where host–pathogen interactions are initiated [7]. This limitation has prompted growing interest in T helper cell responses that coordinate local inflammatory and antimicrobial defenses in the lungs.

$CD4^+$ T cells differentiate into multiple subsets defined by distinct cytokine profiles and effector functions. T helper 1 (Th1) cells, characterized by interferon-gamma (IFN-γ) production, activate macrophages and promote IgG2a/c class-switching, enhancing opsonophagocytic killing [11]. Although capsular polysaccharides are generally considered as T cell-independent antigens, the serotype 1 polysaccharide (PS1) displays zwitterionic properties that allow presentation on MHC class II molecules and activation of $CD4^+$ T cells [12,13]. In contrast, T helper 17 (Th17) cells, defined by the secretion of interleukin-17A (IL-17A) and IL-17F, play a central role in mucosal host defense. These cells promote neutrophil recruitment through the induction of CXC chemokines such as CXCL1 and CXCL2 and stimulate epithelial cells to produce antimicrobial peptides that reinforce the mucosal barrier [14–16].

Increasing evidence suggests that effective immunity to pneumococcal infection involves coordinated activity between multiple T helper cell pathways. Th1 and Th17 responses have been shown to act synergistically in several models of bacterial infection [17,18]. In particular, the relevance of IL-17A in protection against nasopharyngeal pneumococcal carriage has already been established [11,19–21], though its role in protection against invasive pneumococcal pneumonia remains less well defined [22]. Several studies suggest that IL-17A may exert context-dependent effects, being either protective or detrimental, depending on bacterial strain characteristics and capsule thickness [23]. A pathogenic role for IL17A has already been described in inflammatory lung disease, such as cystic fibrosis or severe asthma [24,25], where it can amplify inflammatory signaling pathways, including those driven by IL-13 [26].

Based on these observations, we hypothesize that homologous protection against invasive pneumococcal pneumonia requires a coordinated T cell response that integrates Th1-mediated macrophage activation with Th17-associated mechanisms promoting neutrophil recruitment and mucosal defense. Here, we demonstrate that IL-17A alone is not required for protection against homologous invasive pneumococcal pneumonia. Instead, our results indicate that IL-17-associated immune responses, even in the absence of IL-17A, contribute to protection through enhanced neutrophil recruitment and the induction e of anti-capsular antibodies.

2. Materials and Methods

Animal Studies

Female C57BL/6J mice, 6-8 weeks old, were obtained from the National Division of Veterinary Laboratories (Montevideo, Uruguay). $Il17a^{-/-}$ mice with C57BL/6J genetic background were initially obtained from Dr. Yoichiro Iwakura (University of Tokyo, Japan) and bred at Institut Pasteur Montevideo (IPMONT), Uruguay. $Casp1/11^{-/-}$ mice were also provided by the Institute Pasteur Montevideo (IPMONT), Uruguay. All animals were maintained in individually ventilated cages and handled in class II A2 vertical laminar flow cabinet (ESCO). Mice were anesthetized by intraperitoneal (i.p.) injection of Ketamine (2.2 mg) and Xylazine (0.11 mg). Bacteria were administered intranasally (i.n.) in 50 µl of physiological saline (25 µl per nostril). Mice's survival was monitored daily. For lung sampling, mice were euthanized by cervical dislocation at the indicated time-points. Lungs were collected and homogenized using a cell dissociation sieve-tissue grinder kit

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(Sigma-Aldrich), and serial dilutions were plated onto blood agar plates to determine bacterial loads. S. pneumoniae colonies were detected by the characteristic α-hemolytic green halo. For transcriptional analysis, lungs were stored in TRIzol reagent (Life Technologies). All animal experiments were conducted in accordance with national and institutional regulations and approved by the "Comisión Honoraria de Experimentación Animal" (CHEA)—Universidad de la República, Uruguay (Exp. Nº 07015300081914), in accordance with ARRIVE guidelines.

Bacterial strains and culture conditions

Streptococcus pneumoniae serotype 1 (Pn1, clinical isolate E1585) was obtained from the National Reference Laboratory (Ministry of Health, Uruguay). Working stocks were prepared as previously described [17,27]. Briefly, Todd Hewitt Yeast Broth (THYB) was inoculated with fresh colonies of *S. pneumoniae* grown on blood agar plates and incubated at 37 °C until reaching an optical density at 600nm (OD600) of 0.7-0.9. Cultures were stored at -80 ºC in THYB supplemented with 12% (v/v) glycerol for up to three months. For infections, frozen stocks were thawed, centrifuged (5 min at 2500 x g), washed, and diluted in saline to obtain 2 x 10^4 CFU/50 µl for the sublethal dose or 2 x 10^7 CFU/50 µl for the lethal dose. Bacterial counts in the inocula were determined for every experiment by plating serial dilutions onto blood agar plates.

Adoptive transfer of immune sera to naïve mice

Blood was collected from mice's facial veins or retro-orbital sinus 7 days after administering saline (*control sera*) or the sublethal dose of Pn1 (*immune sera*) to groups of 15 mice each. Serum from each group was pooled separately, immediately aliquoted, and stored at -80ºC until use. For adoptive transfer in vivo assays, groups of seven naïve mice were intravenously (i.v.) injected with 0.5 ml of immune or control sera at day 0 and challenged with the lethal Pn1 dose on day 1. Survival rates were recorded daily for a week. Experiments were independently performed three times.

Depletion of Gr-1$^+$, NK or CD4$^+$

For granulocyte depletion, mice received an i.p. injection of 100 µg of purified anti-Gr-1 antibody (clone RB6-8C5) or an isotype control antibody (clone HB152), 24 h before the i.n. challenge with *S. pneumoniae* [17,28]. Anti-Gr1 injection depletes 96.8% ± 1.2% of pulmonary polymorphonuclear neutrophils (PMNs), as determined by flow cytometry of lung single-cell suspensions (data not shown). Similarly, depletion of natural killer (NK) or CD4 cells was achieved by i.p. administration of 100 µg of purified anti-NK (clone PK136) or anti-CD4 (clone GK 1.5), 24 h before i.n. challenge with *S. pneumoniae*. Depletion efficiency was confirmed by flow cytometry (data not shown). Experiments were independently performed three times.

Detection of IgG and IgM antibodies by ELISA.

Specific IgG, IgM, and IgA antibodies against pneumococcal polysaccharide type 1 (PnPS1), were measured using a modified version of the WHO third-generation ELISA for quantification of human anti-polysaccharide IgG antibodies, as previously described [17]. Briefly, 96-well medium-binding microtiter plates (Greiner) were coated with 0.2 µg/well of type 1-specific pneumococcal polysaccharide (American Type Culture Collection [ATCC], Manassas, Va.) for 5 h at 37ºC, in a humidified chamber. Plates were washed with Tris-buffered saline with 0.01% Brij-35 solution (Wash buffer). Test sera were pre-absorbed with 5 µg/ml cell wall polysaccharide (C-PS, Statens Serum Institute, Copenhagen, Denmark) and 5 µg/ml 22F capsular polysaccharide (ATCC) and incubated for 30 minutes at room temperature (RT). Then, 50 µl of each sample was transferred to the coated microtiter plate and incubated o/n at RT. After washing, 100 µl of diluted peroxidase-conjugated goat anti-mouse IgG or IgA (Southern Biotech, Birmingham, AL) or IgM (SIGMA) was added, and plates were incubated o/n at 4 °C. Finally, the ELISA was developed by adding 100 µl/well of TMB (3,3',5,5'–tetramethylbenzidine, SIGMA), and absorbances at 450 (with reference at 630 nm) were read using a microplate reader (MRX II, Dynex Technologies). A standard curve prepared with a self-made positive reference sample was run in each plate, where test samples were interpolated.

RNA extraction and reverse transcription-quantitative real-time PCR

Lungs were homogenized in Trizol reagent (Life Technologies) as previously described [17,29,30]; total RNA was extracted according to the manufacturer's instructions, quantified, and 1

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μg was treated with DNase I (Life Technologies). First-strand cDNA was synthesized using random primers (Life Technologies), and M-MLV reverse transcriptase (Life Technologies) (cycling: 10 min, 25 °C; 50 min, 37 °C; 15 min, 70 °C). QuantiTect SYBR Green PCR kit (Qiagen) was used for Real-Time PCR assays using a 7900HT instrument (Applied Biosystems) (cycling: 15 min at 95 °C and 40 cycles at 95 °C, 15 s, 60 °C, 1 min: fluorescence acquisition step) with specific primers (0.9 μM, sequences available upon request). Gene expression was normalized to Actb (βactin) using the $2^{-\Delta\Delta Ct}$ method [31]; a cut-off of 33 cycles was used. Results were expressed as the mRNA fold increase relative to the saline-treated group. Experiments were independently performed three times.

Flow cytometry analysis of lung cells

At the indicated time points after the challenge, mice were euthanized, and the pulmonary vasculature was perfused with saline containing 1 mM EDTA to remove intravascular cells. Lung cells were isolated after collagenase/DNase digestion as previously described [17,29]. Cells were resuspended in FACS EDTA buffer (PBS, 0.1% azide, 1% fetal calf serum, 5 mM EDTA) and enumerated using a Countess Automated Cell Counter (Invitrogen, USA) prior to the immunophenotypic analysis by flow cytometry. Neutrophils were defined by forward scatter area (FSC-A) and side scatter area (SSC-A) profile, high Ly6G and CD11b expression, and absence of CD11c expression. Samples were acquired on a FACS Canto II (BD) flow cytometer equipped 488 nm and 635 nm excitation lasers. Data acquisition and analysis were performed using FACS Diva software (BD) (v 6.0). The experiments were conducted independently three times.

Statistical Analysis

Statistical analyses were performed using Graph-Pad Prism (v11.0.0). Differences between groups were evaluated using the non-parametric Mann–Whitney U test, Student T-test, or Kruskal-Wallis test with Dunn's post-test, as appropriate. Differences were considered statistically significant at $p<0.05$ (*) and highly significant at $p<0.01$ (**).

3. Results

3.1. Immune Serum and Neutrophils Are Independently Essential for Protection Against Lethal Pneumococcal Pneumonia

Anti-capsular antibodies promote opsonization of *S. pneumoniae*, facilitating bacterial clearance through phagocytosis. We previously reported that WT mice treated with a sublethal dose of *S. pneumoniae* serotype 1 (Pn1) were 100% protected against a homologous lethal challenge administered 1 or even 8 weeks later. In contrast, naïve WT mice treated with saline (control) and challenged with the same lethal Pn1 dose, developed invasive pneumonia and died 48-72h later [17].

To further elucidate the mechanisms underlying protection against homologous invasive pneumococcal pneumonia, we performed adoptive serum transfer experiments. As previously described, treatment with a sublethal dose of Pn1 induced the elicitation of serum-specific anti-capsular IgG and IgM antibodies, as well as IgG and IgM antibodies against whole-cell pneumococcal antigens, 7 days later (*immune sera*) [17]. Here, we showed that naïve mice receiving *immune sera* prior to a lethal Pn1 challenge achieved 100% survival (Figure 1A). These findings indicate that antibodies generated after sublethal pneumococcal infection are sufficient to confer protection against homologous invasive pneumonia in this experimental model.

Neutrophil (PMN)-mediated opsonophagocytosis is a key mechanism for controlling *S. pneumoniae* infection [32–34]. Consistent with previous observations, neutrophils are massively recruited to the lungs following lethal pneumococcal challenge [17]. To determine whether PMNs were required for protection in this model, neutrophils were depleted using anti-Gr-1 antibodies in sublethally-Pn1-treated WT mice prior to the homologous lethal challenge. PMN depletion resulted in complete loss of protection, with 0% survival in depleted mice (Figure 1B). These findings demonstrate that neutrophils are essential mediators of homologous protection against invasive pneumococcal pneumonia.

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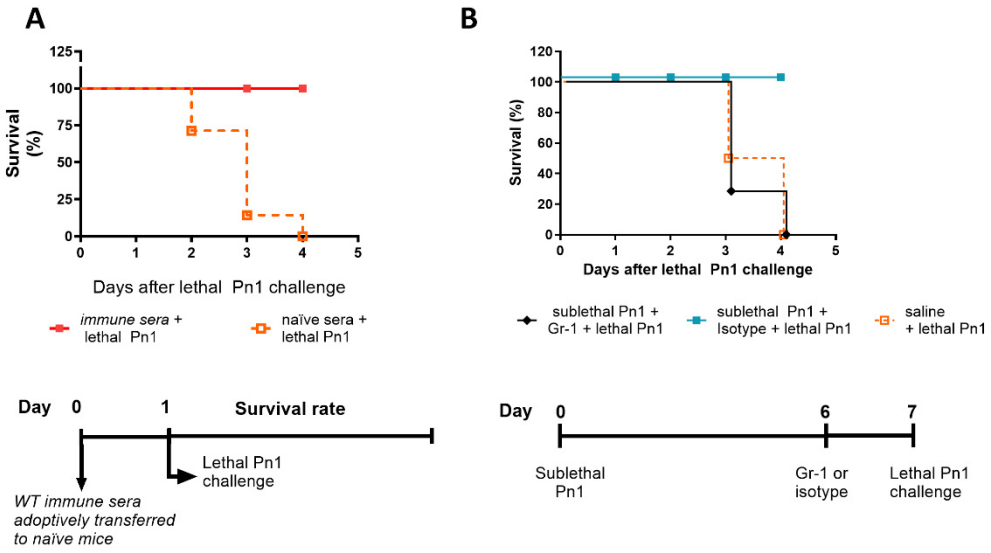

Figure 1. Serum antibodies and PMNs are key components of protection against pneumococcal pneumonia. **A**) Adoptive transfer of immune sera protects against invasive pneumococcal pneumonia. Two groups of naïve C57BL/6 mice (n=7/group) received *immune* (■) or *control* (□) sera (i.p) and were challenged 1 day later with a lethal dose of *S. pneumoniae* serotype 1 (Pn1, 2x10^{7}CFU/50µl, i.n.). Survival rates were recorded daily after the lethal Pn1 challenge. **B**) Gr-1$^+$ cells are essential for protection against pneumococcal pneumonia. Two groups of naïve C57BL/6 mice (n=7/group) were treated with a sublethal Pn1 dose at day 0 (2x10^4 CFU/50µl, i.n.) and were i.p. injected with Gr-1 (◆) or isotype control (■) antibodies at day 6, and finally challenged with a lethal Pn1 dose (2x10^7 CFU/50µl, i.n.) at day 7. Control mice were treated with saline at day 0 and were challenged with a lethal Pn1 dose at day 7 (□). Survival rates were recorded daily after the lethal Pn1 challenge. Experiments were repeated twice.

3.2. CD4$^+$, Il17a, and Casp-1/11 Are Neither Exclusively Essential for Protection

Additionally, we found that administration of specific anti-CD4 or anti-NK-1.1 antibodies prior to the lethal Pn1 challenge did not abrogate protection in WT mice (Figure 2A). However, CD4 depletion resulted in a significant decrease in *Il17a*, *Il17f*, and *Il22* lung mRNA levels, with no change in *Ifng* mRNA levels, compared with sublethal Pn1-treated mice that received an isotype control (Figure 2B). These findings confirm our previous observation, indicating that CD4$^+$ cells are a major source of the rapid IL-17A production detected in the lungs of protected mice [17]. Studies using a serotype 23F strain of *S. pneumoniae* have shown that homologous protection against non-invasive pneumonia depends on antibodies [18,35] and T-helper type 17 (Th17) CD4$^+$ cells [18]. In contrast, our data suggest that although CD4$^+$ cells are major producers of *Il17a*, *Il17f*, and *Il22*, they are not required for homologous protection against invasive Pn1 challenge.

Inflammasomes have been described as critical components of the innate immune response during pneumococcal infection, in part due to NLRP3 sensing of the hemolytic pneumolysin, a major virulence factor of *S. pneumoniae* [36,37]. At the post-transcriptional level, these multiprotein complexes regulate IL-1β and IL-18 production through caspase-1-dependent cleavage of their precursor forms and can ultimately induce an inflammatory form of cell death known as pyroptosis [38]. Because protected WT mice exhibited increased *Casp1* mRNA levels compared with naïve or non-protected mice (Supplementary Figure S1A), we decided to evaluate protection in syngeneic *Casp1/11*$^{-/-}$ mice. Both WT and *Casp1/11*$^{-/-}$ mice treated with a sublethal dose and subsequently challenged with a homologous lethal Pn1 dose showed identical survival rates (Suppl. Figure S1), indicating that *Casp1* is not required for homologous protection against invasive pneumococcal pneumonia.

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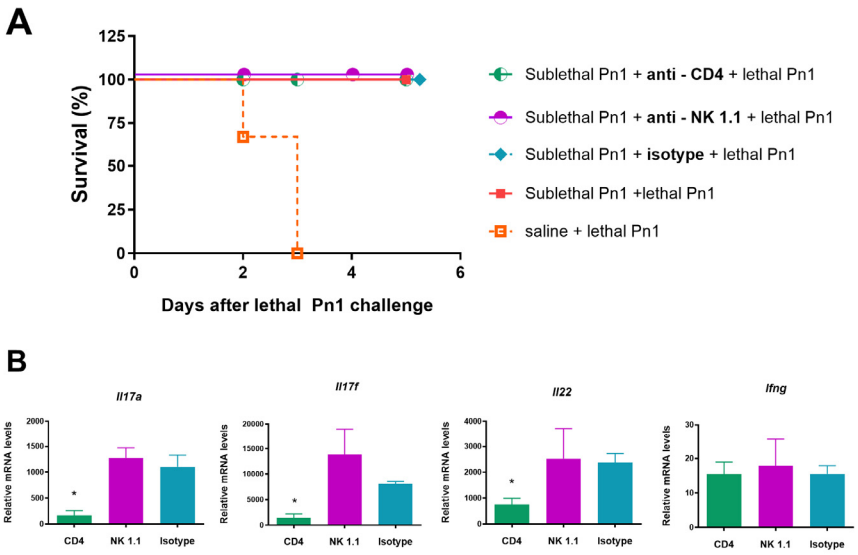

Figure 2. Depletion of CD4 or NK cells did not abrogate protection against homologous pneumococcal pneumonia. C57BL/6 mice were treated with a sublethal Pn1 dose at day 0 (2x10^{4}CFU/50µl, i.n.) and were i.p. injected with anti-CD4 (left half-filled green circle), anti- NK 1.1 (top half-filled purple circle), or isotype control (◆) antibodies at day 6, and finally challenged with a lethal Pn1 dose (2x10^{7}CFU/50µl, i.n.) at day 7. Control mice were treated with saline (□) or sublethal Pn1 (■) at day 0 and were challenged with a lethal Pn1 dose at day 7. A) Survival rates were recorded daily after the lethal Pn1 challenge for each group (n=5 mice per group). **B)** On day 1, after the lethal Pn1 challenge, three mice per group were sacrificed, and total lung RNAs were obtained. Relative mRNA levels for *Il17a, Il17f, Il22*, and *Ifng* were normalized to β-actin as the housekeeping gene and referenced to WT naive mice. *, $p<0.05$ compared to the isotype control group. Man-Whitney Test, n=3/group. Experiments were repeated 2 times.

Il17a was highly up-regulated in mice protected against invasive pneumococcal pneumonia, suggesting a potential association between increased IL-17A levels and protection in this model [17]. Th17 responses have been widely associated with protection against pneumococcal infection [39]. To further evaluate the role of IL-17A in homologous protection against invasive pneumococcal pneumonia, we used syngeneic *Il17a*-deficient mice (*Il17a*$^{-/-}$). *Il17a*$^{-/-}$ control mice (treated with saline) developed acute invasive pneumonia, similar to what happened with WT mice, when challenged with a lethal Pn1 dose, and also died 48-72 h after infection (Supplemental Figure S2). Interestingly, *Il17a*$^{-/-}$ mice previously treated with a sublethal dose of Pn1 exhibited the same survival rates as WT-treated mice, when further challenged with a homologous lethal Pn1 dose (Figure 3A). However, WT mice showed significantly lower lung bacterial loads than *Il17a*$^{-/-}$ mice when evaluated 24 h after the lethal challenge (Figure 3B). These findings indicate that the absence of IL17A delays bacterial clearance from the lungs, although both WT and *Il17a*$^{-/-}$ mice ultimately achieved 100% survival.

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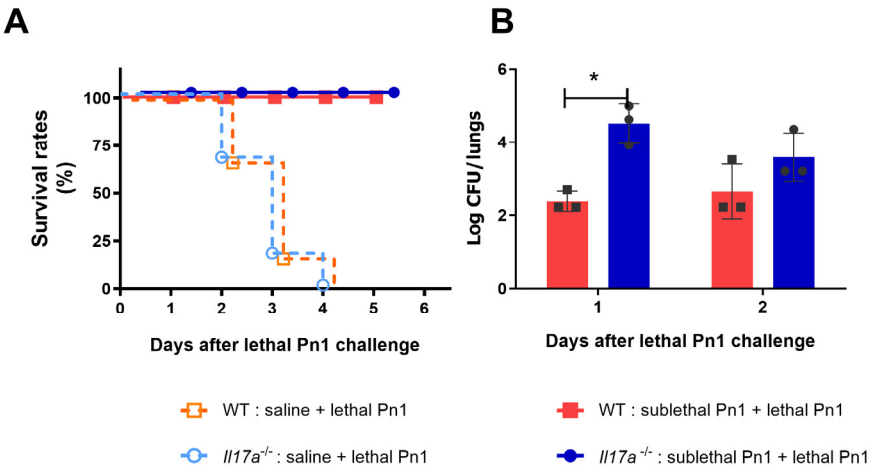

Figure 3. A). Homologous protection against pneumococcal pneumoniae does not involve IL17A. *Il17a*$^{-/-}$ (●) or syngeneic WT (■) mice were treated with a sublethal Pn1 dose (2x10^{4}CFU/50µl, i.n.) and were challenged 7 days later with a lethal Pn1 dose (2x10^{7}CFU/50µl, i.n.). Control *Il17a*$^{-/-}$ (○) or syngeneic WT (□) mice were treated with saline and were challenged with a lethal Pn1 dose at day 7. Survival rates were recorded daily after the lethal challenge (n=6 per group). **B).** 1 and 2 days after lethal Pn1 challenge, *Il17a*$^{-/-}$ (●) or WT (■) protected mice were sacrificed, and lung bacterial loads were assessed. (n=3/group and time-point). *, $p<0.05$, Mann-Whitney Test, n=3/group. Experiments were repeated at least 3 times.

3.3. IL-17f Compensate the Lack of IL17A for Protection

One well-described function of IL-17A is the recruitment of PMNs, so we assessed their recruitment into alveolar spaces. Flow cytometry analysis of BAL samples from *Il17a*$^{-/-}$ and WT mice revealed a similar PMN recruitment in both protected groups (Figure 4), suggesting that mediators other than IL-17A may compensate for its absence in *Il17a*$^{-/-}$ mice.

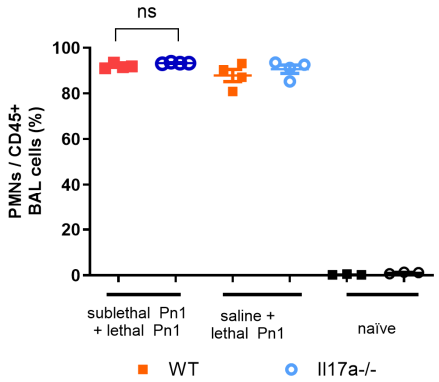

Figure 4. Both WT and *Il17a*$^{-/-}$ mice showed similar PMN recruitment. Bronchoalveolar lavage fluid (BAL) samples were obtained 24h after the lethal challenge with *S. pneumoniae* serotype 1 (Pn1, 2x107 CFU/50µl) of *Il17a*$^{-/-}$ or WT mice, previously treated with saline or sublethal Pn1 dose, 7 days earlier. The naïve group was neither treated with saline nor with a sublethal or lethal dose of Pn1 (n=3). BAL cells were first gated based on cell size (FSC), intracellular particle complexity (SSC), and surface CD45 antigen expression. PMNs were identified as Ly6G$^+$, CD11b$^+$ cells. n=4/group. These results are representative of 3 independent experiments.

We next compared the lung transcriptional profiles between protected WT and *Il17a*$^{-/-}$ mice. Overall, both strains displayed highly similar expression patterns. However, *Il17a*$^{-/-}$ protected mice showed a more rapid increase in *Il17f* mRNA levels than wild-type (WT) mice, as measured 8 hours

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after lethal challenge, although both reached similar levels at 24 hours. Regarding other Th17-associated cytokines, similar increases in *Il21* and *Il22* mRNA levels were observed, while *Il17a* mRNA transcripts were absent in *Il17a*$^{-/-}$ mice (Figure 5A). Moreover, both groups of protected mice showed similar increments in relative mRNA levels for *Ifng* and *Ifng*-related genes (*Cxcl9*, *Cxcl10*, *Cxcl11*) (Figure 5B) and also for the antimicrobial peptide Lipocaline 2 (*Lcn2* - Figure 5C). Regarding the other antimicrobial peptide analyzed (*S100A9*), both mouse strains exhibited a rapid, significant increase at 8 hours, followed by a decrease by 24 hours, similar to the pattern observed for *Il1b* and *Ifnb* mRNA levels (Figure 5C). Previous studies have suggested that type I Interferons contributed to host defense against pneumococcal infection by limiting bacterial dissemination from the lungs to the bloodstream [40]. Accordingly, the rapid induction of *Ifnb* mRNA levels observed in both WT and *Il17a*$^{-/-}$ protected mice (Figure 5C), may contribute to preventing systemic dissemination.

Overall, *Il17a*$^{-/-}$ and WT-protected mice exhibited highly similar transcriptional profiles, characterized by increased expression of Th17-associated genes, *Ifng* and *Ifng*-related genes, and antimicrobial peptides. Thus, despite the absence of IL-17A, *Il17a*$^{-/-}$ mice mount compensatory "Th17-like" responses together with Th1 responses that may account for the protective immunity observed after homologous lethal challenge.

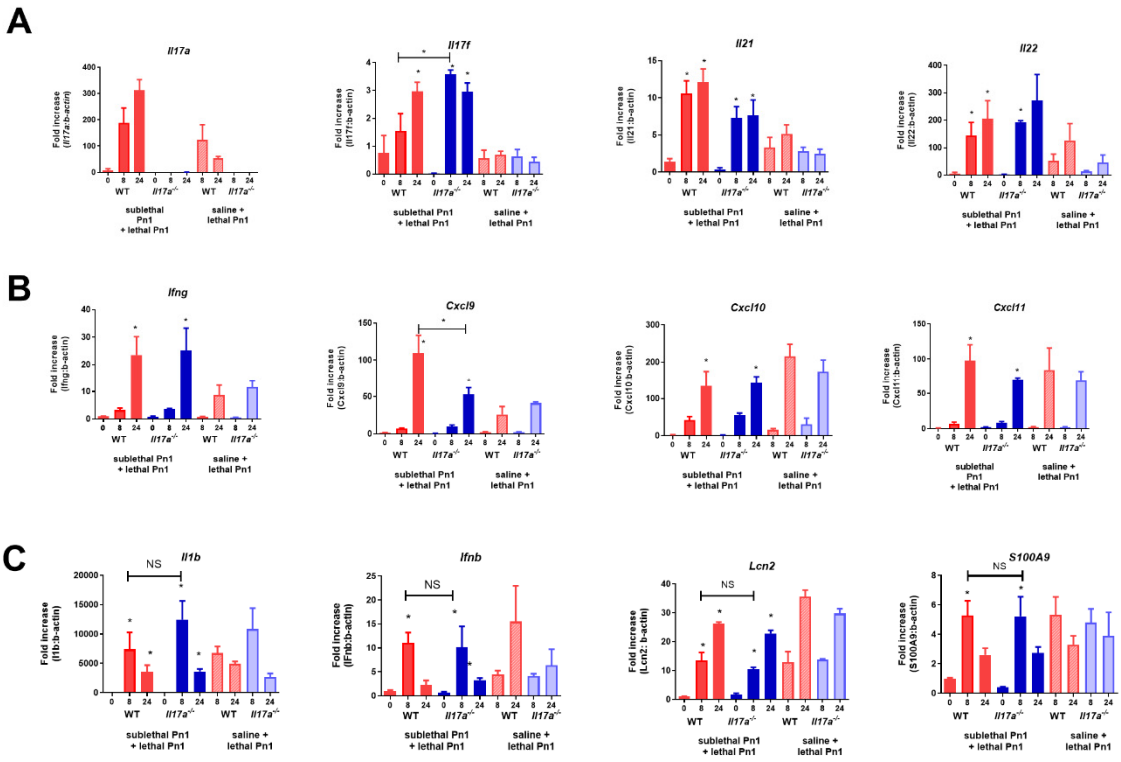

Figure 5. RT-qPCR analysis of the relative mRNA levels of genes encoding *Il17a*, *Il17f*, *Il21*, *Il22* (A), *Ifng*, *Cxcl9*, *Cxcl10*, *Cxcl11* (B), *Il1b*, *Ifnb*, *Lcn2* and *S100A9* (C) in the lungs of WT and *Il17a*$^{-/-}$ mice treated with the sublethal dose of *S. pneumoniae* serotype 1 (Pn1, 2x10^4 CFU/50µl) and sacrificed 8h or 24h after a lethal homologous challenge (Pn1, 2x10^7 CFU/50µl), performed 7 days later. The relative mRNA levels of each gene were normalized to β-actin and expressed relative to naïve WT mice. n=3 animals per time-point, group, and mouse strain. $p<0.05$, ANOVA test with Dunnett's post hoc multiple comparison test versus the control group (time 0h of each strain) or between the groups indicated by the horizontal bar.

3.4. Il17a$^{-/-}$ *Mice Had a Delayed Anti-Capsular Antibody Response*

As already described, serum antibodies are protective against invasive pneumonia, at least in our mouse model. We assessed the elicitation of specific antibodies in serum from *Il17a*$^{-/-}$ treated mice compared to WT mice. Results showed the induction of significant levels of IgG and IgM anti-

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capsular antibodies in *Il17a*$^{-/-}$ treated mice, though with some differences in kinetics compared to WT (Figure 6). At earlier time points (1 and 2 weeks after sublethal Pn1 treatment), WT mice showed increased levels of anti-capsular IgG and IgM antibodies compared to *Il17a*$^{-/-}$ (Figure 6). These results suggest that *Il17a*$^{-/-}$ mice are less efficient than WT in rapidly eliciting anti-capsular antibodies after Pn1 treatment. This could explain the delayed clearance of bacteria from the lungs in *Il17a*$^{-/-}$-treated mice. Nevertheless, at later time points (12 and 24 weeks after treatment), *Il17a*$^{-/-}$ mice showed increased levels compared to WT, meaning that *Il17a*$^{-/-}$ mice do not have an impairment to mount an antibody response that is in line with results obtained by other authors using the same deficient mouse strain [41]

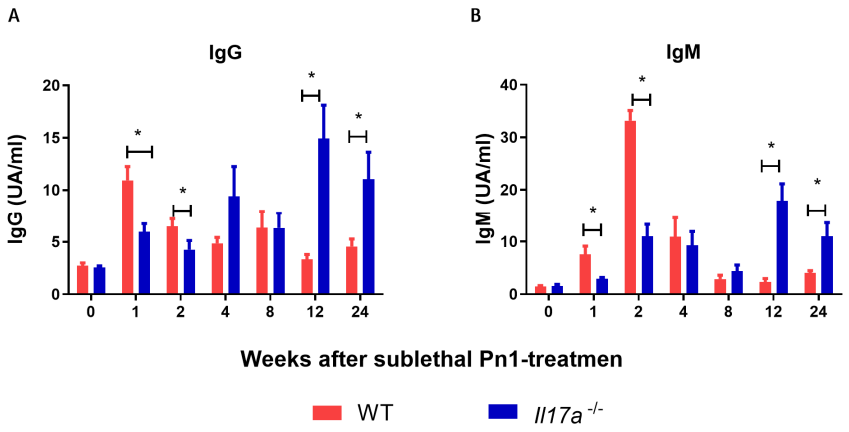

Figure 6. Kinetics of serum anti-capsular IgG (A) or Ig M (B) antibodies elicited after treatment of C57BL/6 WT mice (red bars) or *Il17a*$^{-/-}$ ones (blue bars) with the sublethal dose of *S. pneumonie* serotype 1 (Pn1, 2x10^4 CFU/50µl). Summary data from 3 independent experiments, 7-10 mice per time-point.

4. Discussion

S. pneumoniae is a major human pathogen. Its principal virulence factor is the capsular polysaccharide, partly due to its anti-phagocytic properties. Therefore, available pneumococcal vaccines are based on capsular polysaccharides from the most relevant serotypes and protect against invasive manifestations of pneumococcal disease [42]. Although many authors have studied the immune mechanisms underlying protection against *S. pneumoniae* infection, the relative contributions of humoral and cellular immune responses to protection against invasive pneumococcal pneumonia remain incompletely understood [18]. In this study, we further characterize the immune mechanisms underlying homologous protection against invasive pneumococcal pneumonia using a previously described murine model [17]. To achieve this, we focused on the roles of anti-capsular antibodies, neutrophils (PMNs), CD4$^+$ and NK cells, inflammasome activation, and IL-17A in this protective response.

Our results confirm the important roles of antibodies and PMNs in homologous protection against invasive pneumococcal pneumonia. Sublethal pneumococcal infection, which is rapidly cleared from the lungs, induces serum-specific antibodies that are sufficient to confer protection against a lethal homologous challenge, as demonstrated by an adoptive serum transfer assay (Figure 1A). In parallel, neutrophil depletion abolished protection, as Gr-1-treated mice failed to survive the homologous lethal Pn1 challenge (Figure 1B). It is noteworthy that the lethal Pn1 challenge induces invasive pneumonia and rapid PMN recruitment to the lungs (Figure 4). In mice that did not receive prior sublethal exposure to Pn1, bacteria are not efficiently cleared from the lungs, and animals succumb to infection. Although neutrophils are essential for bacterial clearance, they can also contribute to tissue damage by releasing inflammatory mediators such as superoxide and neutrophil elastase [43]. Excessive lung inflammation is responsible, in part, for the lethality observed during pneumococcal pneumonia [7]. The inflammatory response may facilitate bacterial dissemination to

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the bloodstream, contributing to the severity of invasive pathology [44,45]. In our model, antibodies present in *immune sera* likely promote opsonization of pneumococci, facilitating their efficient phagocytosis by recruited neutrophils. In contrast, in mice receiving naïve serum, recruited PMNs are unable to phagocytose pneumococci; therefore, the inflammatory response contributes to the observed lethal outcome. There are multiple ways to induce inflammation, and the more rapidly bacteria are cleared from the lungs, the better the prognosis. Moreover, in Gr-1-treated mice, despite the presence of anti-pneumococcal antibodies, the absence of functional PMNs contributes to the observed lethal outcome.

Overall, PMNs are pivotal determinants of protection and pathogenesis against *S. pneumoniae* infection, as others have already described [46]. Here, we showed that their recruitment is critical in protection against homologous invasive pneumococcal pneumonia.

Previous studies have demonstrated that Th17 cells play a protective role in pulmonary infections [47]. Regarding *S. pneumoniae*, we previously described an association between IL17A and homologous protection against invasive pneumococcal pneumonia [17]. Moreover, some authors have described a dual role for IL-17A during pneumococcal pneumonia that may depend on the serotype, particularly the thickness of its capsule [23]. These authors described a detrimental role for IL17A in a mouse model of pneumococcal pneumonia induced by a serotype 3 strain. They propose that PMNs cannot effectively phagocytose bacteria because of the thickness of the bacteria's capsule. Hence, the accumulation of PMNs in the lungs not only induces lung damage but also fails to clear the bacteria [23]. Contrary to serotype 3 strains, which have a thick capsule and a high expression of capsular polysaccharide, serotype 1 has a lower expression of its capsular polysaccharide [48,49], which may facilitate antibody-mediated opsonophagocytosis.

Additionally, our data suggest that IL17A is not strictly required for protection, as sublethally Pn1-treated *Il17a*$^{-/-}$ mice were also 100% protected against a homologous lethal challenge. *Il17a*$^{-/-}$ protected mice exhibited compensatory "*Th17-like*" responses, characterized by increased expression of *Il17f*, *Il21*, and *Il22*, along with induction of Th1-associated genes, including *Ifng* and other *Ifng*-related genes. These findings suggest the existence of compensatory cytokine pathways that maintain protective immunity in the absence of IL-17A.

IL-17A and IL-17F are both members of the IL-17 family of cytokines, with the highest homology (58%) among family members, and are mainly produced by Th17 cells [15,47,50,51]. It has already been described that IL-17F induces differential PMN recruitment and/or activation, similar to the described functions of IL-17A [52], so this cytokine could be compensating. It could account for the similar PMN recruitment observed in protected *Il17a*$^{-/-}$ mice compared to WT. Additionally, prior administration of IL-17F has been shown to protect against *S. pneumoniae* infection [32]. Notably, others have reported decreased PMN recruitment in *Il17ra*$^{-/-}$ mice during pneumococcal infection [23], which contrasts with our findings of similar PMN recruitment in *Il17a*$^{-/-}$ and WT mice. Therefore, it would be informative to assess protection in *Il17a*$^{-/-}$ *Il17f*$^{-/-}$ double knockout mice or *Il17ra*$^{-/-}$ mice. IL-22, another Th17 cytokine produced in *Il17a*$^{-/-}$ mice, has been shown to act on non-hematopoietic cells that express IL-22 receptors, such as epithelial cells and some fibroblasts in the lung, parenchymal tissue of the gut, skin, and liver [47].

Finally, our data suggest that IL-17A may contribute to the early clearance of bacteria from the lungs, since *Il17a*$^{-/-}$ protected mice had higher lung bacterial loads than WT-protected mice. Additionally, our data suggest that IL-17A might facilitate the rapid elicitation of anti-capsular antibodies, as *Il17a*$^{-/-}$ mice exhibited a delayed anti-capsular response. It has already been described that Th17 cells trigger not only B cell proliferation but also promote germinal center (GC) formation and isotype switching to IgG1, IgG2a, IgG2b, and IgG3. Interestingly, IL-17A drove class-switch recombination to IgG2a and IgG3, whereas IL-21 promoted the switch to IgG2b and IgG1 [53]. Besides, pneumococcal capsular polysaccharide 1 is zwitterionic [54], meaning that it can be presented on MHC class II molecules by antigen-presenting cells [12,13,55]. Therefore, it could stimulate CD4^{+} T cells. As *Il17a*$^{-/-}$ mice showed a delayed anti-capsular response and no secondary

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anti-capsular responses after homologous challenge (data not shown), we hypothesize that IL17A might be directly involved in this response.

Together, our results reveal that protection against invasive pneumococcal pneumonia does not depend on a single cytokine pathway but instead arises from a coordinated immune network integrating antibody-mediated opsonization, neutrophil recruitment, and overlapping Th1- and Th17-related responses. Within this network, IL-17A enhances early bacterial clearance and antibody responses but is not strictly required for survival, underscoring the functional redundancy of IL-17–related pathways in host defense against pneumococcal infection.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Caspase-1 is not required for homologous protection against pneumococcal pneumonia; Figure S2: Survival and bacterial loads in WT and *Il17a*$^{-/-}$ mice following lethal or sublethal *Streptococcus pneumoniae* serotype 1 challenge.

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