

BASIC SCIENCE ARTICLE



Suppressive effect of therapeutic antibiotic regimen on antipneumococcal Th1/Th17 responses in neonatal mice

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BACKGROUND: Antibiotics are commonly used in human neonates, but their impact on neonatal T cell immunity remains poorly understood. The aim of this study was to investigate the impact of the antibiotic piperacillin with the beta-lactamase inhibitor tazobactam on neonatal CD4⁺ and CD8⁺ T cell responses to *Streptococcus pneumoniae*.

METHODS: Splenic and lung cells were isolated from the neonatal mice receiving piperacillin and tazobactam or saline (sham) and cultured with *S. pneumoniae* to analyze T cell cytokine production by ELISA and flow cytometry.

RESULTS: Antibiotic exposure to neonatal mice resulted in reduced numbers of CD4⁺/CD8⁺ T cells in the spleen and lungs compared to control mice. Upon in vitro stimulation with *S. pneumoniae*, splenocytes and lung cells from antibiotic-exposed mice produced lower levels of IFN- γ (Th1)/IL-17A (Th17) and IL-17A cytokines, respectively. Flow cytometric analysis revealed that *S. pneumoniae*-stimulated splenic CD4⁺ T cells from antibiotic-exposed mice expressed decreased levels of IFN- γ and IL-17A compared to control mice, whereas lung CD4⁺ T cells produced lower levels of IL-17A. However, no significant difference was observed for IL-4 (Th2) production.

CONCLUSIONS: Neonatal mice exposure to piperacillin and tazobactam reduces the number of CD4⁺ and CD8⁺ T cells, and suppresses Th1 and Th17, but not Th2, responses to *S. pneumoniae*.

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IMPACT:

- Exposure of neonatal mice with a combination of piperacillin and tazobactam reduces CD4⁺/CD8⁺ T cells in the spleen and lungs.
- Antibiotic exposure suppresses neonatal Th1 and Th17, but not Th2, responses to *Streptococcus pneumoniae*.
- Our findings may have important implications for developing better therapeutic strategies in the neonatal intensive care unit

INTRODUCTION

Bacterial infections remain a leading cause of mortality and morbidity among human neonates worldwide, particularly in low- and middle-income countries.^{1–3} Therefore, antibiotics are often prescribed to prevent and treat infections in neonates, most notably sepsis.^{4–8} In the neonatal intensive care unit, pregnant women and neonates/infants receive empiric antibiotic therapy, which can reach up to 40% of all admissions, and be as high as 90 to 98% for very low or extremely low birth weight infants.^{9–11} The most commonly used antibiotic combination for the treatment of neonates with suspected clinical sepsis is ampicillin and gentamicin.¹² With the increase in multidrug-resistant infections, a combination of piperacillin and tazobactam has emerged as one of the alternative empirical antibiotics for presumed neonatal sepsis.^{13–15} Piperacillin is a β -lactam antibiotic that belongs to the class of extended-spectrum semisynthetic penicillin antibiotics and has a wider spectrum of activity than natural penicillins, such as aminopenicillins and semi-synthetic penicillinase-resistant penicillins.^{16,17} It is often available in combination with the beta-lactamase inhibitor tazobactam to provide coverage against

penicillinase-producing bacteria.^{16,17} Li et al. showed that piperacillin–tazobactam dose strategies using 44.44/5.56 mg/kg/dose every 8 or 12 h achieves the pharmacodynamic target in about 67% of the neonates/infants.¹³ Furthermore, parenteral injection of piperacillin–tazobactam in very low-birthweight infants, who were included for nosocomial sepsis, suspected necrotizing enterocolitis, or infection after abdominal surgery, resulted in: (1) no clinical adverse events, such as phlebitis, rash, or stool changes; (2) no long-lasting effect on the gut microbiota; and (3) favorable clinical outcome for 17 out of 23 (67%) infants.¹⁴

CD4⁺ T cells, also known as T helper (Th) cells, are a major subpopulation of T cells that include functional subsets, such as Th1, Th2, and Th17 cells, based on their cytokine production pattern.¹⁸ Th1 cells produce copious amounts of signature cytokine interferon (IFN)- γ and promote cell-mediated immunity against intracellular pathogens.¹⁸ Th2 cells, on the other hand, are characterized by the production of interleukin (IL)-4, IL-5, IL-9, IL-10, and IL-13 and are associated with host defense against parasites and pathogenesis of asthma and allergy.¹⁹ Th17 cells are a class of Th cells that produce IL-17A and IL-17F and play an

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important role in the pathogenesis of immune-mediated diseases as well as pathogen defense.²⁰

Antibiotic exposure with other risk factors can contribute to dysbiosis, i.e., disturbance in composition and/or function of the microbiota.^{7,8} Accumulating evidence indicates that the evolving microbiota in neonates plays an important role in educating or imprinting the immune system and that neonatal dysbiosis exerts detrimental effects on antibody-mediated and T cell immunity.^{7,8} Of note, neonates are disproportionately more affected by aberrant T cell responses as a result of antibiotic therapies when compared to adults, resulting in more significant altered immune responses to infections.²¹ Recent studies mainly using animal models have begun to shed light on how antibiotic treatment regimens modulate neonatal/infant T cells against pathogens and vaccines.^{22–27} Treatment of mouse neonates with a combination of ampicillin, streptomycin, and clindamycin has been reported to alter the number of T cell populations in the blood and spleen.^{23,26} More importantly, following an infection with vaccinia virus, mouse neonates delivered by the dams exposed to a combination of ampicillin, streptomycin, and clindamycin via drinking water during pregnancy and lactation exhibited reduced antigen-specific expression of IFN- γ by splenic CD8+ T cells compared to unexposed mice.²³ Furthermore, splenic CD8+ T cells from these exposed neonates partially restored their IFN- γ production upon stimulation with lipopolysaccharide (via Toll-like receptor 4) in vitro as well as in vivo.²⁴ In contrast, when stimulated with phytohemagglutinin, the peripheral blood mononuclear cells (PBMCs) from neonate piglets exposed to amoxicillin via oral gavage produced increased levels of IFN- γ compared to control piglets.²⁶ More importantly, the PBMCs from these piglets challenged with heat-killed *Salmonella Typhimurium* displayed upregulated expression of genes for IFN- γ , IL-6, and IL-2.²⁶ In line with this, when immunized with vaccine (PCV13) against the bacterial pathobiont *Streptococcus pneumoniae*, splenic CD4+ T cells from mouse neonates treated with ampicillin and neomycin via drinking water displayed enhanced IFN- γ responses on ex vivo restimulation with vaccine antigen, underscoring that exposure to antibiotic regimens in early life may lead to enhanced antigen-specific T cell immunity.²⁷ Overall, these findings suggest a differential effect of antibiotics on the function of splenic and blood CD4+ and CD8+ T cells characterized by T cell cytokine profile (e.g., IFN- γ), which may depend on the type of antibiotics and animal model. Therefore, it is important to evaluate the effect of specific antibiotic types, such as piperacillin–tazobactam, which are used in the neonatal intensive care unit, on T cell responses to specific pathogens using appropriate animal models that mimic treatment protocols analogous to those used in the human neonatal intensive care unit.

Alterations in neonatal T cell responses as a result of antibiotic use have been reported in several studies mostly involving viral pathogens.^{22–27} However, it is still rather unknown how this impacts T cell responses ensued by major pathogens that are part of the commensal human microbiota, including *S. pneumoniae*. *S. pneumoniae* causes several diseases in human adults and neonates/infants, including pneumonia and sepsis, and is responsible for the deaths of around 1 million children per year globally.^{28,29} Although antibody-mediated immunity is crucial for protection against *S. pneumoniae*, accumulating evidence suggests a key role for T cell immunity, especially Th17 immunity, in preventing/reducing pneumococcal infection/carriage in the respiratory tract.^{30–35} Furthermore, Th1 and Th2 responses have been shown to play an important role in host defense against pneumococcal infections.^{36–39} Additionally, CD8+ T cells are reported to enhance resistance against lung infection by *S. pneumoniae* serotype 3 in mice.⁴⁰ Thus, both CD4+ and CD8+ T cells are crucial for protective immunity to infections with *S. pneumoniae*. Antibiotic usage-driven modulation of peripheral and mucosal T cell immunity to *S. pneumoniae* remains unexplored. In this study, we investigated the impact of a combination

of piperacillin and tazobactam, whose effect on neonatal T cells is unknown, on antigen-specific T cell responses using mouse neonates and ultraviolet (UV)-killed *S. pneumoniae*. Splenic and lung cells were isolated from the neonatal mice receiving piperacillin and tazobactam or saline (sham) and cultured with killed *S. pneumoniae* to analyze T cell cytokine production by enzyme-linked immunosorbent assay (ELISA) and flow cytometry.^{32,41,42} Our findings provide important insights into how piperacillin–tazobactam usage can modulate *S. pneumoniae*-specific splenic and lung T cell, particularly Th1/Th17, responses.

MATERIALS AND METHODS

Neonatal mouse model

Specific pathogen free (SPF) female Swiss mice (around 2 weeks pregnant) were bought from the JANVIER LABS, France and quarantined and housed in a Minimal Disease Unit at the animal facility at Oslo University Hospital, Rikshospitalet, Oslo, Norway. Each pregnant mouse was kept in an IVC cage that are environmentally enriched with impellers and paper nest building and given standard feed and water ad libitum. After delivery, mouse neonates stayed with their dams till experimental completion. Each mouse litter (12–18 in number) with their respective dams constituted an experimental group. The 10-day-old neonates in different groups were intraperitoneally injected with a combination of piperacillin and tazobactam (≈ 10 mg/mouse) or saline (sham) every 8 h for 5 consecutive days. The mouse equivalents of the antibiotic dose used in human infants were calculated based on normalization of dose to body surface area as described previously.⁴³ To avoid litter bias, we randomized littermates across the experimental groups (treated and control) before commencement of antibiotic treatment. The experiment was performed twice. Following antibiotic treatment for 5 days, neonatal mice were euthanized by anesthetizing with 4–5% isoflurane (Isofluran; IsoFlo Vet 100%; Zoetis) and then inoculating with an intraperitoneal injection of pentobarbital (Mebumal 10%; dose rate of 0.05–0.5 ml per mouse).

Animal ethics

All mouse experiments were approved by the Norwegian Food Safety Authority, Oslo, Norway (Project license number FOTS – 21062) and performed in accordance with the guidelines of the Norwegian Animal Welfare Act (10 June 2009 no. 97), the Norwegian Regulation on Animal Experimentation (REG 2015-06-18-761), and the European Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes.

Streptococcus pneumoniae

The *S. pneumoniae* TIGR4 strain was used in this study.⁴⁴ The strain was suspended in trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ) and 15% glycerol and stored in -80°C freezer. For the use of bacteria, stock cultures were diluted and grown at 37°C to an optical density of 0.5 at 600 nm in a 5% CO_2 incubator. The bacterial cells were harvested by centrifugation at $5000 \times g$ for 10 min at 4°C and washed in endotoxin-free Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) and UV-inactivated for 30 min. The UV-treated pneumococcal suspension was aliquoted and frozen at -80°C .

Cell isolation

The neonatal mice were euthanized and their lungs and spleens were collected. Each spleen was mashed on a $70\ \mu\text{m}$ cell strainer (ThermoFisher Scientific, Rockford, IL) with the plunger of a 3 ml syringe and washed with the washing buffer (PBS, 0.5% bovine serum albumin (BSA), and 5 mM EDTA). The cell suspension was lysed with red blood cell (RBC) lysis buffer and washed twice. The lung cell isolation was performed as described previously.⁴⁵ Briefly, the lungs were digested in 10 mg/ml collagenase XI (Sigma-Aldrich, Israel) in RPMI 1640 (Sigma-Aldrich, United Kingdom) for 1 h at 37°C . The lung cell suspension was treated with lysis buffer (eBioscience, San Diego, CA) to lyse contaminating RBCs. The lung cells were washed with washing buffer (PBS, 0.5% BSA, and 5 mM EDTA). Hemocytometer and trypan blue were used to count viable cells.

Antigenic stimulation of the splenocytes and lung cells

In all, 2.5×10^6 splenocytes in $500\ \mu\text{l}$ or 5×10^5 lung cells in $200\ \mu\text{l}$ of complete RPMI medium (10% heat-inactivated fetal bovine serum (FBS),

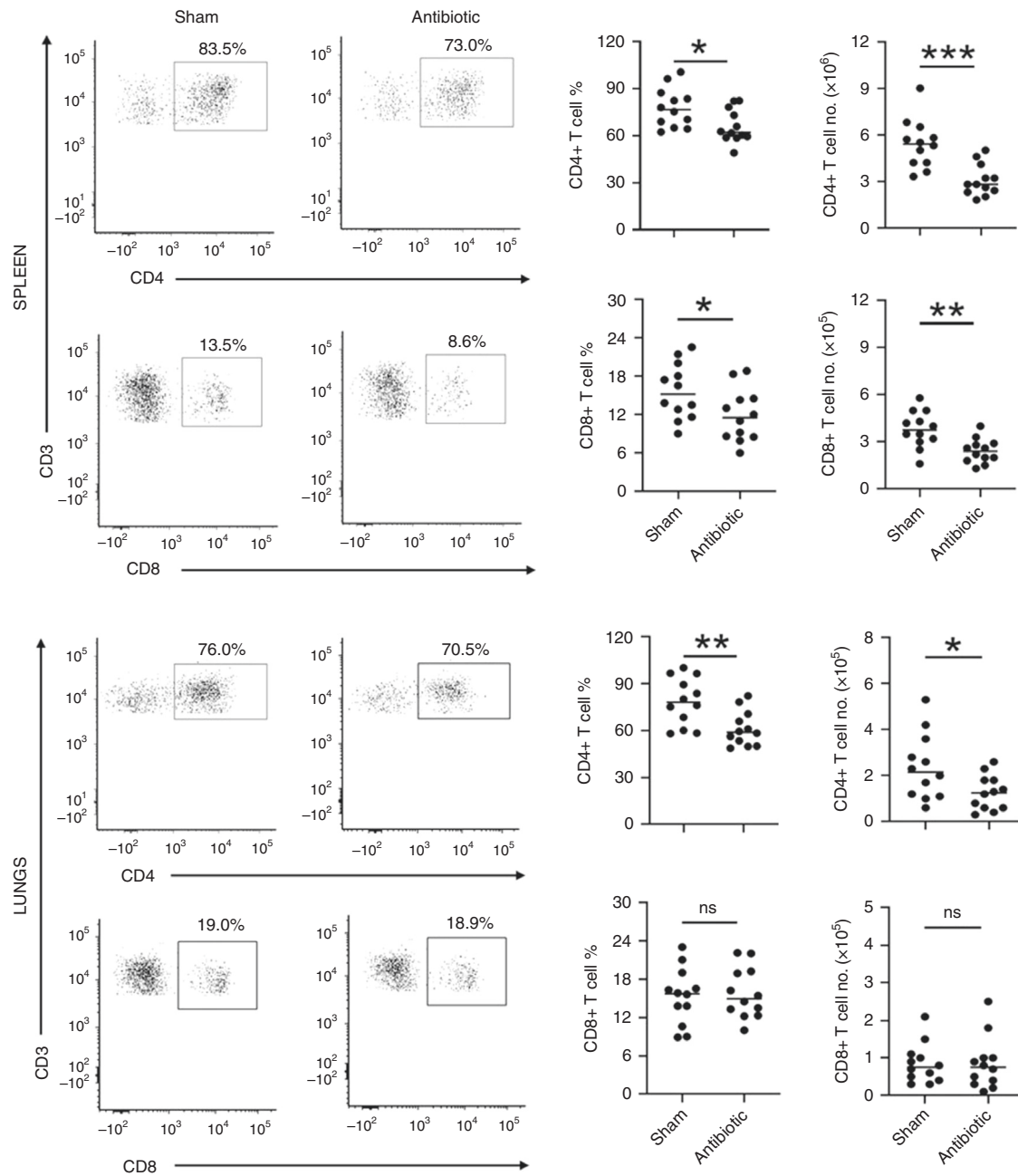


Fig. 1 Impact of piperacillin and tazobactam regimen on CD4+ and CD8+ T cell numbers. Splenocytes and lung cells isolated from the mice treated with piperacillin–tazobactam or saline (sham) were stained with antibodies specific for CD3, CD4, and CD8 T cell markers and analyzed by flow cytometry. Detailed gating strategy for CD3, CD4, and CD8 T cell populations has been illustrated in Supplementary Fig. 1. CD3+ cells were gated and displayed in terms of CD3+CD4+ T cells and CD3+CD8+ T cells. The frequencies (percentages) and absolute numbers of T cell subsets (CD4 and CD8) were determined. Representative flow cytometric dot plot images (left) and a summary of the percentages and numbers of T cells (right). Each experimental group (antibiotic or sham) had 12 mice. Data are shown as mean \pm SD and are representative of two independent experiments with similar results. Each symbol represents data from an individual mouse, and the horizontal bars are mean values for the groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns = non-significant. Unpaired Student's t test.

25 μ g/ml gentamicin, L-glutamine, and sodium bicarbonate; Sigma-Aldrich, UK) were cultured at 37 $^{\circ}$ C and stimulated with UV-killed *S. pneumoniae* TIGR4 (10^5 colony-forming units (CFU)/ml) for 72 h. Culture supernatants were frozen at -80° C for further cytokine analysis by commercial ELISA kits. The concentrations of IFN- γ , IL-4, and IL-17A were measured by Ready-SET-Go ELISA kits (eBioscience, San Diego, CA), whereas the concentration of IL-4 was measured with an ELISA kit from Invitrogen (Vienna, Austria), in accordance with the instructions of respective manufacturers. The cytokine detection limit of the ELISA kits for IL-17, IL-4, and IFN- γ were 4, 3.9, and 15 pg/ml, respectively.

Flow cytometry

To analyze the expression of the various surface markers, the freshly prepared lung and spleen single-cell suspensions were stained using anti-CD4-Phycoerythrin (PE), anti-CD8-Fluorescein isothiocyanate (FITC), anti-CD3-PE-Cy7, or with respective isotype controls (eBioscience, San Diego, CA). For T cell intracellular cytokine staining, 2.5×10^6 splenocytes in 500 μ l or 5×10^5 lung cells in 200 μ l of complete RPMI medium (10% heat-inactivated FBS, 25 μ g/ml gentamicin, L-glutamine, and sodium bicarbonate; Sigma-Aldrich, UK) were cultured at 37 $^{\circ}$ C and stimulated with UV-killed *S. pneumoniae* TIGR4 (10^5 CFU/ml) for 72 h. Culture supernatant was

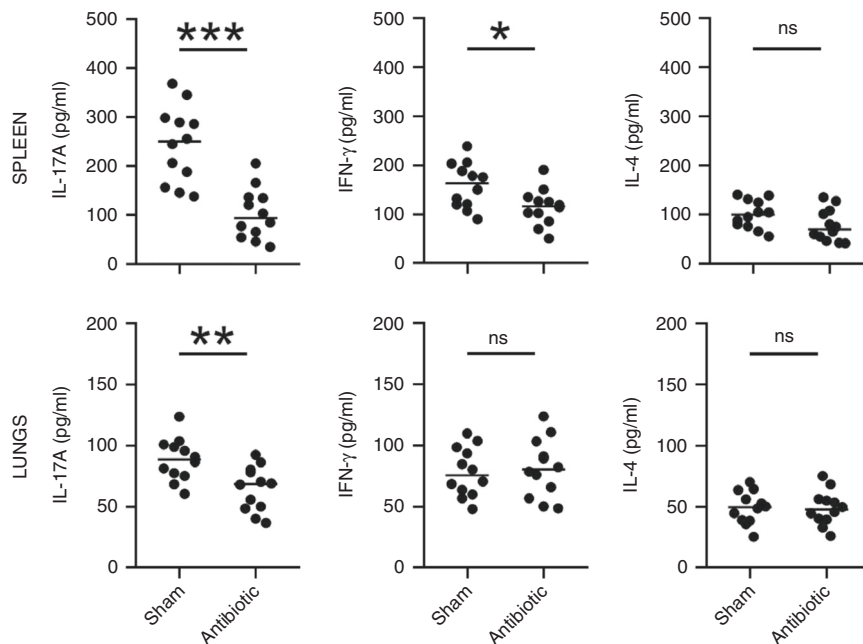


Fig. 2 Cytokine production by splenocytes and lung cells of mice exposed to piperacillin–tazobactam. Splenocytes and lung cells from antibiotic- or saline-treated mice were stimulated with killed *S. pneumoniae* for 72 h, and IL-17A, IFN- γ , and IL-4 levels in the culture supernatants were measured by ELISA. Each experimental group (antibiotic or sham) had 12 mice. Data are shown as mean \pm SD and are representative of two independent experiments with similar results. Each symbol represents data from an individual mouse, and the horizontal bars are mean values for the groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns = non-significant. Unpaired Student's *t* test.

discarded and the splenic and lung cells were incubated in 48-well plates at 37 °C and treated with a cell stimulation cocktail for 18 h, according to the instructions of the manufacturer (eBioscience, San Diego, CA). Following incubation, the cells were washed (Dulbecco's PBS containing 0.5% BSA and 1 mM EDTA) and incubated with FcR-blocking antibodies (anti 16/32; eBioscience) for 15 min at 4 °C. The cell surface markers were first stained with anti-CD3-PECy7, anti-CD8-FITC, and anti-CD4-PE or respective isotype control antibodies (eBioscience, San Diego, CA). The cells were fixed with IC fixation buffer (Invitrogen, CA) and permeabilized with permeabilization buffer (eBioscience, San Diego, CA) according to the manufacturer's instructions, which was followed by intracellular staining with anti-IL-17A-allophycocyanin (APC), anti-IFN-APC, anti-IL-4-APC, or isotype control antibodies (eBioscience, San Diego, CA), and Fluorescence minus one (FMO) control was also used (Supplementary Figs. 2 and 3). Finally, the cells were washed, resuspended in Dulbecco's PBS containing 0.5% BSA and 1 mM EDTA, and analyzed by flow cytometry. Sample data were collected using a BD LSR II flow cytometer (BD Biosciences, San Diego, CA), and the data were analyzed using the FCS Express software (De Novo Software, Los Angeles, CA).

Statistics

Unpaired Student's *t* test was used for comparing two groups (GraphPad Prism Software, version 7, Graph Pad, San Diego, CA). A *p* value < 0.05 was considered significant.

RESULTS

Piperacillin–tazobactam regimen reduces the number of neonatal T cells

We found that intraperitoneal antibiotic injections in mouse neonates resulted in a significant reduction in the frequencies (percentages) and absolute numbers of CD4+ T cells in the spleen and lungs compared to those neonates receiving saline (sham) (Fig. 1). Although CD8+ T cells decreased in the spleen of antibiotic-exposed neonates, they remained unchanged in the lungs (Fig. 1). Thus, exposure to piperacillin–tazobactam in neonates reduced the number of CD4+/CD8+ and CD4+ T cells in the spleen and lungs, respectively.

Neonatal exposure to piperacillin–tazobactam suppresses Th1 and Th17, but not Th2, immune responses to *S. pneumoniae*

Since Th17 cell responses play a crucial role in antipneumococcal immunity,^{30–32,36,41} and antibiotic usage impairs Th17 immunity to fungal pathogens in neonates,⁴⁶ we attempted to assess whether therapeutic piperacillin–tazobactam regimen in mouse neonates modulate Th17 responses to *S. pneumoniae*. For this, we isolated splenocytes and lung cells from antibiotic-exposed mice, stimulated them with UV-killed *S. pneumoniae* in vitro, and measured Th17 responses characterized by the production of IL-17A by CD4+ T cells. We found that splenocytes from antibiotic-exposed neonates produced significantly reduced quantities of IL-17A cytokines compared to control mice (Fig. 2). In line with these results, lung cells from the mice that received antibiotic exhibited diminished production of IL-17A (Fig. 2). Of note, without stimulation with the killed *S. pneumoniae*, IL-17A production levels by splenocytes from the neonates treated with piperacillin–tazobactam or saline did not show any significant difference (data not shown). Using flow cytometric intracellular cytokine staining, we assessed the production of pneumococcal antigen-specific IL-17A by CD4+ and CD8+ T cells. Flow cytometric analysis showed that splenic CD4+ T cells from antibiotic-exposed mice expressed lower levels of IL-17A than those from control mice (Fig. 3). Similar results were observed for lung CD4+ T cells (Fig. 3). In contrast, both splenic and lung CD8+ T cells failed to show significant difference between exposed and control groups of mice (Fig. 3). Thus, piperacillin–tazobactam usage in neonates suppressed splenic and lung Th17 immunity to *S. pneumoniae*.

After having known its suppressive activity on Th17 responses, we explored whether or not piperacillin–tazobactam can inhibit antigen-specific neonatal Th1/Th2 immunity to pneumococci. Cytokine analysis of the splenocyte and lung cell culture stimulated with the killed *S. pneumoniae* revealed that splenocytes, but not lung cells, from antibiotic-exposed mice produced reduced quantities of IFN- γ compared to control mice (Fig. 2). Flow cytometric

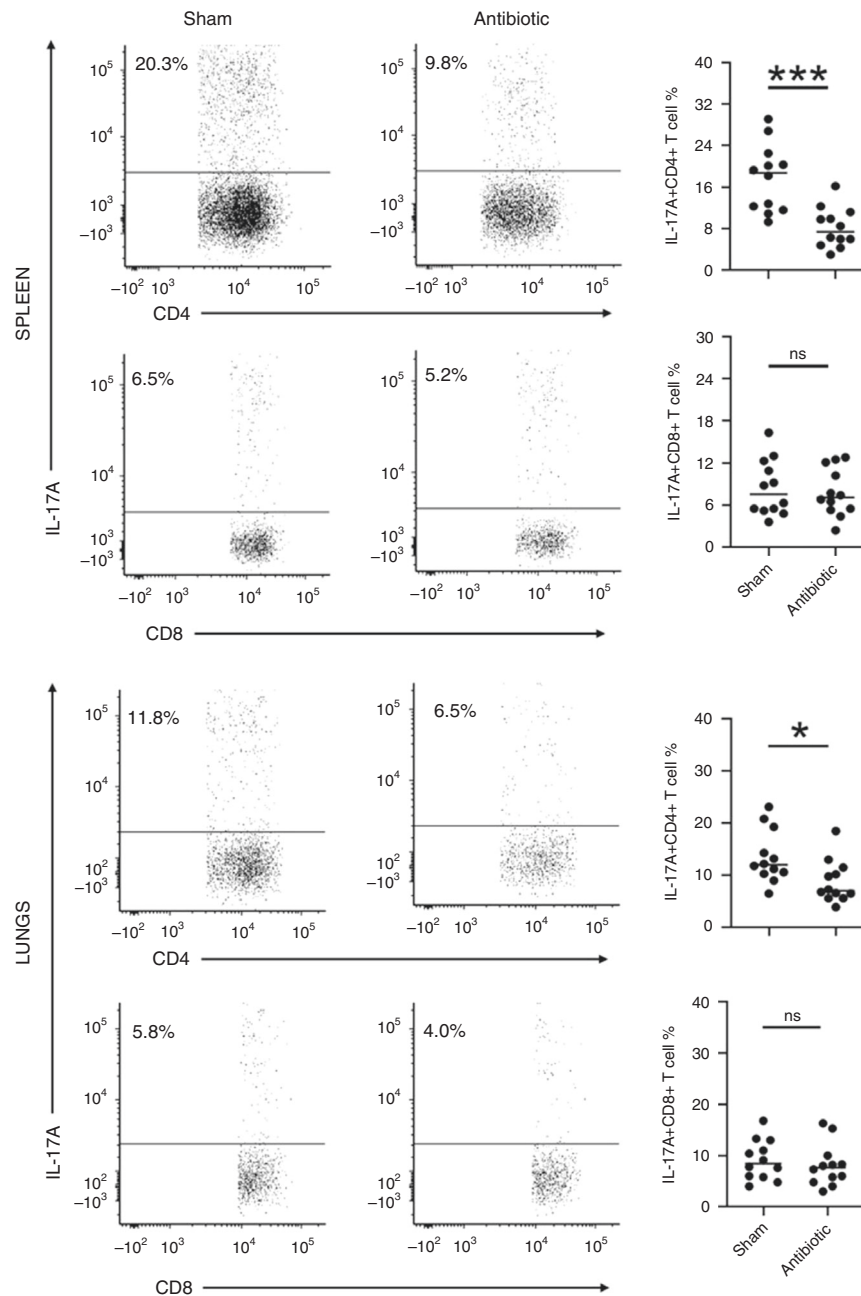


Fig. 3 Secretion of IL-17A by CD4+ and CD8+ T cells from antibiotic-treated mice. IL-17A production in the spleen and lungs by CD4+ and CD8+ T cells was analyzed by flow cytometric intracellular cytokine analysis. Representative flow cytometric dot plot images (left) and a summary of the percentages of IL-17A+ T cells (right). CD3+ cells were gated and displayed in terms of CD3+CD4+ T cells and CD3+CD8+ T cells. Detailed gating strategy for CD4+ and CD8+ T cell populations, and intracellular cytokine expression has been illustrated in Supplementary Fig. 1. Each experimental group (antibiotic or sham) had 12 mice. Data are shown as mean \pm SD and are representative of two independent experiments with similar results. Each symbol represents data from an individual mouse, and the horizontal bars are mean values for the groups. * $P < 0.05$; *** $P < 0.001$. ns = non-significant. Unpaired Student's *t* test.

analysis further showed that splenic CD4+, but not CD8+, T cells from antibiotic-exposed mice expressed lower levels of IFN- γ (Fig. 4). However, the frequencies of the lung CD4+ and CD8+ T cells expressing IFN- γ were similar between exposed and unexposed groups (Fig. 4). On the other hand, there was no significant difference between splenic and lung cells from the treated and control groups for IL-4 production (Fig. 2). Additionally, similar frequencies of lung and splenic CD4+IL-4+ and CD8+IL-4+ T cells from both groups were observed (Fig. 5). Of note, IFN- γ and IL-4 production levels by unstimulated splenocytes between piperacillin-tazobactam or saline were similar (data not shown). In

conclusion, these findings indicate that piperacillin-tazobactam usage inhibits neonatal Th1 responses characterized by IFN- γ production against *S. pneumoniae*, whereas Th2 (IL-4) responses remain unaffected.

DISCUSSION

Using neonatal mice and killed *S. pneumoniae* for antigen stimulation as a model, we evaluated the impact of a combination of piperacillin and tazobactam on antigen-specific splenic and lung CD4+ and CD8+ T cell responses. This experimental model is

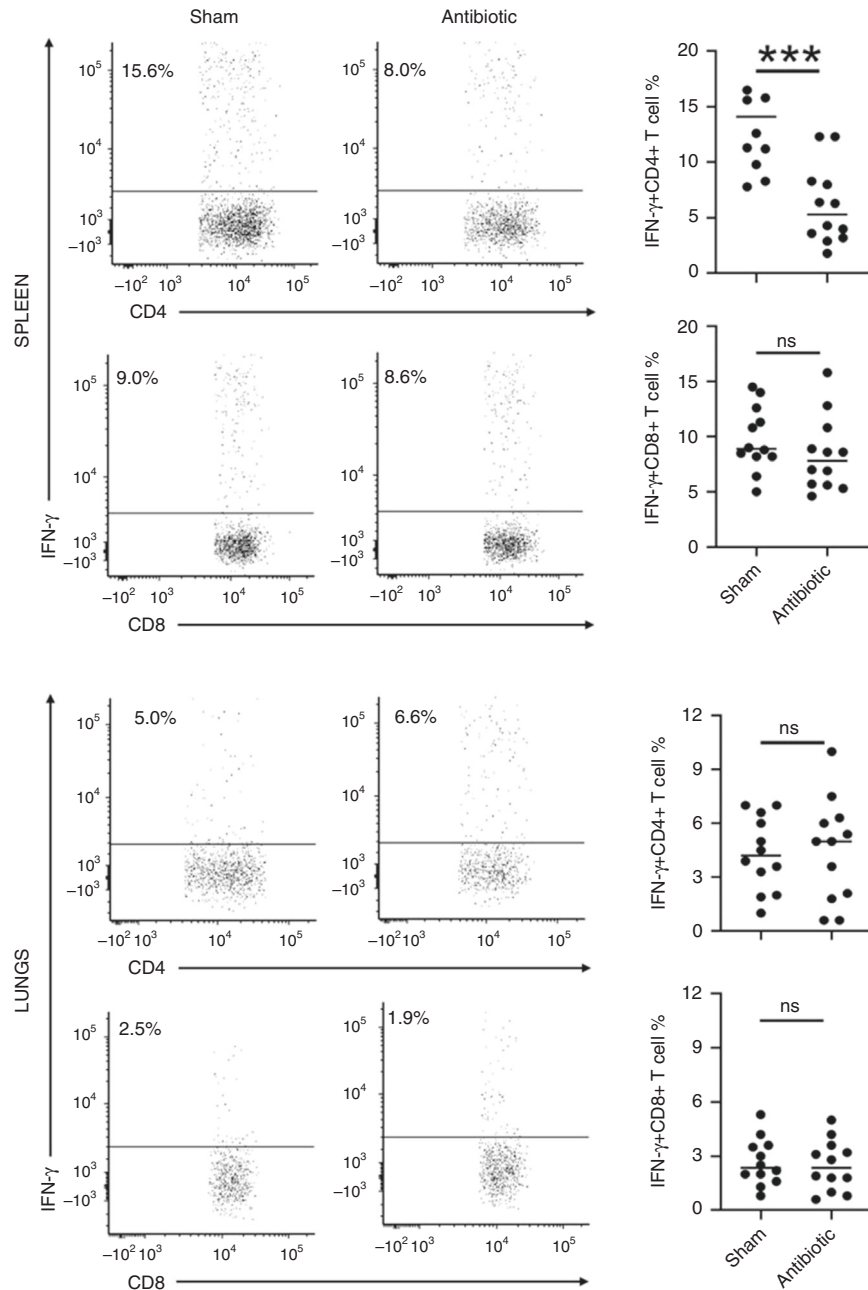


Fig. 4 Antibiotic exposure alters Th1 responses. IFN- γ production by splenic and lung CD4+ and CD8+ T cells was analyzed by intracellular cytokine analysis by flow cytometry. Representative flow cytometric dot plot images (left) and a summary of the percentages of IFN- γ + T cells (right). CD3+ cells were gated and displayed in terms of CD3+CD4+ T cells and CD3+CD8+ T cells. Detailed gating strategy for CD4+ and CD8+ T cell populations, and intracellular cytokine expression has been illustrated in Supplementary Fig. 1. Each experimental group (antibiotic or sham) had 12 mice. Data are shown as mean \pm SD and are representative of two independent experiments with similar results. Each symbol represents data from an individual mouse, and the horizontal bars are mean values for the groups. *** $P < 0.001$. ns = non-significant. Unpaired Student's t test.

relevant due to its closeness to the therapeutic protocols that are commonly followed in the neonatal intensive care unit, including antibiotic dose and parenteral drug administration. The main findings of this study indicate that exposure of piperacillin and tazobactam to mouse neonates results in a reduction in the numbers of CD4+/CD8+ T cells in the spleen and lungs, suppresses antigen-specific splenic and lung Th17 responses, and diminishes splenic Th1, but not Th2, responses to *S. pneumoniae*.

A main finding of this study was the suppressive effect of piperacillin–tazobactam on Th1/Th17 responses, which is

characterized by IFN- γ /IL-17A production by CD4+ T cells, against *S. pneumoniae*. In accordance with our findings, in a mouse model of vaccinia virus infection, neonates from the dams treated with ampicillin, streptomycin, and clindamycin showed decreased antigen-specific expression of IFN- γ by splenic CD8+ T cells.²³ Furthermore, neonatal CD8+ T cells from these antibiotics-treated dams, who were not subjected to infection, had an impaired IFN- γ and tumor necrosis factor- α cytokine production pattern following T cell receptor (TCR) stimulation in vitro.²³ Not only that but splenic CD8+ T cells developing in infant mice with gut microbiota dysbiosis failed to generate optimal TCR signaling

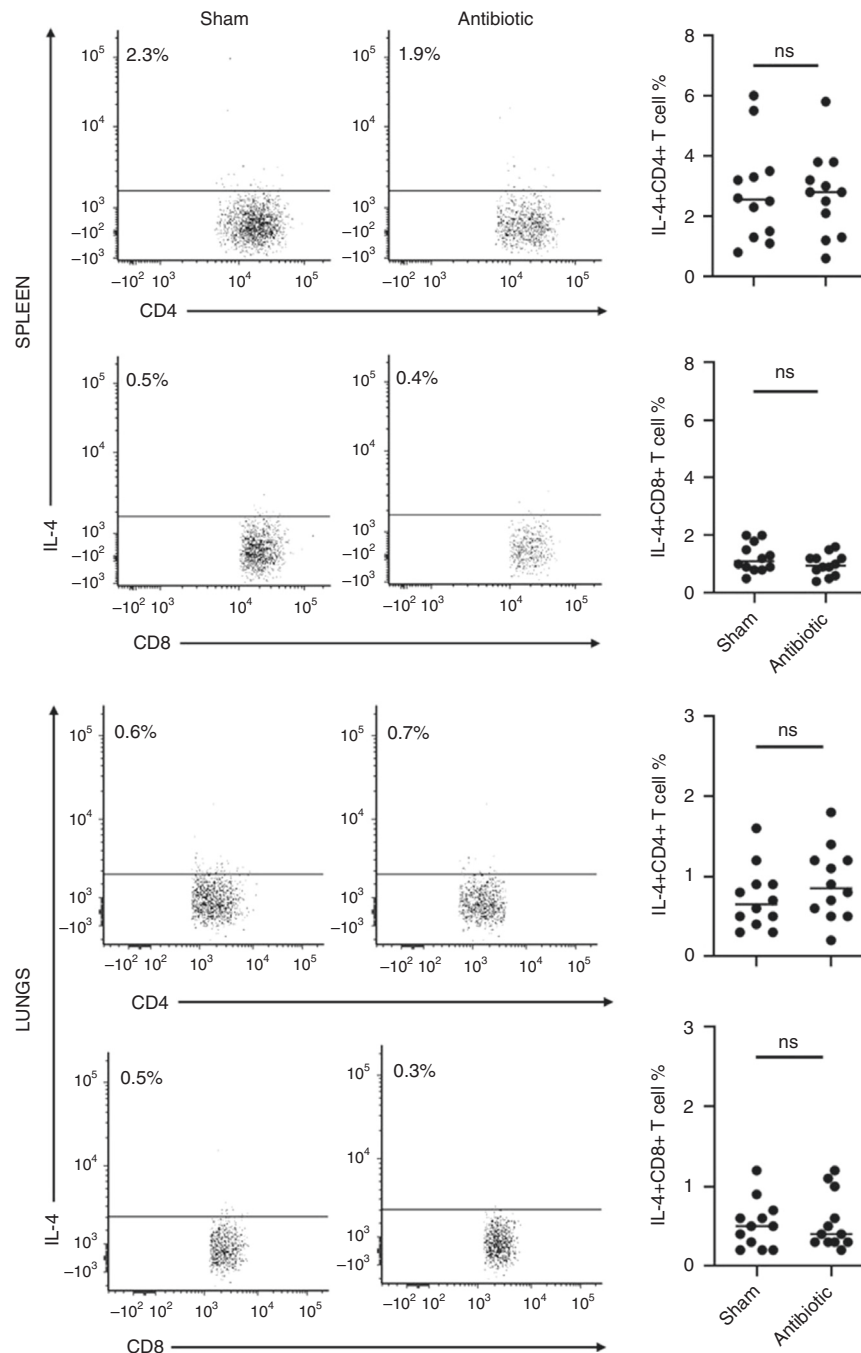


Fig. 5 Effect of antibiotic treatment on Th2 responses. IL-4 production by splenic and lung CD4+ and CD8+ T cells was analyzed by intracellular cytokine analysis by flow cytometry. Representative flow cytometric dot plot images (left) and a summary of the percentages of IL-4+ T cells (right). CD3+ cells were gated and displayed in terms of CD3+CD4+ T cells and CD3+CD8+ T cells. Detailed gating strategy for CD4+ and CD8+ T cell population and intracellular cytokine expression has been illustrated in Supplementary Fig. 1. Each experimental group (antibiotic or sham) had 12 mice. Data are shown as mean \pm SD and are representative of two independent experiments with similar results. Each symbol represents data from an individual mouse, and the horizontal bars are mean values for the groups. ns = non-significant. Unpaired Student's *t* test.

and effector function.²⁴ However, it remains unclear how microbiota dysbiosis impairs the function of T cells. It could be that altered production of short-chain fatty acids induced by the microbiota impairs T cell effector function as shown in case of regulatory T cells.²⁵ Moreover, inhibition of Th17 immunity in rat neonates to a fungal (*Candida albicans*) infection by a combination of meropenem and vancomycin usage has also been reported.⁴⁶ These data indicate a suppressive effect of antibiotic regimens on Th1 and Th17 immune responses. On the other hand, amoxicillin-

exposed neonatal piglets following immunization with heat-killed *S. Typhimurium* challenge showed gene upregulation of IFN- γ .²⁶ More importantly, splenocytes or purified CD4+ T cells that were isolated from antibiotic-exposed mouse infants following immunization with pneumococcal vaccine PCV13 and restimulated with vaccine antigen produced higher levels of IFN- γ than those cells from unexposed mice, underscoring an enhancing cytokine (IFN- γ) recall response by CD4+ T cells.²⁷ These discrepancies when compared to our findings from this study could be due to

differences in animal models, type, dose, and duration of antibiotics, route of drug administration, and nature of microbial antigens/pathogens. Furthermore, in the present study, we calculated the mouse equivalents of the human neonatal antibiotic doses based on normalization of dose to body surface area rather than body weight, which is an accurate method for interspecies dose conversion and often represented in mg/m².⁴³ This is because the body surface area correlates well across multiple mammalian species with several physiological parameters, including oxygen utilization and basal metabolism.⁴⁷ Furthermore, in most previous animal studies, antibiotics were administered to pregnant dams or dams with neonates by mixing the antibiotics in drinking water and maintaining treatment regimens during weaning. Hence, actual delivery doses may vary among dams. Also, murine neonates survive on dam's milk and do not drink the water that contains antibiotics. In contrast, we ensured delivery of precise doses of piperacillin–tazobactam by injecting it via parenteral route, despite being technically challenging and tedious, which is opted for antibiotic delivery in human neonates. Overall, following these appropriate treatment protocols, our study provides new knowledge on suppressive activities of piperacillin and tazobactam on splenic and pulmonary Th1/Th17 immunity to *S. pneumoniae*, which is a clinically relevant human pathogen. However, we did not use a methodology that enables to discriminate between vascular and lung cells isolated from the lungs, which will be addressed in our future studies. It is important to mention that, since mouse neonates were treated with short-term antibiotic regimens, inferences pertaining to persistence of these observations in the long term cannot be made. Our study evaluated the action of piperacillin–tazobactam combination on antibacterial T cell responses in neonates, which has not been addressed in previous studies despite multiple reports of differential effects of different classes of antibiotics on immune responses to microbial pathogens.^{7,8,23,24,48,49} Considering the fact that Th1/Th17 immunity is crucial for host defense against pneumococcal infections, the suppression of Th1/Th17 responses by piperacillin and tazobactam as measured in the short-term can impair clearance of *S. pneumoniae*.

There are certain pitfalls in extrapolating mouse research findings to humans, which need consideration. Analogous to having multiple anatomical and physiological similarities and dissimilarities, there exist spatio-temporal similarities and differences between the mouse and human gut microbiota.⁵⁰ Although mice and humans share many common microbial taxa in the gut, they differ in their proportion and abundance.⁵⁰ Additionally, the composition of the murine gut microbiota is influenced by their genetic constitution, rearing facilities, and commercial suppliers. As host–microbiota interactions shape the immunity to pathogens, we therefore need to be cautious while translating our research findings from mouse model to humans. In addition, we explored the impact of piperacillin and tazobactam for short-term (5 days). While such a regimen is commonly used in clinical practice, shorter or longer treatments are not an exception and may have variable outcomes.

CONCLUSIONS

Our results specifically highlight a suppressive effect of the short-term usage of piperacillin and tazobactam on antigen-specific neonatal CD4⁺ and CD8⁺ T cell responses, particularly Th1 and Th17, to *S. pneumoniae* using a neonatal mouse model. This could have important implications for the development of effective and safe therapeutic strategies for neonates and infants. However, it should be noted that piperacillin–tazobactam is not routinely used for management of neonatal invasive pneumococcal infections. Future studies are required to focus on how impairment of neonatal T cell responses by specific types of antibiotics could have long-term consequences, such as increased susceptibility to

pneumococcal infections, and the underlying mechanisms by which antibiotics modulate T cell immunity.

DATA AVAILABILITY

We confirm that the data supporting the findings of this study are available within the manuscript and its Supplementary Figures.

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AUTHOR CONTRIBUTIONS

S.S.: study design, data acquisition and analysis, drafting and approval of the manuscript. N.K.B.: data acquisition, drafting and approval of the manuscript. F.C.P.: study design, data analysis, drafting and approval of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

We did not use patients in this study and so patient consent was not required.

ADDITIONAL INFORMATION

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