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Coordinated Th1- and Th17-Related Responses Support Antibody- and Neutrophil-Mediated Protection Against Pneumococcal Pneumonia

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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 inadequately understood. Using a murine model of homologous protection against invasive pneumococcal pneumonia, we explored the relative contributions of humoral and cellular immunity using adoptive serum transfer, immune cell depletion, and lung transcriptional profiling. Our findings indicated that passive transfer of immune serum provided robust protection, while neutrophil depletion significantly compromised bacterial control, highlighting that both 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. We associate this delay with compensatory upregulation of IL-17F and increased expression of Th1-associated genes in the lungs. 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

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1. Introduction

Streptococcus pneumoniae (Pn) is a leading cause of community-acquired pneumonia and remains 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 one million deaths annually worldwide [5]. Pneumococcal diseases encompass a wide spectrum of clinical manifestations, including pneumonia, meningitis, septicemia, and otitis media [6].

S. pneumoniae frequently colonizes the human nasopharynx, especially in children. In most cases, asymptomatic colonization is cleared by the host immune response within

weeks in adults or months in children [7]. However, under certain conditions, such as viral infections or immunosuppression, bacteria may disseminate from the nasopharynx to other anatomical sites. This dissemination can result in pneumococcal pneumonia when bacteria invade the lungs or, in some cases, lead to meningitis after spreading to the central nervous system [8,9]. More than 100 serotypes have been identified, which differ in their capsular polysaccharides and their ability to colonize or cause invasive pneumococcal disease (IPD) [10,11].

In particular, *S. pneumoniae* serotype 1 (Pn1) remains one of the most prevalent invasive serotypes globally, especially in West and Sub-Saharan Africa [10,12] and Asia [13]. In South America, particularly in Uruguay, this serotype was among the most prevalent associated with IPD, at least prior to widespread vaccination [14–18]. The introduction of pneumococcal conjugate vaccines (PCVs), which target capsular polysaccharides, has substantially reduced the incidence of invasive pneumococcal disease caused by vaccine serotypes, including serotype 1 [19]. Although vaccine-induced protection has traditionally been attributed to serotype-specific opsonizing antibodies, increasing evidence suggests that $CD4^+$ T cell-mediated immunity also contributes to protection against pneumococcal infection, especially at mucosal sites such as the lungs [20].

Historically, protective immunity against pneumococcus has been largely attributed to antibody-mediated opsonophagocytosis. While this mechanism is essential for clearing bacteremia, it may be insufficient to fully control infection at mucosal surfaces, where host–pathogen interactions are initiated [8]. 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 characterized by distinct cytokine profiles and effector functions. T helper 1 (Th1) cells, defined by their production of interferon-gamma (IFN-γ), activate macrophages and promote IgG2a/c class-switching, thereby enhancing opsonophagocytic killing [21]. Although capsular polysaccharides are generally considered T cell-independent antigens, the serotype 1 capsular polysaccharide (PnPS1) displays zwitterionic properties that allow for presentation on MHC class II molecules and activation of $CD4^+$ T cells [22,23]. In contrast, T helper 17 (Th17) cells, which secrete interleukin-17A (IL-17A) and IL-17F, play a central role in mucosal host defense. These cells promote neutrophil recruitment by inducing the production of CXC chemokines such as CXCL1 and CXCL2, and by stimulating epithelial cells to produce antimicrobial peptides that reinforce the mucosal barrier [24–26].

Growing evidence suggests that effective immunity to pneumococcal infection involves coordinated activity among multiple T helper cell pathways. Th1 and Th17 responses have been shown to act synergistically in several models of bacterial infection [27,28]. The importance of IL-17A in protection against nasopharyngeal pneumococcal carriage is well established [21,29–31], though its role in protection against invasive pneumococcal pneumonia is less clearly defined [32]. Several studies suggest that IL-17A can exert context-dependent effects, being either protective or detrimental depending on bacterial strain characteristics and capsule thickness [33]. A pathogenic role for IL17A has been described in inflammatory lung diseases such as cystic fibrosis and severe asthma [34,35], where it can amplify inflammatory signaling pathways, including those driven by IL-13 [36].

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 that promote 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 of anti-capsular antibodies.

2. Materials and Methods

2.1. Animal Studies

Female C57BL/6J mice (6–8 weeks old) were obtained from the National Division of Veterinary Laboratories (Montevideo, Uruguay). *Il17a*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice on a C57BL/6J background were provided by Institut Pasteur Montevideo, Uruguay (IPMont). All animals were housed in individually ventilated cages and handled in a class II A2 vertical laminar flow cabinet (ESCO), as previously described [27,37]. Anesthesia was induced by intraperitoneal (i.p.) injection of ketamine (2.2 mg) and xylazine (0.11 mg), as previously described [27,37]. Bacteria were administered intranasally (i.n.) in 50 µL of physiological saline (25 µL per nostril). Survival was monitored daily. For lung sampling, euthanasia was performed by cervical dislocation at the specified time points. Lungs were collected and homogenized using a cell dissociation sieve–tissue grinder kit (Sigma-Aldrich, St. Louis, MO, USA), and serial dilutions were plated on blood agar to determine bacterial loads. *S. pneumoniae* colonies were identified by their characteristic α-hemolytic green halo. For transcriptional analysis, lungs were stored in TRIzol reagent (Life Technologies, Carlsbad, CA, USA); for flow cytometry, lungs were kept on ice until processing, as described below. All animal experiments were conducted in compliance with national and institutional regulations and approved by the Comisión Honoraria de Experimentación Animal (CHEA)—Universidad de la República, Uruguay (Exp. N° 07015300081914), in accordance with ARRIVE guidelines.

2.2. 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 [27,38]. Briefly, Todd Hewitt Yeast Broth (THYB) was inoculated with fresh colonies of *S. pneumoniae* grown on blood agar and incubated at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.7–0.9. Cultures were stored at −80 °C in THYB with 12% (*v*/*v*) glycerol for up to 3 months. For infections, frozen stocks were thawed, centrifuged (5 min at 2500× *g*), washed, and diluted in saline to obtain 2×10^4 CFU/50 µL for the Pn1 sublethal dose (priming) or 2×10^7 CFU/50 µL for the Pn1 lethal dose (challenge). Bacterial counts in the inoculum were determined for every experiment by plating serial dilutions onto blood agar plates.

2.3. Adoptive Transfer of Immune Sera to Naïve Mice

Blood was collected from the facial veins or retro-orbital sinus of mice 7 days after the administration of saline (control serum) or a sublethal dose of Pn1 (immune serum). Serum from each group (n = 15/group) was pooled, aliquoted, and stored at −80 °C 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 serum on day 0 and challenged with a lethal Pn1 dose (2×10^7 CFU) on day 1. Survival was monitored daily for one week. Experiments were independently performed three times.

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

For granulocyte depletion, mice received an i.p. injection of 100 µg purified anti-Gr-1 antibody (clone RB6-8C5) or an isotype control antibody (clone HB152), 24 h before intranasal challenge with *S. pneumoniae* (2×10^7 CFU), as previously described [27,39]. Similarly, depletion of natural killer (NK) or CD4 cells was achieved by i.p. injection

of 100 µg of purified anti-NK (clone PK136) antibody, anti-CD4 (clone GK1.5) antibody, or isotype control (clone HB152) 24 h before challenge. Purified antibodies were kindly provided by Dr. Jean Claude Sirard (Institut Pasteur, Lille, France) and used as instructed. Depletion efficiency was confirmed by flow cytometry (Figure S1).

2.5. Detection of IgG and IgM Antibodies by ELISA

Antibodies specific to pneumococcal polysaccharide type 1 (PnPS1) were measured using a mouse-adapted version of the 3rd-generation WHO ELISA protocol to quantify serotype-specific IgG levels, as previously described [27]. Briefly, 96-well microtiter plates were coated overnight at 4 °C with PnPS1 antigen (American Type Culture Collection [ATCC], Manassas, VA, USA; 2 µg/mL). Serum samples were pre-absorbed with cell wall polysaccharide (C-PS, Statens Serum Institute, Copenhagen, Denmark; 5 µg/mL) and type 22F polysaccharide (ATCC; 5 µg/mL) for 30 min at room temperature. After extensive washing with Tris-buffered saline containing 0.01% Brij-35, HRP-conjugated antibodies against mouse IgG, IgM, or IgA were added. The reaction was developed using TMB substrate (3,3′,5,5′–tetramethylbenzidine, Sigma-Aldrich, St. Louis, MO, USA), stopped with 1M H_2SO_4, and absorbance was measured at 450 nm (reference 630 nm). Standard curves generated from in-house reference sera were used for quantification.

2.6. RNA Extraction and Reverse Transcription–Quantitative Real-Time PCR

Lungs were homogenized in TRIzol reagent (Life Technologies, Carlsbad, CA, USA) as previously described [27,40,41]. Total RNA was extracted according to the manufacturer's instructions, quantified, and 1 µg was treated with DNase I (Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized using random primers (Life Technologies, Carlsbad, CA, USA) and M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA, USA) (cycling: 10 min at 25 °C; 50 min at 37 °C; 15 min at 70 °C). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Venlo, The Netherlands) and a 7900HT instrument (Applied Biosystems, Foster City, CA, USA) (cycling: 15 min at 95 °C, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min; fluorescence acquisition at 60 °C) with specific primers (0.9 µM, sequences available upon request). Gene expression was normalized to Actb (β-actin) using the $2^{-\Delta\Delta Ct}$ method [42], with a cut-off of 33 cycles. Results were expressed as mRNA fold increase relative to the saline-treated WT group. Experiments were independently performed three times.

2.7. Flow Cytometry Analysis of Lung Cells

At the indicated time points after challenge, mice were euthanized, and the pulmonary vasculature was perfused with saline containing 1 mM EDTA to remove intravascular cells. Lung cells were isolated following collagenase/DNase digestion, as previously described [27,40]. Cells were resuspended in FACS EDTA buffer (PBS, 0.1% azide, 1% fetal calf serum, 5 mM EDTA) and counted using a Countess Automated Cell Counter (Invitrogen, Waltham, MA, USA) prior to 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 with 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.

2.8. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (v11.0.0). Differences between groups were evaluated using the non-parametric Mann–Whitney U test, Student's *t*-test, or Kruskal–Wallis test with Dunn's post-test, as appropriate and as described in each

figure legend. 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 facilitate opsonization of *S. pneumoniae*, promoting bacterial clearance via phagocytosis. We previously reported that wild-type (WT) mice primed with a sublethal dose of *S. pneumoniae* serotype 1 (Pn1) were completely protected against a homologous lethal challenge given 1 or even 8 weeks later. In contrast, naïve WT mice treated with saline (control) and subsequently challenged with the lethal Pn1 dose developed invasive pneumonia and died within 48–72 h [27].

To further elucidate the mechanisms underlying protection against homologous invasive pneumococcal pneumonia, we performed adoptive serum transfer experiments. As previously described, priming with a sublethal dose of Pn1 induced serum-specific anti-capsular IgG and IgM antibodies, as well as antibodies against whole-cell pneumococcal antigens [27]. Serum collected 7 days after priming was designated as immune serum. Here, we show that naïve mice receiving immune serum prior to a lethal Pn1 challenge achieved complete survival (Figure 1A). These findings demonstrate that antibodies generated 7 days after sublethal pneumococcal infection are sufficient to confer protection against homologous invasive pneumonia, at least when administered 24 h before the challenge.

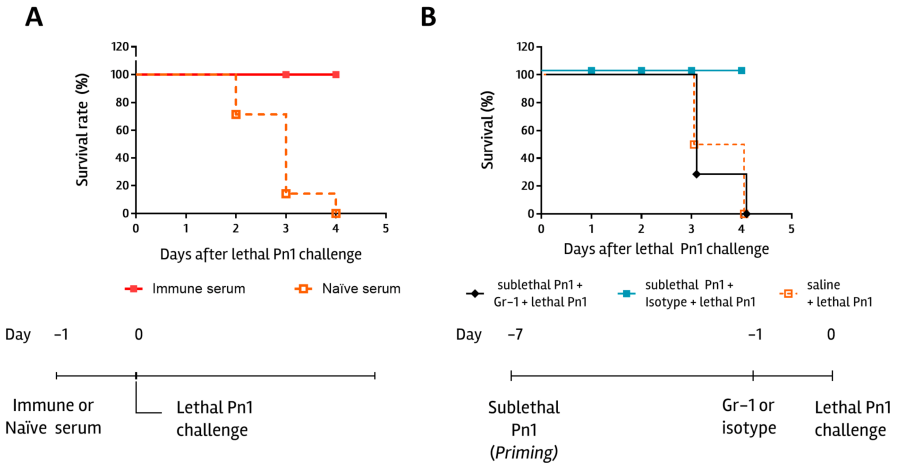

Figure 1. Serum antibodies and PMNs are key components of protection against pneumococcal pneumonia. (**A**) Adoptive transfer of immune serum protects against invasive pneumococcal pneumonia. Two groups of naïve C57BL/6 mice (n = 7/group) received immune (■) or naïve (□) serum (i.p.) and were challenged 1 day later with a lethal dose of *S. pneumoniae* serotype 1 (Pn1, 2×10^7 CFU/50 µL, i.n.). Survival rates were recorded daily after challenge. (**B**) Gr-1$^+$ cells are essential for protection against pneumococcal pneumonia. Two groups of naïve C57BL/6 mice (n = 7/group) were primed with a sublethal Pn1 dose at day −7 (2×10^4 CFU/50 µL, i.n.) and were i.p. injected with Gr-1 (◆) or isotype control (■) by day 6, and challenged with a lethal Pn1 dose (2×10^7 CFU/50 µL, i.n.) 1 day later (day 0). Control mice were treated with saline (day −7) and challenged on day 0 (□). Survival rates were recorded daily after challenge. These results are representative of 2 independent experiments.

Neutrophil (PMN)-mediated opsonophagocytosis is a key mechanism in controlling *S. pneumoniae* infection [43–45]. Consistent with previous studies [27], we confirmed massive neutrophil recruitment to the lungs following a lethal pneumococcal challenge. To determine whether PMNs were required for protection in this model, neutrophils were depleted using anti-Gr-1 antibodies in sublethally Pn1-primed WT mice before the

homologous lethal challenge. PMN depletion resulted in complete loss of protection, with no survival in depleted mice (Figure 1B). These findings confirm that neutrophils are essential mediators of homologous protection against invasive pneumococcal pneumonia.

3.2. CD4⁺, Il17a, and Casp-1/11 Are Not Exclusively Essential for Protection

Additionally, we found that administration of anti-CD4 or anti-NK-1.1 antibodies before the lethal Pn1 challenge did not eliminate protection in WT mice (Figure 2A). However, CD4 depletion before the challenge resulted in a significant decrease in *Il17a* (Figure 2B), *Il17f* (Figure 2C), and *Il22* (Figure 2D) lung mRNA levels, with no change in *Ifng* mRNA levels (Figure 2E), compared with sublethally Pn1-primed mice receiving isotype control. These findings confirm our previous observation that $CD4^+$ cells are a major source of the rapid IL-17A production detected in the lungs of protected mice [27]. Studies using a serotype 23F strain of *S. pneumoniae* showed that homologous protection against non-invasive pneumonia depends on antibodies [28,46] and T-helper type 17 (Th17) $CD4^+$ cells [28]. Our data indicate that, while $CD4^+$ cells can be considered major producers of *Il17a*, *Il17f*, and *Il22*, they are not required for homologous protection against invasive Pn1 challenge. To further validate these findings, future studies should consider sustained depletion of $CD4^+$ T cells and/or NK cells, starting before the sublethal Pn1 dose and maintained throughout the experiment via subsequent antibody injections, rather than only a single dose before the challenge, as used here.

Inflammasomes have been described as critical components of the innate immune response during pneumococcal infection, in part due to NLRP3 sensing of hemolytic pneumolysin [47,48] or NLRP6 sensing of lipoteichoic acid, both of which are key virulence factors of *S. pneumoniae*. At the post-translational level, assembly of these complexes triggers *caspase-1* activation, which mediates the maturation of IL-1β and IL-18 while concurrently initiating *gasdermin D*-dependent pyroptosis [49]. The non-canonical *caspase-11* pathway often augments this canonical signaling. We assessed *Casp1* expression in protected WT mice and found that they exhibited higher *Casp1* mRNA levels than naïve or non-protected mice (Supplementary Figure S2A). We then evaluated protection in syngeneic *Casp1/11*$^{-/-}$ mice. Both WT and *Casp1/11*$^{-/-}$ mice primed with a sublethal Pn1 dose and subsequently challenged with a homologous lethal dose showed identical survival rates (Supplementary Figure S2B). Consequently, these results indicate that inflammasome-mediated signaling is dispensable for this robust homologous protection.

Il17a was highly upregulated in mice protected against invasive pneumococcal pneumonia, suggesting a potential association between increased IL-17A levels and protection in this model [27]. Th17 responses have been widely associated with protection against pneumococcal infection [50]. 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 WT control mice, when challenged with a lethal Pn1 dose, and also died 48–72 h after infection (Supplementary Figure S3A). Interestingly, *Il17a*$^{-/-}$ mice previously primed with a sublethal dose of Pn1 exhibited the same survival rates as primed WT mice when subsequently challenged with a homologous lethal Pn1 dose (protected WT and *Il17a*$^{-/-}$ mice; Figure 3A). However, protected WT mice showed significantly lower lung bacterial loads than protected *Il17a*$^{-/-}$ mice when evaluated 24 h after the lethal challenge (Figure 3B). These findings suggest that the absence of IL17A delays bacterial clearance from the lungs, although both WT and *Il17a*$^{-/-}$ mice ultimately achieved 100% survival.

3.3. IL-17f Could Be Compensating for the Lack of IL17A for Protection

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

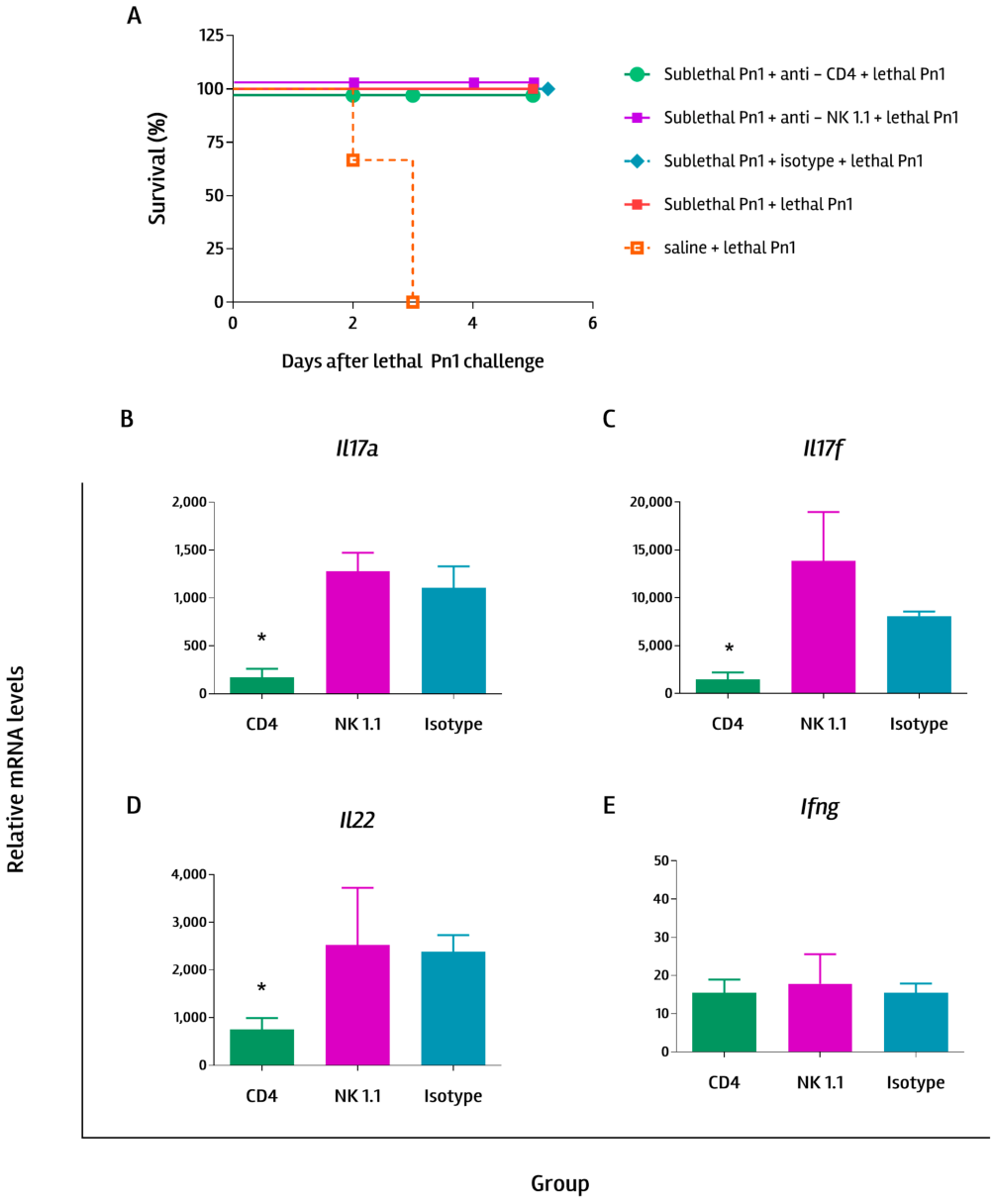

Figure 2. Depletion of CD4 or NK cells did not abrogate protection against homologous pneumococcal pneumonia. C57BL/6 mice were primed with a sublethal Pn1 dose at day −7 (2×10^4 CFU/50 µL, i.n.); i.p. injected with anti-CD4 (●), anti- NK 1.1 (■), or isotype control (◆) antibodies at day −1; and finally challenged with a lethal Pn1 dose (2×10^7 CFU/50 µL, i.n.) at day 0. Control mice were primed on day −7 with a sublethal dose of Pn1 (■) or saline (□) and challenged at day 0. (**A**) Survival rates were recorded daily after challenge for each group (n = 5 mice per group). (**B**–**E**) Three mice per group were sacrificed and total lung RNA was obtained 24 h after challenge. Relative mRNA levels for *Il17a* (**B**), *Il17f* (**C**), *Il22* (**D**), and *Ifng* (**E**) were normalized to β-actin as the housekeeping gene and referenced to the WT naïve group. * $p < 0.05$ compared to the isotype control group. Mann–Whitney test, n = 3/group. These results are representative of 2 independent experiments.

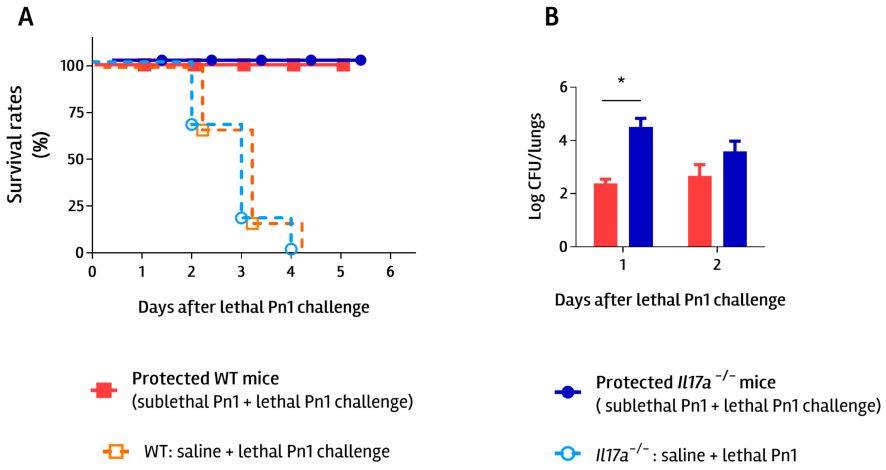

Figure 3. (**A**). Homologous protection against pneumococcal pneumoniae does not involve IL17A. *Il17a*$^{-/-}$ (●) and syngeneic WT (■) mice were primed with a sublethal Pn1 dose (2 × 10^4 CFU/50 µL, i.n.) and challenged 7 days later with a lethal Pn1 dose (2 × 10^7 CFU/50 µL, i.n.). Control *Il17a*$^{-/-}$ (⭘) and syngeneic WT (□) mice were treated with saline and challenged 7 days later. Survival rates were recorded daily after challenge (n = 6 per group). (**B**). 1 and 2 days after challenge, protected *Il17a*$^{-/-}$ (●) and WT (■)mice were sacrificed, and lung bacterial loads were assessed (n = 3/group and time-point). * $p < 0.05$, Mann–Whitney test, n = 3/group. These results are representative of 3 independent experiments.

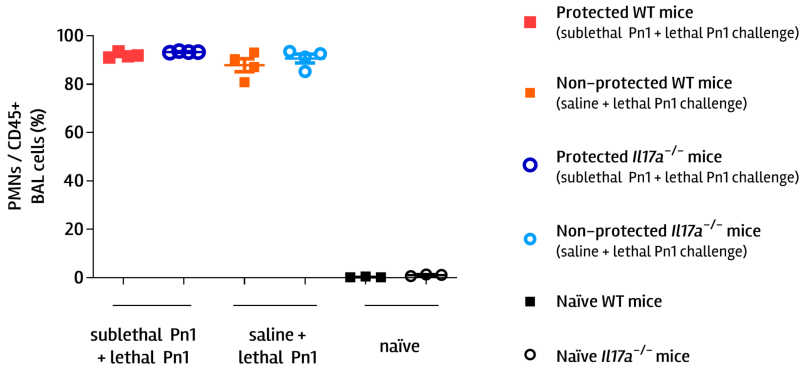

Figure 4. Both WT and *Il17a*$^{-/-}$ mice showed similar PMN recruitment. *Il17a*$^{-/-}$ and syngeneic WT mice were primed with a sublethal Pn1 dose (2 × 10^4 CFU/50 µL, i.n.) and challenged 7 days later with a lethal Pn1 dose (2 × 10^7 CFU/50 µL, i.n.). Control *Il17a*$^{-/-}$ and syngeneic WT mice were treated with saline and challenged 7 days later. WT and *Il17a*$^{-/-}$ naïve groups were also included in the analysis (n = 3 per group). Bronchoalveolar lavage fluid (BAL) samples were obtained 24 h after challenge. 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, except for naïve control groups. These results are representative of 3 independent experiments.

We next compared the lung transcriptional profiles between protected WT and *Il17a*$^{-/-}$ mice, defined as those primed with a sublethal Pn1 dose and surviving the subsequent challenge with a lethal Pn1 dose on day 7. Overall, both strains displayed highly similar expression patterns. However, protected *Il17a*$^{-/-}$ mice showed a more rapid increase in *Il17f* mRNA levels than wild-type (WT) mice, as measured 8 h after lethal challenge, although both reached similar levels at 24 h (Figure 5A). For other Th17-associated cytokines, similar increases in *Il21* (Figure 5C) and *Il22* (Figure 5D) mRNA levels were observed, while *Il17a* mRNA transcripts were absent in *Il17a*$^{-/-}$ mice (Figure 5A). Additionally, both groups of protected mice showed similar increments in relative mRNA levels for *Ifng* (Figure 5E) and *Ifng*-related genes (*Cxcl9*, *Cxcl10*, *Cxcl11*; Figure 5F–H) as well as for the antimicrobial peptide *Lipocaline 2* (*Lcn2*; Figure 5K). For *S100A9*, both mouse strains

exhibited a rapid, significant increase at 8 h, followed by a decrease by 24 h (Figure 5L), similar to the pattern observed for *Il1b* and *Ifnb* mRNA levels (Figure 5I,J). Previous studies suggest that type I interferons contributed to host defense against pneumococcal infection by limiting bacterial dissemination from the lungs to the bloodstream [51]. Accordingly, the rapid induction of *Ifnb* mRNA levels observed in both protected WT and *Il17a*$^{-/-}$ mice (Figure 5J) may help prevent systemic dissemination.

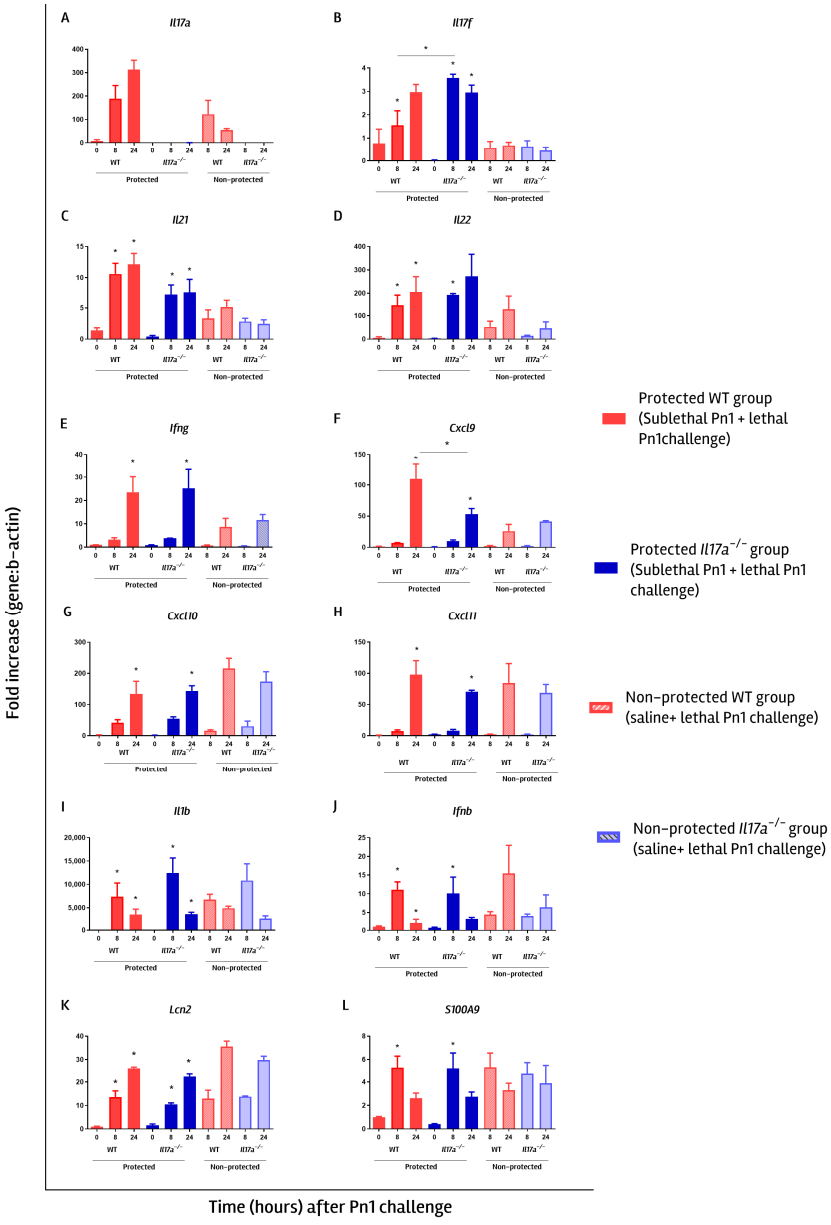

Figure 5. RT-qPCR analysis of the relative mRNA levels of genes encoding *Il17a* (**A**), *Il17f* (**B**), *Il21* (**C**), *Il22* (**D**), *Ifng* (**E**), *Cxcl9* (**F**), *Cxcl10* (**G**), *Cxcl11* (**H**), *Il1b* (**I**), *Ifnb* (**J**), *Lcn2* (**K**) and *S100A9* (**L**) in the lungs of WT (red bars) and *Il17a*$^{-/-}$ (blue bars) mice primed with the sublethal dose of *S. pneumoniae* serotype 1 (Pn1, 2×10^4 CFU/50 µL) and sacrificed 8 h or 24 h after a lethal homologous challenge (Pn1, 2×10^7 CFU/50 µL), performed 7 days later. WT and *Il17a*$^{-/-}$ control mice received saline and were challenged 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. * indicates $p < 0.05$, ANOVA test with Dunnett's post hoc multiple-comparisons test versus the control group (time 0 h of each strain) or between the groups indicated by the horizontal bar.

Overall, protected *Il17a*$^{-/-}$ and WT 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, our data suggest that *Il17a*$^{-/-}$ mice mount compensatory "Th17-like" responses together with Th1 responses that may account for the protective immunity observed after homologous lethal challenge.

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

Our results suggest that serum antibodies are protective against invasive pneumonia, at least in our mouse model (Figure 1A). We then compared the induction of specific antibodies in serum from *Il17a*$^{-/-}$ and WT mice, both of which were primed with the sublethal Pn1 dose. Results showed significant induction of IgG and IgM anti-capsular antibodies in primed *Il17a*$^{-/-}$ mice, though with some differences in kinetics compared to WT (Figure 6). At earlier time points (1 and 2 weeks after sublethal Pn1 priming), WT mice showed higher 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 generating anti-capsular antibodies after priming, which could explain the delayed clearance of bacteria from the lungs in *Il17a*$^{-/-}$ mice (Figure 3B). However, at later time points (12 and 24 weeks after sublethal Pn1 dose), *Il17a*$^{-/-}$ mice showed increased antibody levels compared to WT, indicating that *Il17a*$^{-/-}$ mice are not impaired in their ability to mount an antibody response, consistent with findings in other studies using this deficient mouse strain [52]. Further studies are required to elucidate the mechanism underlying the delayed anti-capsular response observed in *Il17a*$^{-/-}$ mice.

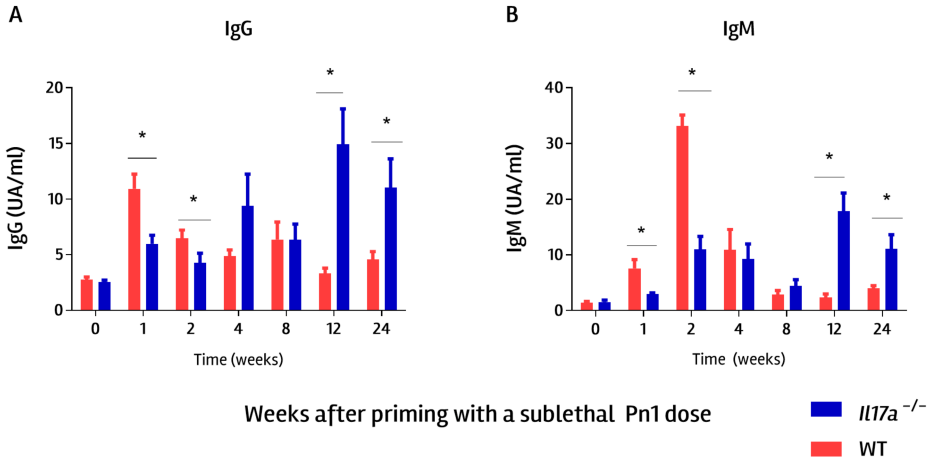

Figure 6. Kinetics of anti-capsular antibody responses induced after sublethal *S. pneumoniae* infection. Serum levels of IgG (**A**) and IgM (**B**) antibodies specific to pneumococcal polysaccharide 1 (PnPS1) were measured by indirect ELISA in C57BL/6 WT (red bars) and *Il17a*$^{-/-}$ (blue bars) mice following intranasal priming with a sublethal dose of *S. pneumoniae* serotype 1 (Pn1; 2×10^4 CFU/50 µL). Data represent the mean ± SEM of three pooled independent experiments (n = 7–10/mice per time point). * indicates $p < 0.05$ by Mann–Whitney test.

4. Discussion

S. pneumoniae is a major human pathogen whose principal virulence factor is its capsular polysaccharide, largely due to its anti-phagocytic properties. Consequently, current pneumococcal vaccines are based on capsular polysaccharides from the most clinically significant serotypes and are effective at preventing invasive manifestations of pneumococcal disease [53]. While the immune mechanisms underlying protection against *S. pneumoniae* infection have been extensively investigated, the relative contributions of humoral and

cellular immunity to defense against invasive pneumococcal pneumonia are not fully defined [28]. In this study, we further elucidated these mechanisms using a well-established murine model of homologous protection against invasive pneumococcal pneumonia [27], with a particular focus on the roles of anti-capsular antibodies, neutrophils (PMNs), $CD4^+$ and NK cells, inflammasome activation, and IL-17A.

Our results reinforce the pivotal roles of antibodies and neutrophils 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 subsequent lethal homologous challenge, as demonstrated by passive serum transfer (Figure 1A). In parallel, neutrophil depletion completely abrogated protection, as Gr-1-treated mice failed to survive the homologous lethal Pn1 challenge (Figure 1B). Lethal Pn1 challenge induced invasive pneumonia and rapid PMN recruitment to the lungs (Figure 4). In mice without prior sublethal Pn1 priming, bacteria were not efficiently cleared from the lungs, and animals succumbed to infection. While neutrophils are essential for bacterial clearance, they can also contribute to tissue damage by releasing inflammatory mediators such as superoxide and neutrophil elastase [54]. Excessive lung inflammation is partly responsible for the lethality observed during pneumococcal pneumonia [8] and may promote bacterial dissemination to the bloodstream, exacerbating disease severity [55,56]. In our model, antibodies in immune serum likely facilitated opsonization and efficient phagocytosis of pneumococci by recruited neutrophils. In contrast, in mice receiving naïve serum, recruited PMNs failed to clear bacteria, and the resulting inflammation likely contributed to mortality. Rapid clearance of bacteria from the lungs is a key determinant of outcome. Moreover, in Gr-1-treated mice, despite the presence of anti-pneumococcal antibodies (induced by prior sublethal Pn1 priming), the absence of functional neutrophils is directly associated with loss of protection.

Overall, our findings and those of others [57] highlight that neutrophils are central determinants of both protection and pathogenesis against *S. pneumoniae* infection. Our data specifically demonstrate that their recruitment is essential for protection against homologous invasive pneumococcal pneumonia.

Previous studies have shown that Th17 cells play a protective role in pulmonary infections [58]. With respect to *S. pneumoniae* serotype 1, we previously described an association between IL17A and homologous protection against invasive pneumococcal pneumonia [27]. However, other authors have described a context-dependent, dual role for IL-17A during pneumococcal pneumonia that may depend on serotype, particularly on capsule thickness [33]. 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, recruited lung PMNs are unable to effectively phagocytose bacteria, resulting in lung damage without efficient bacterial clearance [33]. In contrast, serotype 1 expresses less capsular polysaccharide [59,60], which may enable more effective antibody-mediated opsonophagocytosis.

Importantly, our results indicate that IL17A is not strictly required for protection: sublethally Pn1-primed *Il17a*$^{-/-}$ mice, as well as WT mice, were completely protected against a homologous lethal challenge. Protected *Il17a*$^{-/-}$ mice exhibited what we consider Th17-like responses, with increased expression of *Il17f*, *Il21*, and *Il22*, as well as Th1-associated genes (including *Ifng* and other *Ifng*-related genes) (Figure 5). These observations support the existence of compensatory cytokine pathways that can maintain protective immunity in the absence of IL-17A.

IL-17A and IL-17F are both members of the IL-17 family of cytokines, sharing the highest sequence homology (58%) among family members, and are primarily produced by

Th17 cells [25,58,61,62]. It has been shown that IL-17F can induce PMN recruitment and activation similarly to IL-17A [63], which could explain the comparable PMN recruitment observed in protected *Il17a*$^{-/-}$ and WT. Administration of IL-17F has been demonstrated to protect against *S. pneumoniae* infection [43]. Interestingly, other studies have reported decreased PMN recruitment in *Il17ra*$^{-/-}$ mice during pneumococcal infection [33], which contrasts our findings. Future studies assessing protection in *Il17a*$^{-/-}$ *Il17f*$^{-/-}$ double knockout mice or *Il17ra*$^{-/-}$ mice would clarify these mechanisms. Additionally, IL-22, another Th17 cytokine upregulated in *Il17a*$^{-/-}$ mice, acts on non-hematopoietic cells such as lung epithelial cells and fibroblasts, further contributing to mucosal defense [58].

Our data also suggest that IL-17A contributes to the early clearance of bacteria from the lungs, as protected *Il17a*$^{-/-}$ mice exhibited higher lung bacterial loads than protected WT mice (Figure 3B). Additionally, IL-17A may facilitate the rapid induction of anti-capsular antibodies, since *Il17a*$^{-/-}$ mice showed a delayed anti-capsular response (Figure 6). There is a very interesting report suggesting a still-unappreciated role for Th17 cells and their signature cytokines in mediating B-cell differentiation and class switch recombination [64]. Based on their results, they propose that Th17 cells not only promote B cell proliferation but also support germinal center (GC) formation and isotype switching to IgG subclasses [64]. Specifically, IL-17A drives class-switch recombination to IgG2a and IgG3, while IL-21 promotes switching to IgG2b and IgG1 [64]. Notably, pneumococcal capsular polysaccharide 1 (PnPS1) is zwitterionic [65], enabling its presentation on MHC class II molecules and activation of CD4^{+} T cells [22,23,66]. Although polysaccharide antigens are typically considered T-independent type 2 antigens, the zwitterionic properties of PnPS1 may be related to the delayed anti-capsular response observed in *Il17a*$^{-/-}$ mice following priming with a sublethal dose of Pn1. We therefore hypothesize that IL17A might be directly involved in this anti-capsular response. The initial deficit in anti-capsular antibody responses observed in *Il17a*$^{-/-}$ mice may represent a signaling bottleneck. In WT mice, early IL17A production orchestrates rapid leukocyte recruitment and optimizes the lymphoid microenvironment, facilitating prompt extrafollicular B-cell activation and early antibody secretion. In the absence of IL-17A, this early spatial organization is disrupted, and this could lead to a localized lag phase in polysaccharide antigen recognition and subsequent B-cell activation. Anti-capsular antibody titers in *Il17a*$^{-/-}$ reached levels comparable to those in WT mice at later time points after priming with a sublethal Pn1 dose (Figure 6). This delayed resolution is consistent with compensatory upregulation of alternative Th17-associated cytokines—namely *Il17f*, *Il21*, and *Il22*—alongside *Ifng*, as observed in our *Il17a*-deficient model. While these alternative pathways require more time to accumulate and reach functional thresholds than the immediate primary IL-17A response, they ultimately provide the necessary cooperative signaling to rescue the humoral response. Specifically, the compensatory rise in *Il21*, a potent driver of B-cell proliferation and plasma cell differentiation, would likely serve as a critical mechanism that forces the delayed anti-capsular antibody production to match WT levels over time. This hypothesis still has to be demonstrated and opens a new interesting area of research. We are deepening our investigation of IL-17A-mediated polysaccharide responses through ongoing experiments to uncover the precise mechanisms involved.

Taken together, our findings demonstrate that protection against invasive pneumococcal pneumonia is mediated not by a single cytokine pathway, but by a coordinated immune network that integrates antibody-mediated opsonization, neutrophil recruitment, and overlapping Th1- and Th17-related responses. Within this network, we propose that IL-17A enhances early bacterial clearance and antibody responses but is not strictly required for survival, highlighting the functional redundancy of IL-17–related pathways in host defense against pneumococcal infection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/immuno6020041/s1, Figure S1: Depletion controls for PMNs, CD4, or NK depletion studies; Figure S2: *Caspase-1* is not required for homologous protection against pneumococcal pneumonia; Figure S3: Survival and bacterial loads in WT and *Il17a*$^{-/-}$ mice after being challenged with *Streptococcus pneumoniae* serotype 1.

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